ABSTRACT

SABERI BOSARI, SAHAND. Microfluids and Quantitative Image Processing for High-Content Analysis of Neuronal Degeneration and Particle-based Library Screening. (Under the direction of Dr. Adriana San Miguel Delgadillo).

Biological systems and their interactions are extremely complicated and complex. The study of these systems is proportionally challenging and requires various tests and large datasets to obtain a comprehensive understanding of distinct pathways involved and answer elusive questions regarding them. In order to circumvent challenges associated with investigating biological phenomena and elucidate these complex systems, various scientific fields and industries sought to develop hardware and software equipment and techniques to facilitate acquisition of large datasets and ensure high-content analysis of the recordings. These tools that range from flow cytometers and high-end optics to sophisticated machines learning and image analysis methods were created to maximize the amount of information extracted from each assay. Large, consistent, reliable, and in-depth datasets provide the scientists the materials needed to elucidate the unknowns about biological phenomena by tracking subtle changes that may be hidden from human vision.

Among other fields, the technologies incorporated in image-based assays have been greatly improved since images acquired from biological samples contain rich dynamic information regarding the phenomenon of interest. Thus, various high-end technologies such as high-quality optics, automated microscopes, and digital cameras has been developed to guarantee acquisition of high-grade images in high-throughput manner. However, generation of large pools of images without the capability of properly analyzing them and discovering subtle phenotypic changes and patterns diminishes the purpose of creating big quantitative data sets. Thus, algorithms were generated to process and analyze images in circumstance and events where complicated biological
phenotypes overwhelm the human analysis capacity. However, despite the developments and advances in developing automated tools, there are biological fields with experiments that are extensively image-based and do not have access to equipment to conduct high-throughput high-content assays. Thus, we sought to facilitate assays in the fields with no or limited access to cutting-edge automated equipment by introducing high-throughput high-content technologies.

In the first project (Chapter 2), we sought to characterize age-induced phenotypes occurring to synaptic domain by quantitatively investigating morphological alterations occurring to presynaptic sites. To obtain a comprehensive insight regarding the machinery of synaptic degeneration, microfluidic devices for high-throughput assays and machine learning techniques for high-content analysis were implemented. Degenerative phenotypes occurring to synaptic domain can be subtle or have complex patterns which may be imperceptible to human vision. Thus, for better visualization of phenotypes, rich dynamic information of presynaptic sites such as the localization, distribution, and morphology that is stored in each fluorescent image should be extracted. These layers of information and data can only be accessed and uncovered by analyzing large sets of data quantitatively. In this work, we developed a microfluidic device to address the need for life-long high-throughput microscopy in *C. elegans* and track subtle age-induced phenotypes occurring to the synaptic domain of DA9 neuron. This technology was coupled with a machine learning and computer-based algorithms to process the images acquired. The computer vision provided the quantitative tools required to extract metrics such as the size, fluorescent intensity, and distribution of the synaptic sites. With these metrics, unique subtle degenerative patterns of aging in synaptic domain was identified and tracked.

In the second project (Chapter 3), we sought to deep phenotype and characterize subtle complicated neurodegenerative morphological changes occurring to PVD neuron by extracting
quantitative metrics from fluorescent images to describe structure and morphology of the neuron. Thus, with the aid of computer vision, a Convolutional Neural Network based algorithm coupled with a feature extraction algorithm were trained and developed to automate image segmentation and analysis. Access to these quantitative metrics led to a more comprehensive and vivid understanding of neurodegenerative phenotypes and their unique patterns induced by aging process and exposure to acute cold-shock. In addition, this pipeline enabled us to extract sufficient and detailed information regarding morphology of PVD to an extent where subtle degenerative pattern differences induced by aging and acute cold-shock were identified. This tool was also used to predict biological status of nematodes based on individual’s PVD neurodegeneration profiles.

In the last project (Chapter 4), we sought to fill the absence of an affordable flexible platform to conduct high-throughput library screening of bioactive compounds with high-sensitivity and specificity. The key for an accurate target selection is to screen through large pool of candidates and identify the potential hits based on their fluorescent pattern. Thus, an affordable microfluidic platform was developed to fully automate screening libraries of bioactive compounds on solid phase particles. The microfluidic platform developed was coupled with computer-based algorithms to detect the particles in the device, process the images acquired, extract quantitative metrics, and select the target based on the criteria defined by the operator. This automated platform enabled high-throughput screening while achieving high accuracy and sensitivity in isolating targets with complicated fluorescent patterns and minimizing human involvement.
Microfluids and Quantitative Image Processing for High-Content Analysis of Neuronal Degeneration and Particle-based Library Screening

by

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BIOGRAPHY

Sahand Saberi Bosari was born in Tehran, Iran on 1994 and enjoyed playing soccer, watching cartoons, and playing video games as any kid does. He then moved to Shanghai, China with his family when he was 12. With the encouragement of his family especially his father, he decides to study first and second grade of middle school in only one year. This decision was among the first signs of his love and passion to science and higher education. In addition, by completing 2 grades in one year, he became the youngest member of each class he has attended until this biography is being drafted. Upon his return from 3 years of living in beautiful city of Shanghai, he starts his high school studies in Tehran where a 4-year marathon of extensive work to get accepted into prestigious higher education program was ahead of him. In the last year of his preparation for entrance exam, the average daily time spent by him to study different courses reaches to 11 hours. His hard-work and desire for higher education in a prestigious program landed him on the rank of 892 out of few hundred thousand of applicants who attended the exam and granted him an admission to Chemical Engineering program and The University of Tehran.

Sahand’s interest and passion in research, science, and education led him to be ranked in top 5 students of his class. In addition, he performed a thorough literature review about the applications of microfluidic devices in biotechnology which was advised by Dr. Salehi. He also collaborated and helped Dr. Rouein Halladj and Dr. Sima Askari with their research regarding SAPO-34 catalyst. Upon graduation from The University of Tehran, he moved to Raleigh on 2015 to start his graduate studies at North Carolina State University at department of Chemical and Biomolecular Engineering. At North Carolina State University, Sahand pursued his Ph.D. under Dr. Adriana San Miguel’s supervision and worked on microfluids and quantitative image processing for high-Content analysis of neuronal degeneration and particle-based library
screening. During the executing this project, he got the chance to collaborate with Dr. Stefano Menegatti from Chemical and Biomolecular Engineering department and Dr. Kevin Flores from Mathematics department.
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# TABLE OF CONTENTS

LIST OF TABLES .............................................................................................................................. xii

LIST OF FIGURES .......................................................................................................................... xiii

Chapter 1: Motivation and introduction .........................................................................................1

1.1 Motivation for integrating high-throughput automated platforms to study biology .......... 1

1.2 *C. elegans*, a powerful model organism to investigate age-induced phenotypes .......... 4

1.3 Challenges associated with traditional and qualitative approaches toward studying *C. elegans* and screening bioactive compounds .............................................................................. 6

1.4 Microfluidic platforms and computer-based quantitative techniques for high-throughput high-content biology ......................................................................................................................... 8

1.5 Overview of the projects and broader impact ........................................................................ 12

1.6 References .................................................................................................................................. 16

Chapter 2: A microfluidic platform for lifelong high-resolution and high throughput imaging of subtle aging phenotypes in *C. elegans* ................................................................. 28

2.1 Abstract ...................................................................................................................................... 28

2.2 Introduction ................................................................................................................................. 29

2.3 Results and discussion .............................................................................................................. 33

2.3.1 On-chip high-throughput age-synchronization for drug-free lifelong culture .......... 34

2.3.2 Loading and trapping nematodes for high-resolution imaging ......................................... 38

2.3.3 Temperature induced immobilization .................................................................................. 40
2.3.4 Evacuation .................................................................................................................. 41
2.3.5 Analysis of drug-free synaptic aging .............................................................................. 41
2.3.6 Qualitative analysis of synaptic changes .......................................................................... 42
2.3.7 Image processing and quantitative analysis of the data .................................................. 43
2.3.8 Average puncta size, mean of average puncta intensity, and puncta density .................. 44
2.3.9 Overall synaptic degradation ......................................................................................... 47
2.4 Conclusion ......................................................................................................................... 49
2.5 Materials and methods ...................................................................................................... 51
2.5.1 Device fabrication ........................................................................................................... 51
2.5.2 Strain, media, culture ...................................................................................................... 51
2.5.3 Sterilization .................................................................................................................... 52
2.5.4 Microscopy ..................................................................................................................... 53
2.5.5 On-chip culture ............................................................................................................. 53
2.5.6 Maintaining the nematodes in well-fed in the main chamber ...................................... 53
2.5.7 Bacterial aggregation ..................................................................................................... 54
2.5.8 Bacterial aggregation in the media flask ........................................................................ 55
2.5.9 Bacterial aggregation in tubing, fittings, and on chip .................................................... 59
2.6 References ......................................................................................................................... 62
2.7 Supplementary information ............................................................................................... 68
2.7.1 Experimental setup ....................................................................................................... 69
Chapter 3: Deep learning-enabled phenotyping reveals distinct patterns of neurodegeneration induced by aging and cold-shock ........................................... 77

3.1 Abstract .......................................................................................................................... 77

3.2 Background ...................................................................................................................... 78

3.3 Results and discussion ..................................................................................................... 80

3.3.1 Training the Mask R-CNN algorithm to perform complex image segmentation ...... 80

3.3.2 Deep phenotyping of age induced PVD neurodegeneration ...................................... 83

3.3.3 Acute cold-shock induces neurodegeneration in PVD neuron .................................. 87

3.3.4 Post cold-shock recovery can eliminate PVD dendritic protrusions ......................... 91

3.3.5 Pre cold-shock culture temperature affects neurodegeneration severity .................... 94

3.3.6 Predicting biological status using deep quantitative classification ......................... 97

3.4 Conclusions .................................................................................................................... 102

3.5 Materials and methods .................................................................................................. 106

3.5.1 Worm culture ............................................................................................................. 106

3.5.2 Microscopy ................................................................................................................ 106

3.5.3 Image segmentation and analysis ............................................................................. 107

3.5.4 Aging assay ............................................................................................................... 107

3.5.5 Cold-shock assay ....................................................................................................... 108
Chapter 4: Affordable microfluidic beadsorting platform for automated selection of porous particles functionalized with bioactive compounds

4.1 Abstract ............................................................................................................. 128

4.2 Introduction ..................................................................................................... 129

4.3 Results and discussion .................................................................................. 133

4.3.1 Diverse fluorescence patterns for protein binding detection ......................... 133

4.3.2 Flexible automated detection, analysis, and sorting of beads .......................... 135

4.3.3 Sorting beads with homogenous fluorescence patterns .................................. 139

4.3.4 Sorting beads with “halo”-like fluorescence pattern ....................................... 141

4.3.5 Screening through a ChemMatrix-peptide library to sort “halo”-like fluorescence pattern ........................................................................................................... 145

4.4 Conclusions ..................................................................................................... 147

4.5 Materials and methods ................................................................................... 149

4.5.1 Device fabrication ......................................................................................... 149

4.5.2 Preparation of fluorescently labeled beads ..................................................... 149

4.5.3 Experimental setup ....................................................................................... 150
4.5.4 Image processing ................................................................. 151

4.6 References .................................................................................. 153

4.7 Supplemental information ............................................................... 158

4.7.1 Library synthesis ....................................................................... 162

4.7.2 Conjugation of fluorescent dyes to protein ................................ 162

4.7.3 Incubation of beads against IgG and CHO HCP ......................... 163

4.7.4 Sequencing of selected beads ..................................................... 163

Chapter 5: Conclusion ....................................................................... 165

5.1 Summary and overview of the projects .......................................... 165

5.2 Future work/ Applications beyond scope of this dissertation ............ 170

5.2.1 Project 1 .................................................................................. 170

5.2.2 Project 2 .................................................................................. 173

5.2.3 Project 3 .................................................................................. 178

5.3 References .................................................................................... 180
LIST OF TABLES

Table 1 Device designs tested to optimize progeny evacuation.............................................38

Table 2 Loading efficiency of imaging section in different runs.................................................40
LIST OF FIGURES

Figure 2.1 Microfluidic platform for high-resolution high-throughput imaging of subcellular aging phenotypes. a) Photograph of microfluidic platform. b) Entire experimental imaging system. Media flow through the device was controlled by a custom built pneumatic pressure box that regulates pressure in the stock media bottles. Confocal fluorescence microscopy was carried out on an inverted microscope equipped with a motorized stage. c) Nematodes increase in both width and length throughout the aging process. d) Nematodes are usually on their lateral side, which is not a desired orientation for microscopy of synaptic sites located at the dorsal axon. This device favors a desired dorsal-down orientation.

Figure 2.2 Overview of the platform design specifications. a) The device is made of two main sections: a main chamber and an imaging section. The main chamber is comprised of a chamber surrounded with hundreds of auxiliary channels fabricated to evacuate the progeny and keep the population age-synchronized. 72 traps are designed in the imaging section where nematodes are individually trapped for imaging. b) Detailed specifications of different designs. The auxiliary channels are designed to be shallower than the rest of device and tapered to prevent adult population from exiting the chamber. The imaging channels are also designed to be tapered, lowering the motility of worms. A height restriction at the beginning of channels favored dorsal down orientation. The table is the demonstration...
of which devicewas used within each specific period of population’s lifespan. Scale bar is 5 mm. .................................................................35

**Figure 2.3** Operational loop of the platform. a) There are 2 operational regimes carried out, depending on the desired task. Daily progeny evacuation was carried out once a day while nematodes were reproductively active. Nematodes were flown into the main chamber, eggs and larvae were evacuated, and the population was evacuated from the platform and transferred to the culture media. Complete imaging cycles were performed on day 3, 6, 9, and 12 when periodic microscopy was scheduled. b) Population in the main chamber during progeny evacuation. Larvae and eggs are evacuated from platform. c) Progeny evacuation (red arrow shows progeny getting evacuated). d) Nematodes were transferred to the imaging section and trapped in each imaging compartment. e) Worms loaded in each trap. Scale bars are 2, 1, 2, and 0.5 mm, respectively.................................37

**Figure 2.4** High-resolution microscopy of synapses located at the DA9 axon. a) Puncta formation along dorsal asynaptic region, comissure, and dendrites is exhibited in aged individuals (Lateral view,yellow dashed boxes). Migration of synaptic sites toward the cell body and dendrite initiated at day 9 and aggravated by day 12. b) Major morphological changes occur throughout the aging process (dorsal view). Small synaptic sites tend to form in the vicinity of larger sites (Red dashed boxes). Uneven and irregular distribution of sites along axonal cord (cyan dashed box). The axonal cord seems to bend and form a curve in aged nematodes (green line). Scale bar is 50 µm........................................43
Figure 2.5 Quantitative analysis of acquired images. a) Characterization of synaptic aging throughout the population’s lifespan. Intensity, size, and density of synapses were chosen as representative metrics. b) Binning of the results acquired for average intensity, size, and density. The Gaussian distribution was calculated and plotted for each data set by Origin Lab. $p > 0.05$ (NS), $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), and $p < 0.001$ (***) (N3=35, N6=25, N9=28, N12=41). Error bars are SEM. All p-values were calculated by multiple comparison one-way ANOVA using MATLAB.

Figure 2.6 Deep phenotyping of subtle changes of the DA9 dorsal axon synaptic sites.

a) p-Values calculated from one-way ANOVA comparison to day 3. p-Values were corrected using the multiple comparison method (Tukey–Kramer). b) Heatmap of $Z$ = scores calculated at each time point, using day 3 as the baseline (purple boxes represent metrics discussed in Fig. 5) (brown box represents the metric for mean of the interpunctal distance) (black box represents the metric for maximum integrated intensity).

Figure 2.7 Characterization of synaptic aging. Binning of p-values calculated at each time point. $p > 0.05$ (NS), $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), and $p < 0.001$ (***)

Figure 2.8 Flask bacterial aggregation in the presence of different surfactants.

Figure 2.9 delflic defective strain incubated at 4 °C showed lowest aggregation after 3 days. The OP50 strain sample incubated at 20 °C started to have aggregations from day 2 and the aggregates tend to get larger on day 3.
**Figure 2.10** Time points in on-chip culture. The bacterial cells tend to aggregate in the

tubing as well as in the device. The bacterial aggregation in tubing is

washed to the device and caused major clogging. ..................................................61

**Supplemental Figure 2.1** Immobilization schematic. a) The setup for immobilization process

where dry ice is placed on the top of the platform. b) The

immobilization process flow. .................................................................68

**Supplemental Figure 2.2** Image segmentation and synaptic detection. Maximum projection

images taken for each worm are fed to the segmentation

algorithm. SVM segmentation algorithm performs unsupervised

image processing by exploiting machine learning techniques.

The overlay of the original image and the segmented image

is shown in the right column. The red segments are the regions

that the algorithm eliminated as background or unwanted parts.

The yellow regions are the points that algorithm detected as

synapses..............................................................69

**Supplemental Figure 2.3** Platform degassing. The device is degassed by flowing

pressurized air through it. The high-flowrate will be constantly

flown until all air bubbles are evacuated............................................70

**Figure 3.1** Quantitative analysis of PVD neurodegeneration by deep learning.

a) Schematic of PVD neuron with menorah-like dendritic branches.

Fluorescence images of PVD anterior and posterior to the cell body.

b) Schematic of quantitative analysis pipeline to study PVD

neurodegeneration. c) Aging and acute cold-shock induce neurodegeneration
on PVD dendrites. These two stressors increase the formation of bubble-like protrusions along the dendritic arbors of PVD.

**Figure 3.2** Deep learning approach successfully identifies beads in the PVD neuron. 

- **a)** Schematic of segmentation pipeline. Raw 2048×2048 images are fed to the trained Mask R-CNN model to perform instance segmentation. Yellow arrows point to neuronal beads. 
- **b)** Illustration for true positive, true negative, false positive, and false negative cases used to quantify the performance of instance segmentation. 
- **c-d)** The performance of the algorithm was examined by defining precision and recall of segmentation where 12 validation images were used. Error bars are Standard Error of Mean (SEM). 
- **e)** Images showing the algorithm successfully distinguishes bubble-like protrusions (beads) from fat droplets.

**Figure 3.3** Deep learning allows quantitative analysis of aging induced morphological changes in PVD. 

- **a)** Qualitative inspection of PVD at 3 time points of their life-span shows an increase in number of beads throughout the dendrites. Protrusion formation was identified in both anterior and posterior parts of the PVD neuron. Yellow arrows point to neuronal beads. 
- **b-d)** Average number of beads, average of mean bead size, and average inter-bead distance of both anterior and posterior regions of PVD throughout aging. Lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. 
- **e-f)** Average
number of beads, and average inter-bead distance of anterior versus posterior regions of PVD throughout the aging process. Error bar is SEM. ..........................86

**Figure 3.4** PVD neuronal structure undergoes morphological changes upon exposure to acute cold-shock. **a)** Schematic of acute cold-shock assay. Nematodes were cultured at 20°C until day two of adulthood, split into four plates, and cold-shocked for 4, 8, 16, or 24 hours. Fluorescence microscopy was conducted before exposure to cold-shock and after specific periods of shock. **b-d)** Average number of beads, average of mean bead size, and average inter-bead distance of anterior and posterior regions of PVD as nematodes experienced various duration of cold-shock. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. **e)** Illustration of distinct beading patterns in aging and acute cold-shock based on the inter-bead distance. Inter-bead distance decreases with aging while it increases with cold-shock. **f-g)** Average number of beads and average inter-bead distance of anterior versus posterior parts of PVD upon cold-shock. Error bar is SEM. .................................................................90

**Figure 3.5** PVD neurodegenerative phenotypes caused by acute cold-shock are reversible. **a)** Schematic of post cold-shock rehabilitation treatment assay. Nematodes were cultured at 20 °C until day 2 of adulthood and were exposed to cold-shock for 16 hrs. To perform recovery, the population was split into three plates at either 15 °C, 20 °C, or 25 °C for one day. **b-d)** Average number of beads, average of mean bead size, and average inter-bead distance of PVD neuron as nematodes
experienced cold-shock for 16 hrs. and undergo rehabilitation at 3 different temperatures. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. e-f) Average number of beads, and average inter-bead distance of anterior versus posterior regions of PVD as nematodes experienced cold-shock for 16 hrs. and rehabilitation at 3 different temperatures. Error bar is SEM.

Figure 3.6 Populations cultured at different temperature before being exposed to cold-shock show different susceptibility to neurodegeneration. a) Schematic of the experimental setup to study the effect of pre cold-shock cultivation temperature. Animals were cultured at 20 °C until young adulthood and then transferred to 15 °C, 20 °C, or 25 °C for 3.5, 2.5, and 1.5 days, respectively. Cold-shock was then performed for 16 hrs. and rehabilitation was performed for one day at the pre cold-shock temperature. b-d) Average number of beads, average of mean bead size, and average inter-bead distance of PVD for populations that undergo cold-shock as described in part a). The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. e-f) Average number of beads, and average inter-bead distance of anterior versus posterior part of PVD for populations that undergo cold-shock as described in part a). Error bar is SEM.

Figure 3.7 Biological status of a nematode can be predicted based on PVD neuron’s health. a) Average of mean bead size versus the average number of beads for young
and aged nematodes. Age-induced PVD degeneration patterns are complex and two metrics are not sufficient to accurately classify the two populations. b) Principle component analysis (PCA) for young and aged adults does not enable distinguishing young and aged groups, based on the two first principal components. c) Schematic of the pipeline for computer-based machine learning models to predict the nematode’s biological status based on the morphological structure of PVD. Raw images are fed to Mask R-CNN algorithm to obtain binary mask, which is then used to extract the 46 metrics. Multiple models were trained based on these 46 metrics, and tested on separate data sets. d-f) Classification accuracy for young vs. aged, cold-shocked vs control, and cold-shocked vs. aged nematodes. SDE=Subspace discriminant ensemble, KNN=K-nearest neighbor, SVM=Support vector machine.

Supplemental Figure 3.1 Age-induced degeneration causes morphological variation on PVD structure. a) Total area of PVD neuron covered with beads. b) Percentage of beads with size lower than 100 pixels. c) Average of the mean intensity of the beads. d) The percentage of inter-bead distances lower than 300 pixels. e) 90th percentile of beads fluorescence intensity. Lines are 25th percentile, mean, and 75th percentile. Whisker is the standard deviation. f-h) Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.
**Supplemental Figure 3.2** PVD neuronal structure undergoes morphological changes upon exposure to acute cold-shock. **a)** Total area of PVD neuron covered with beads. **b)** Percentage of beads with size lower than 100 pixels. **c)** Average of the mean intensity of the beads. This line is a transcriptional line; thus, the change is the representative of the fluctuation in concentration on promoter along beads. **d)** The percentage of inter-bead distances lower than 300 pixels. **e)** 90\(^{th}\) percentile of beads fluorescence intensity. The lines are 25\(^{th}\) percentile, mean, and 75\(^{th}\) percentile. Whisker is standard deviation. **f-h)** Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.

**Supplemental Figure 3.3** PVD neurodegenerative phenotypes caused by acute cold-shock is reversible and can be alleviated by post shock rehabilitation. **a)** Total area of PVD neuron covered with beads. **b)** Percentage of beads with size lower than 100 pixels. **c)** Average of the mean intensity of the beads. This line is a transcriptional line; thus, the change is the representative of the fluctuation in concentration on promoter along beads. **d)** The percentage of inter-bead distances lower than 300 pixels. **e)** 90\(^{th}\) percentile of beads fluorescence intensity. The lines are 25\(^{th}\) percentile, mean, and 75\(^{th}\) percentile. Whisker is the standard deviation. **f-h)** Average of mean bead size,
percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.  

**Supplemental Figure 3.4** Histogram distribution of individual bead size for recovery assay of a population cultured at 20 °C.  

**Supplemental Figure 3.5** Populations cultured at different temperature before being exposed to cold-shock respond in various ways to this external stressor.  

- **a)** Total area of PVD neuron covered with beads.  
- **b)** Percentage of beads with size lower than 100 pixels.  
- **c)** Average of the mean intensity of the beads. This line is a transcriptional line; thus, the change is the representative of the fluctuation in concentration on promoter along beads.  
- **d)** The percentage of inter-bead distances lower than 300 pixels.  
- **e)** 90th percentile of beads fluorescence intensity. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation.  
- **f-h)** Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.  

**Supplemental Figure 3.6** Biological status of a nematode can be predicted based on PVD neuron’s health.  

- **a)** Classification accuracy for young vs aged nematodes. The images were acquired from anterior part.  
- **b)** Classification accuracy for cold-shocked vs control nematodes.
The images were acquired from posterior part. c) Classification accuracy for cold-shocked vs aged nematodes. The images were acquired from posterior part. d) Classification accuracy for posterior vs anterior images of nematodes.

**Figure 4.1** Microfluidic device for automated screening of bioactive compounds.

a) Schematic of experimental setup. b) Photograph of the microfluidic platform.

c) Device schematic. Beads enter the device through the bead inlet and are trapped in the imaging zone by on-chip valves. Beads are then directed to positive or negative outlets depending on their fluorescence profile. d) The width and length of imaging zone is 350 µm while the height is 400 µm. The PDMS membrane separating flow and valve channels is 140 µm. Scale bar is 1 cm.

**Figure 4.2** Various classes of beads with different fluorescence profiles. Beads in classes 1-3 exhibit homogeneous fluorescence patterns in green, red, and green/red. Class 4 and 7 were prepared by adsorbing red and green Streptavidin onto biotin beads producing broad halo pattern in red and green channel respectively. Classes 4-5 were prepared by adsorbing red-Streptavidin-BSA and red-Streptavidin-AD onto biotin beads producing medium and narrow halo pattern respectively. The scale bar is 200 µm. *Contrast has been modified in the Unlabeled class image for visibility.*

**Figure 4.3** Screening process flowchart. Beads are flown through the device while the loading valve is closed and the imaging valve open. Once a bead is detected in the imaging zone, the imaging valve closes trapping the bead. If the bead is
assessed to be negative, the loading valve will open up and allow the bead to flow through the negative outlet. If the bead is assessed to be positive, the negative outlet valve closes and the positive outlet valve opens while the loading valve opens and allows the bead to flow through the positive outlet.

**Figure 4.4** Detection and sorting of beads with homogeneous fluorescence patterns. a,c,e) Dot plot of samples screened with the goal of sorting class 1,2, and 3 beads respectively. The values for decision hyperplanes (lines) were established based on preliminary data acquired for each class. The purple point in figure (c) occurred due to having class 1 and class 2 beads entering the imaging zone together. Both beads were sorted as negative. b,d,f) Bar plots of the platform performance in sorting class 1,2, and 3 beads respectively. “Positive added” is the known number of positive beads added to the flask. “Positive Retrieved” represents the true positives collected at the outlet. “False Positive” and “False Negative” are the beads sorted incorrectly.

**Figure 4.5** Capability of platform in sorting halo fluorescence patterns. a,c) The 3D plot of samples screened to sort class 6 and 5 beads respectively. The values for decision hyperplanes (surfaces) were established based on preliminary data acquired for each class. b,d) the bar plot of the platform performance in sorting class 6 and 5 beads respectively. “Positive Added” is the known number of positive beads added to the flask. “Positive Retrieved” represents the true positives collected at the outlet. “False Positive” and “False Negative” are the beads sorted incorrectly. e) Unsupervised K-mean clustering of data extracted from images taken from class 5 and 6 beads. ~30 beads from each class were
imaged and processed. f) The ground truth labels for points used in the unsupervised K-mean clustering. These labeled data were used to calculate the accuracy of unsupervised clustering of data points acquired from class 5 and 6 beads.  

Supplemental Figure 4.1 Flowchart of image segmentation. ........................................143  

Supplemental Figure 4.2 Comparison of speed of bead sorting between trained operators and the microfluidic device. Operators were asked to plate 96 well-plates with single beads and perform fluorescence microscopy of each well. .................................................................159  

Supplemental Figure 4.3 Edman sequencing of positive control bead collected in well F3 from library screening. All amino acid residues were correctly assigned by the Edman data analysis, except for the third and last residues. The Arginine (Arg) peak is the only peak whose intensity does not decrease from the 2nd chromatogram to the 3rd chromatogram, thereby indicating that Arginine is actually located in position 3, as expected. Since the last three amino acids in all sequences of the library and in the spiked IgG-binding peptide HWRGWV-GSG are Gly-Ser-Gly, we can assume that the last amino acid is Glycine..........................160
Supplemental Figure 4.4 Edman sequencing of a positive control bead collected in well F12 from library screening.
Chapter 1: Motivation and introduction

1.1 Motivation for integrating high-throughput automated platforms to study biology

In the past few decades, the world’s population has increased, and this growth has led to higher demand for food, products, and services. Traditional approaches toward manufacturing and production were heavily dependent on human work power which has failed to elevate the throughput of processes and maintain the quality of products while addressing the growth in demand. To fulfill the increase in demands, numerous tools, equipment, and technologies with the main focus of being automated and high-throughput were developed to optimize the outcome. Industrial companies have been pioneers in shifting to high-throughput production lines where human work force has been mostly replaced by automated equipment and robotic arms. The switch to automation was carried out to 1- Reduce worker fatigue and effort especially for repetitive and labor-intensive tasks 2- Increase the efficiency and consistency of the products 3- Elevate productivity and reliability 4- Reduce the operational cost by improving the throughput and 5- Optimize the overall performance of the production line.

The implementation of automation and process optimization has not been limited to the production lines of industrial companies and the research and development sections of corporations as well as academic laboratories have also been using state of art technologies to perform high-throughput assays. In research and development fields, high-throughput automated assays are preferred to be coupled with high-content quantitative data analysis to obtain a comprehensive perspective and understanding about the project and phenomenon under investigation. Thus, in addition to hardware development to automate the data acquisition, algorithms and software needs to be developed to analyze the recordings and extract meaningful results and conclusions from them. Computer vision-based techniques such machine learning or
artificial intelligence has been extensively integrated to assays and tests to provide the research the access needed to layer of information deeply hidden in data sets which can be invisible to human\textsuperscript{2–10}.

Among different scientific and industrial fields, the spectrum of studies of complicated biological systems could benefit the most from integration of high-throughput high-content equipment and techniques. Investigating complex convoluted biological pathways and their interactions in animals accurate and efficiently is challenging and requires collection of large data sets and thorough deep analysis of the information obtained. The failure in integrating automated high-throughput high-content methods could lead to labor-intensive time-consuming tests where the conclusions drawn are prone to error and bias. In addition, sufficient details needed to explain complicated biological phenomena cannot be obtained without integrating quantitative tools and techniques. Thus, in the absence of quantitative data, the interpretation of data will be left to be done by human observation which lacks reliability and consistency. For instance, Studies have shown that 70\% of researchers are unable to reproduce results from other labs and 50\% of scientists have failed to replicate their own\textsuperscript{11}. A separate study have indicated that 28 Billion dollars has been spent in United State on assays that has not been reproduced\textsuperscript{12}. The root of these issues can be found in not implementing techniques that provide scientists with large amount of high-quality reliable information. To circumvent these challenges, various high-end technologies and equipment such as flow-cytometer, DNA-sequencer, thermal cycler, automated liquid handlers, robotic arms, and bioinformatic software have been developed and implemented in different stages of research such as 1- Sample preparation and media handling 2- Sample maintenance 3- Conducting experiments and data acquisition and 4- Data collection, handling, and analysis.
With the growth in number of investigations in biological fields that incorporate image-based analysis, extensive advancements have been made to develop hardware and software to improve image acquisition, processing, and analysis. High-quality optics and automated microscopes have been designed and created to acquire large pools of images in short periods of time. In addition, sophisticated cameras and light sources have been developed to enhance the quality of images obtained from samples. Number of software and algorithms have also been generated to process the images and analyze the data to extract the information in circumstances where the complexity of the biological systems overwhelm the human analysis\textsuperscript{13–30}.

Despite the emergence of large number of techniques developed for automating assays in biology, there are fields that are deprived of high-throughput high-content methods to conduct tests. This can be as a result of high-acquisition cost of some of this equipment or the lack of specificity, sensitivity, and flexibility offered by commercial tools available. Thus, in this work, we sought to phenotype age-induced degeneration in cellular and subcellular level of nervous system and screen for target peptides by developing high-throughput hardware and high-content image processing and analysis software. We successfully developed affordable microfluidic devices to perform high-resolution high-throughput imaging in \textit{C. elegans} and to screen for bioactive compounds in solid-phase libraries. In addition, we integrated cutting-edge novel computer-based techniques to conduct accurate, efficient, high-throughput, and unbiased quantitative data analysis and image processing. The incorporation of these novel technologies enabled us to perform reliable and robust assays in an automated high-throughput manner. In addition to automation, we tracked and identified subtle age-induced changes occurring to nematodes’ nervous system and identified unique neurodegenerative patterns based on the
quantitative metrics extracted from fluorescent images acquired. These findings and discoveries would not been possible by qualitative approaches due to limited information provided by them.

1.2 C. elegans, a powerful model organism to investigate age-induced phenotypes

*C. elegans* is a transparent round worm about 1 mm in length and 60 microns in width which lives in soil environment. On 1963, Sydney Brenner first suggested investigating this nematode primarily for neuronal development and introduced it as a model organism in 1974. Since 1974, this model organism has been studied extensively due to the advantages offered by it. Among various advantages studying *C. elegans* offers, the relatively short life-span and rapid life-cycle were the ones that made this organism a promising model to investigate aging since 1970s. One of the major questions to be elucidated by this model was the factors alternating life-span and the genes and genetic pathways orchestrating this abnormality. The genetic screens performed on *C. elegans* have led to discovery of genes (*age-1, daf-2, daf-16*) which upon mutation cause significant extension or reduction in the nematodes’ life-span.

In addition to changes in life-span, various organs and tissues in *C. elegans* undergo degeneration during the aging process. For instance, the nematode’s cuticle, hypodermis, muscle, intestine, and reproductive system are among main tissues experiencing age-induced degeneration. Behavioral changes such as slower locomotion and reduction in food uptake rate are also phenotypes occurring during aging. Along with various parts of *C. elegans*’ body, its nervous system also exhibits degeneration in cellular and subcellular level which has been studied extensively in the past. During normal aging, and similar to human neurons, the nematodes’ nervous system do not exhibit a drastic neurodegeneration such as neuronal loss. However, subtle neurodegenerative phenotypes such as soma and axon shrinkage, new branches addition to the neuron, axon waviness and swelling, and somatic outgrowth do occur as a population age.
For instance, previous studies have shown that PVD neuron which is responsible for harsh-touch sensation and thermo sensation experiences morphological variation throughout aging process. The PVD neurons in young healthy adults resemble menorahs (candle like shape) and are formed separately and do not cross each other\textsuperscript{46–53} while aged nematodes exhibit dendritic disorganization, outgrowth, and branching. Previous studies have shown than the menorahs’ structure get deformed and numerous side branches are grow in different chaotic way\textsuperscript{54–59}.

In addition to dendrite outgrowth and disorganization, Lezi E et al reported bubble-like protrusions formation along the neuron during aging which points to existence of separate distinct neurodegenerative mechanisms\textsuperscript{60}. In this study, an increase in frequency of beads forming along dendrites within older population of nematodes was observed. Apart from various neurodegenerative phenotypes, the rate at which each neuron ages is different suggesting a distinct pathway incorporated for degeneration of each individual neuron\textsuperscript{38,61}. Interestingly, based on various surveys conducted, each individual worm in an isogenic population exhibit distinct age-induced phenotypes suggesting the presence of stochasticity in aging process and the need for studying large sample sizes to obtain comprehensive understanding of degeneration process\textsuperscript{41}.

In addition to neuronal degeneration at the cellular level, synapses and synaptic machinery (subcellular) experience age-induced degradation. Previous studies have shown that the number of synaptic vesicles and the size of the presynaptic sites decrease in late stages of worm’s life-span\textsuperscript{62}. Li et al has reported a decrease in density of synaptic vesicle in the DA9 motoneuron by day 18. In addition, a decrease in presynaptic release, vesicle recycling, and synaptic vesicle protein accumulation were also reported by various group as age-induced phenotypes\textsuperscript{38,63–67}. Despite extensive investigations carried out to elucidate mechanisms and pathways involved in
neurodegeneration, there are still large number of questions to be answered requiring further investigation.

1.3 Challenges associated with traditional and qualitative approaches toward studying *C. elegans* and screening bioactive compounds

Despite the advantages associated with studying neurodegeneration in *C. elegans*, there are several challenges with traditional techniques implemented to perform high-resolution microscopy and image analysis. These challenges have led the assays to be labor-intensive, and less efficient in various steps of experiments such as culturing, handling, preparing nematodes for fluorescent microscopy, and data analysis. For instance, a chemical agent that inhibits reproduction, FUdR (Fluorodeoxyuridine), is introduced to nematodes’ growth media to maintain the population of worms age-synchronized\(^6^8\). Exposure to FUdR treatment hampers egg generation or prevents nematodes from having viable progeny depending on the concentration of the drug used by inhibiting the DNA synthesis\(^6^8\). However, studies have shown that exposure to this chemical hinders growth and alters nematodes’ response to dietary restriction\(^6^9\)–\(^7^2\). In addition, it has been reported that FUdR influences the nematode’s longevity under certain conditions by extending the population’s life-span. Artificial manipulation of a population’s life-span caused by FUdR can lead to misrepresentative findings and conclusions regarding natural normal aging process.

The second issue associated with studying age-induced degeneration in cellular and subcellular level is the need for complete immobilization of animal during high-resolution microscopy. Given the small scale of the cellular and subcellular structures of interest (synapses are \(\sim 1 \mu m\)), small \(1 \mu m\) movements of sample can drastically affect the quality of images acquired and the reliability of the post quantitative data analysis. Traditionally, to circumvent this challenge, nematodes are immobilized by being transferred to an agar pad with a drop of anesthetics.
Tetramisole and levamisole are two commonly used anesthetics implemented to immobilize worms. However, studies have reported that exposure to Levamisole poses side effects to acetylcholine receptors leading to potential physiological and functional changes in synaptic levels which is not desirable while investigating it\textsuperscript{73,74}.

Nematodes’ correct orientation during high-resolution microscopy is crucial for acquiring high-quality images. For instance, in order to image presynaptic sites tagged by RAB-3::GFP fusion protein located at the dorsal side of the nematode’s tail, it is desired to get the dorsal side as close as possible to the cover slip. In cases where the ventral side is placed closer to the objective (undesired orientation), the light needs to travel through nematodes’ tissue before hitting the target on the dorsal side, which will decrease the quality of image acquisition. It is challenging to control and dictate the orientation of nematodes and get the dorsal side close to cover slip during high-resolution microscopy while using agar pad due to the tendency of worms to be on their side.

In addition to lack of reliable high-throughput hardware for image acquisition, data handling and analysis using traditional techniques also face certain obstacles. Non-automated qualitative image analysis of complex phenotypes is extremely low-throughput requiring an operator to go through hundreds to thousands of images and interpret the images and draw conclusions based on qualitative observation. In addition, direct involvement of an operator in image processing introduces human error and bias which could lead to conclusions with less confidence and lower quality data. In addition to susceptibility of traditional approaches to error and bias, they are also unable to fully extract rich information embedded in each image requiring more quantitative methods to be sought for. Qualitative image processing and data analysis is limited to binary assessments of phenotypes of interest which is unable to offer comprehensive illustration of biological phenomena. To remedy these challenges, there is a need for automated high-throughput
software to facilitate the assays and increase the accuracy, efficiency, robustness, and reliability of the experiments. Integrating computer vision in biological field such as cell biology has attracted a lot of attention. For instance, many recent cell biological studies have integrated computer vision-based techniques to track cell morphology and subcellular protein distribution. In other studies, computer-based methods have been developed and integrated to perform single-cell tracking or following thousands of subcellular molecular markers. Despite these advances in integrating computer vision in image-based assays and tools for high-throughput experiment, there are numerous fields with no or limited access to these cutting-edge techniques. Thus, in this work, we developed platforms such as microfluidic systems for high-throughput high-resolution microscopy and computer-based data analysis and machine learning techniques to perform quantitative, high-throughput, accurate, unbiased image processing and analysis.

1.4 Microfluidic platforms and computer-based quantitative techniques for high-throughput high-content biology

In the past decades, numerous platforms and equipment have been developed to increase the throughput and accuracy of various experimental assays in academic laboratories. Microfluidic devices were among revolutionary platforms developed in past two decades to facilitate both industrial and academic processes. Two major advantages that has made microfluidic devices an upper hand compared to conventional systems are low cost of fabrication and reagent consumption. In addition, these miniaturized devices possess high portability, accuracy, and precision in controlling the operational parameters. Considering these advantages, microfluidic platforms are now being implemented in various fields such as biological sciences, soft matter, reaction and synthesis, drug screening, etc.
The relatively low flow-rates within micron-size channels of microfluidic platforms ensure Reynolds numbers in Laminar flow regime leading to a more predictable media behavior in the device and facilitating liquid handling which is crucial for high-throughput assays\textsuperscript{85–88}. Microfluidic platforms can be fabricated using different techniques based on the application and resolution needed. These techniques can be categorized as 1- wet and dry etching 2- thermoforming 3- polymer ablation and 4- polymer casting. Each of these general techniques can be performed in various ways\textsuperscript{89–91}. For instance, 1- physical dry etching 2- chemical dry etching and 3- reactive ion etching (RIE) are three different subgroups of dry etching method each implemented for specific application\textsuperscript{92}. In addition to variety of techniques for microfluidic fabrication, these platforms can be made of different materials. Depending on the field where these platforms are being implemented in, microfluidic devices can be made of substrates such as glass, metal, polymers or even natural substrates such as collagen\textsuperscript{93,94}.

Academic researchers have been extensively integrating PDMS based microfluidic devices to their experiments to perform assays not feasible without a platform or to automate labor-intensive time-consuming tasks and increase the throughput and accuracy of their assays. One of the common techniques used to fabricate microfluidic devices has been photo-lithography followed by soft-lithography\textsuperscript{95,96}. In this approach, the substrate which can be made of silicon is spin-coated with photoresist and baked to solidify. To obtain the pattern of interest, UV light is shined through the photo-mask, baked, and developed to wash off the unwanted segments of the photoresist. The PDMS is then poured on top of the substrate with finalized patterns on to perform the soft-lithography.

The accessibility and relatively simple fabrication protocol offered by this technique has made it a common approach toward developing microfluidic device. Various groups investigating
C. elegans have also been integrating microfluidic devices in their assays\textsuperscript{97–106}. For instance, Lu et al designed a microfluidic device for high-throughput microscopy of L4 nematodes. This platform is composed of parallel rows connected to individual imaging channels where nematodes are flown through, trapped and partially immobilized for microscopy\textsuperscript{107}. Hulme et al designed a parallel series of chambers each accommodating a single nematode to perform life-long culture of nematodes on-chip. The chambers were connected to a tapered channel to transfer the worms into, partially immobilize them and perform microscopy. Xian et al fabricated WormFarm for high-throughput life-long culture of populations on worms\textsuperscript{108}. They were able to cultivate various strains and populations of worms in their chambers and determine their life-span in a FUdR free environment. The custom designed auxiliary channels surrounding the chambers facilitated progeny evacuation and eliminated the need for FUdR. Mondal et al have developed a high-throughput imaging arrays capable of whole body microscopy of thousands worms in each imaging round\textsuperscript{102}. The platform is comprised of parallel chambers each connected to arrays of imaging channels were worms are loaded into. This platform was implemented to study protein aggregation in various lines on C. elegans. These studied exhibited the advantages offered by integrating high-throughput automated tools to study C. elegans. However, there are large number of opportunities and studies where novel microfluidic platforms could be integrated to further facilitate the experiments leading to better understanding of fundamentals in C. elegans.

Microfluidic devices have been promising tools as an automated high-throughput hardware incorporated in C. elegans investigations. However, process automation and optimization should not be limited to hardware-based innovations and developments. Data collection, handling, analysis and informatics which emerge post result acquisition should also be improved by introducing cutting-edge technology to increase the throughput, accuracy, and reliability of
assessments. In addition to providing unbiased accurate assessments, computer-based data analysis further facilitates elucidating complex biological phenomena by accessing to quantitative rich information embedded in images or data sets. For instance, San Miguel et al integrated computer-based algorithms in both data collection and analysis steps. They developed a Graphical User Interface (GUI) in MATLAB to automate the entire image acquisition, image storage, and microfluidic operation\textsuperscript{109}. The integration of this fully automated algorithm with microfluidic platform eliminated the need for an operator to perform the forward genetic screen experiments. In addition, they developed a custom trained Support Vector Machine (SVM) algorithm to process the images acquired and segment the fluorescently tagged synapses. The utilization of this machine learning technique enabled generating binary masks of synapses which were then used to extract 76 different metrics describing the morphology of the synaptic domain. Based on these metrics, deep phenotyping of presynaptic site of DA9 neuron was performed. Hakim et al developed a platform called WorMachine which is comprised of image processing, deep learning, and machine learning techniques to perform assays such as supervised classification of binary-sex phenotype, scoring continuous-sexual phenotypes, quantifying the effects of two different RNA interference treatments, and measuring intracellular protein aggregation\textsuperscript{110}. In this work, MATLAB image processing toolbox has been implemented to segment images, remove noises and clean images, and identify worms. Deep learning and machine learning techniques were then used to remove faulty worms, analyze morphology, and perform clustering and classification. Kaltdorf et al combined a machine learning technique with an image segmentation workflow to develop an automated method to classify synaptic vesicles. Clear Core Vesicles (CCV) and Dense Core Vesicles (DCV) are two types of synaptic vesicles with various roles and functions\textsuperscript{111}. This technique enabled reliable distinguishing of CCVs and DCVs in electron tomograms of \textit{C. elegans}. 

11
neuromuscular junctions using features extracted from images. Wang et al implemented deep reinforcement learning of cell movement in the early stage of *C. elegans* embryogenesis\(^{112}\). In this work, they investigated cell movement by integrating deep reinforcement learning embedded in a modeling system to analysis 3D time-lapse microscopy images. They were able to implement this technique to characterize cell movement in two real developmental process.

All of these studies demonstrated the importance of integrating computer-based technologies in data collection, handling, and analysis. The addition of these techniques in image acquisition step has increased the throughput of the process while minimizing a human operator involvement. The binary masks obtained from segmentation could then be used to extract various features describing the phenomena in more comprehensive and quantitative fashion. Extracting quantitative rich information from images facilitates identifying and tracking subtle changes while performing high-content deep phenotyping. Deep quantitative phenotyping enabled by computer and machine learning based techniques has unveiled patterns and traits which were not to be recognized using traditional conventional methods. Despite the developments made by integrating novel cutting-edge computer-based techniques to automate data analysis, there is a need for new methods and techniques to further facilitate biological assays.

**1.5 Overview of the projects and broader impact**

In this work, we sought to provide the areas in biological sciences with high-throughput high-content equipment and tools required to investigate complicated and convoluted bio related systems in more quantitative fashion. The tools developed in this dissertation are comprised of microfluidic devices to facilitate data acquisition and computer-based software to access hidden layers of information stored in each fluorescent image and extract rich data. This approach enabled us to obtain large sets of fluorescent images and process them to obtain more comprehensive
understanding and dynamic information about degenerative changes occurring to synaptic sites and PVD neuron. For instance, in the first project we tracked plasticity of presynaptic domains located at nematodes’ tail as a population ages based on quantitative metrics extracted from images acquired from confocal fluorescent microscopy. We fabricated a microfluidic platform to perform life-long high-resolution high-throughput imaging of subtle phenotypes on-chip in a drug free environment. A computer-based quantitative analysis was then integrated to extract rich information and features from images obtained using this platform. The results obtained from quantitative characterization of synaptic morphology enabled us to identify subtle but significant changes in presynaptic structure such as increase in size and fluorescent intensity of presynaptic sites. By coupling our novel microfluidic platform with machine learning-based image segmentation, we were able to track and identify age-induced synaptic degeneration patterns which were hidden from human observations.

In our second project, to further facilitate studies related to cellular degeneration in *C. elegans* and elucidate complicated mechanisms and phenotypes, we integrated a cutting-edge machine-learning based technique to quantify age and stressor induced neurodegeneration in PVD neuron. In this project, a Convolutional Neural Network-based (MaskRCNN) algorithm was trained and calibrated to process the images acquired from PVD neuron and segment the bubble-like protrusions forming along the neuron as a sign of neurodegeneration. The segmentation pipeline was accompanied by a feature extraction algorithm to quantify the structural changes occurring to this neuron under various conditions. Integrating this technology enabled us to quantify age-induced neurodegenerative phenotypes occurring to PVD. In addition, we discovered the neurodegenerative effect of acute cold-shock on PVD which had been unknown. Our powerful
approach was able to surface distinct neurodegenerative patterns of beading induced by aging and acute-cold shock which could not been identified by qualitative methods.

In our last approach, we developed an affordable automated tool for academic laboratories where we sought to address the challenges associated with selection of solid porous particles functionalized with bioactive compounds. The capability to identify ligands that bind to specific proteins is a fundamental need in protein purification and drug delivery industries. Traditional manual screening techniques are extremely labor-intensive while commercial automated platforms developed for this task are either too expensive or lack the specificity needed to screen for complex fluorescent patterns. This, an affordable fully automated microfluidic system was designed and fabricated to screen libraries for desired peptide-protein binding based on particles’ fluorescent pattern. We achieved high throughput, accuracy, and sensitivity in target selection and isolation. The three hardware and software developed in this work not only provided the assays with an automated high-throughput tools, but also enabled quantitative high-content analysis and assessment of the images and data sets. These quantitative analysis in cases led to discoveries which were hidden or unknown to human based observations. These platforms can be directly integrated to the experimental assays in various labs working on C. elegans or bioactive compound screening related studies to further facilitate the investigations. In addition, the flexibility and customizability of these technologies allow them to be modified in a way to address more diverse and specific issues and challenges. Thus, in this work, we sought to deep phenotype to obtain a more comprehensive understanding of biological systems by developing microfluidic systems for high-throughput image acquisition and computer-based algorithms for high-content data analysis. This combination enabled us to access rich and imperceptible to human vision information embedded in large pools of images acquired and conduct deep phenotyping to unveil subtle
degenerative patterns emerging in nervous system. This quantitative approach has shifted the data interpretation based on qualitative human observation to quantitative one based on information extracted from the images leading to a more reliable and comprehensive conclusions.
1.6 References


31. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of


Cells **40**, 90–99 (2017).


96. Ma, Y., Thiele, J., Abdelmohsen, L., Xu, J. & Huck, W. T. S. Biocompatible macro-


105. Cornaglia, M. *et al.* An automated microfluidic platform for *C. elegans* embryo arraying,


Chapter 2: A microfluidic platform for lifelong high-resolution and high-throughput imaging of subtle aging phenotypes in C. elegans

2.1 Abstract

Aging produces number of changes in neuronal structure and function throughout a variety of organisms. These aging-induced changes encompass a wide range of phenotypes, from loss of locomotion ability to defective production of synaptic vesicles. C. elegans is one of the primary systems to elucidate phenotypes associated with aging processes. Conventional aging studies in C. elegans are typically labor-intensive, low-throughput, and incorporate Fluorodeoxyuridine (FUdR) as a sterilization agent to keep the population age-synchronized throughout the assay. However, FUdR exposure induces life-span extension, and can potentially mask the phenotypes associated with the natural aging process. In addition, studying cellular or subcellular structures requires anesthetics or adhesives to immobilize nematodes while acquiring high-resolution images. In this platform, we are able to maintain a population (~1000 worms) age-synchronized throughout its life-span and perform a series of high-resolution microscopy in a drug free environment. The device is comprised of two main interconnected sections, one with the purpose of filtering progeny while keeping the parent population intact, and one for trapping nematodes in individual compartments for microscopy. Immobilization is carried out by decreasing the temperature of the device where nematodes are trapped by placing a heat sink on top of the chip. We were able to perform periodic high-resolution microscopy of fluorescently tagged synapses located at dorsal side of the nematode’s tail throughout the worms’ life-span. To characterize the subtle phenotypes that emerge as nematodes age, computer vision was implemented to perform
automated unbiased detection of synapses and quantitative analysis of aging-induced synaptic changes.

2.2 Introduction

Human life expectancy has increased over the past century, and this pattern is expected to continue in next few decades\(^1\). The main reason for this lifespan extension is improvement in healthcare. As a consequence of this life prolongation, the senior population, which is most susceptible to age-induced diseases including Alzheimer’s, Parkinson’s, and Huntington’s, is growing\(^2\). Thus, the fraction of people vulnerable to these illnesses is expected to increase in the near future, making aging studies crucial. However, studying this process directly in humans poses numerous difficulties, particularly the long timescale involved in the aging process. Model organisms, on the other hand, enable the study of aging in simpler systems with faster life cycle.

*\textit{C. elegans},* the first eukaryote to have its genome fully sequenced, is one of the premier model organisms to study aging\(^3-5\). Its relatively short life-span (~20 days) enables rapid experimentation. These nematodes are self-fertilizers, which makes preparing a large isogenic population in 2-3 days, simple\(^6\). Their transparent bodies enable fluorescence microscopy of structures of interest with fluorescent tags\(^7,8\) in live, intact animals. *\textit{C. elegans}* is amenable to easy genetic manipulation, especially with emerging novel gene editing techniques that have increased the efficiency and throughput of targeted insertion of markers and mutations\(^9\). Due to all these advantages, *\textit{C. elegans}* has become a widely used model of aging, particularly after the discovery of mutations that dramatically extend lifespan\(^10\). Several aspects involved in aging and longevity have been studied in this model organism, such as DNA damage, mitochondrial dysfunction, autophagy impairment, and
nutrient sensing dysregulation\textsuperscript{11,12}. Some efforts have also been devoted to explore neurodegeneration induced by aging, as \textit{C. elegans} is also very important in neuroscience studies, mainly due to its fully mapped neuronal connections\textsuperscript{11,13–18}. Aging induces phenotypes reminiscent of functional deterioration in the nervous system: motility decline, long-term associative memory deterioration, and changes in neuronal morphology and synaptic patterning\textsuperscript{14,15,17–19}.

The nature of life-long aging studies in \textit{C. elegans}, particularly those that require large sample numbers, makes them time consuming and labor-intensive. One of the key requirements of these studies is to maintain an age-synchronized population. To achieve this criterion, most aging assays commonly use 5-Fluorodexyuridine (FUdR) as a sterilizing agent, which prevents nematodes from laying viable progeny. However, studies have shown that FUdR interferes with longevity pathways and causes an artificial lifespan extension, potentially affecting the aging phenotype of interest\textsuperscript{20,21}. As an alternative to FUdR, nematodes can be transferred from plate to plate on a daily basis to avoid mixing of progeny and parent population. This method suffers from being extensively time consuming and low throughput. In addition, aging studies that require high-resolution microscopy of cellular and subcellular features (such as neurons or synapses), also typically use anesthetics like Levamisole to achieve complete immobilization for imaging. However, Levamisole is a potent cholinergic agonist which paralyzes nematodes by binding to acetylcholine receptors, potentially inducing changes at the synaptic level\textsuperscript{22–24}.

To overcome these issues, various microfluidic platforms have been developed for imaging and aging studies\textsuperscript{25}. Xian et al. developed WormFarm, which enables long-term culture of an age-synchronized population without introducing FUdR\textsuperscript{26}. Hulme et al.
designed a platform with 16 chambers connected to imaging channels where nematodes can be cultured and imaged individually\textsuperscript{27}. However, these platforms do not allow for high-resolution imaging of subcellular features. Various techniques for immobilization that avoid anesthetics on-chip include mechanical membrane deflection, cooling, CO\textsubscript{2} exposure, and temperature-driven gelation of a polymer. Integrating each of these methods with microfluidic devices has resulted in different levels of on-chip animal immobilization\textsuperscript{28–30}. A large number of microfluidic devices have also been developed to perform fluorescence microscopy\textsuperscript{31}. Some examples include a device to perform high-throughput imaging and tracking of protein aggregation in a population\textsuperscript{32,33}, a multi-channel device to monitor cellular features in multiple L4 animals in parallel\textsuperscript{34}, platforms to image neuronal activity\textsuperscript{35,36}, and a platform to perform whole population phenotyping while tracking single individuals for high-content studies\textsuperscript{37}.

Despite the capabilities these systems provide, there is a need for a platform to track age-induced subtle phenotypes throughout the population’s lifespan by conducting high-resolution high-throughput microscopy. In this study, we developed a platform to characterize aging of cellular and subcellular structures throughout a population’s lifespan in a drug free environment (Figure 2.1.b). We focus on subtle changes in synaptic patterning in the dorsal axon of the DA9 neuron, a widely used model of synaptic patterning in C. elegans\textsuperscript{16,38}. We developed a platform that incorporates two interconnected sections: one responsible for evacuating progeny to keep the population age-synchronized, and a parallel array of channels for trapping and imaging individual worms (Figure 2.1.a)\textsuperscript{34}. In the first chamber, eggs and larvae are filtered out through hundreds of auxiliary channels surrounding the chamber while retaining the adult population, thus maintaining a
population age-synchronized while avoiding the use of FUdR. These auxiliary channels are shallower than the rest of the chamber in order to prevent the adult worms’ evacuation from the device while eggs are filtered out. Progeny removal is carried out once a day to ensure age-synchronization is maintained throughout the lifespan of the population. In the adjacent chambers, animals are loaded and immobilized for imaging. Three devices with different specifications were fabricated to accommodate the nematodes’ size increase that occurs as they age (Figure 2.1.c), thus avoiding the possibility of worm injury in the system. High-resolution microscopy is performed once every three days by transferring nematodes to the imaging section. Nematodes are trapped individually in imaging compartments specially designed to restrict the motility of worms and favor a dorsal down orientation, thus improving the image quality of DA9 pre-synaptic sites (Figure 2.1.d). Animals are then immobilized by cooling, placing a heat sink on the top of the device. By integrating this platform with a quantitative image analysis algorithm, we were able to quantitatively determine the phenotypic changes in fluorescent tagged presynaptic sites that occur as a result of aging. This novel platform enables high-resolution high-throughput imaging of small cellular and subcellular features as a population ages in a drug free environment. The high-throughput aspect of this platform enabled sampling a large set of animals from a large population, thus acquiring information from a set with a phenotypic distribution representative of the entire population. The integration of quantitative analysis with high-throughput data acquisition enabled extracting rich information from images of sub-cellular features, leading to high-content deep characterization of subtle age-induced phenotypes.
Figure 2.1 Microfluidic platform for high-resolution high-throughput imaging of subcellular aging phenotypes. a) Photograph of microfluidic platform. b) Entire experimental imaging system. Media flow through the device was controlled by a custom-built pneumatic pressure box that regulates pressure in the stock media bottles. Confocal fluorescence microscopy was carried out on an inverted microscope equipped with a motorized stage. c) Nematodes increase in both width and length throughout the aging process. d) Nematodes are usually on their lateral side, which is not a desired orientation for microscopy of synaptic sites located at the dorsal axon. This device favors a desired dorsal-down orientation.

2.3 Results and discussion

To characterize the aging behavior of DA9 dorsal cord synapses at different time-points throughout a population’s lifespan, periodic high-resolution microscopy of an age-synchronized population was performed in the platform. A population of ~1000 worms was cultured throughout its lifespan while progeny were filtered out of the device to keep the population age-synchronized. Once every three days, nematodes were transferred to the imaging section and high-resolution fluorescence microscopy was performed. Images were segmented, quantified, and analyzed to extract the relevant information incorporated in each image.
2.3.1 On-chip high-throughput age-synchronization for drug-free lifelong culture

C. elegans’ lifespan on-chip at 25 °C is ~16 days and they lay eggs for 5 days, from day 2 (egg laying adult) to day 7. It takes 2 days for an egg to reach the egg-laying adulthood phase. Morphological similarities among day 3 and day 5 animals make distinguishing them almost impossible. Thus, aging studies require all individuals within a population to be the same age (i.e., age-synchronized) throughout the assay. To achieve this goal throughout the lifespan of the population, worms were grown in liquid culture flasks for 24 hours at 25 °C and transferred daily to the device for progeny filtration. The main chamber is surrounded by hundreds of auxiliary channels (Figure 2.2.a) which allow progeny removal, thus keeping the population age-synchronized in the absence of FUdR. The auxiliary channels were precisely designed to address the complexity of mechanically filtering progeny, as the width difference between eggs and adult animals is only ~20 µm. In addition, the nematodes’ motility poses an additional difficulty, since they can squeeze through small openings. An ideal sieve should be wide enough to let the progeny pass while also being tapered and long to prevent evacuation of adult animals. Thus, the auxiliary channels surrounding the chamber were designed to be tapered, long, and shallower than the main chamber. This configuration prevents animals from squeezing through while allowing egg removal.
**Figure 2.2** Overview of the platform design specifications. a) The device is made of two main sections: a main chamber and an imaging section. The main chamber is comprised of a chamber surrounded with hundreds of auxiliary channels fabricated to evacuate the progeny and keep the population age-synchronized. 72 traps are designed in the imaging section where nematodes are individually trapped for imaging. b) Detailed specifications of different designs. The auxiliary channels are designed to be shallower than the rest of device and tapered to prevent adult population from exiting the chamber. The imaging channels are also designed to be tapered, lowering the motility of worms. A height restriction at the beginning of channels favored dorsal down orientation. The table is the demonstration of which device was used within each specific period of population’s lifespan. Scale bar is 5 mm.

As previously mentioned, we developed three devices with different dimensions (Devices 1-3) as the size of the animal continues to increase throughout adulthood. As shown in Table 1, several widths and lengths were tested, and the optimal design was selected. The height of the chamber for animals at the earlier stages (Device 1) was fabricated to be 60 µm in height, compared to 100 µm for later stage animals (Devices 2-3). Similarly, the auxiliary channels were 40 and 80 µm in height, respectively. The auxiliary channel entrance width was 45 µm and tapered down to 35 µm at the exit (Figure
2.2.b) in both devices. The channel length was 600 μm to minimize the probability of adult nematodes leaving the chamber and avoiding a significant increase in pressure drop. The first step in the progeny filtration process involved transferring the population from the liquid culture flask to centrifuge tubes, and washing with M9 buffer 2-3 times. This step reduced the amount of progeny to be removed by 40-50%, thus simplifying egg evacuation. The nematodes were then introduced to the main chamber where bidirectional flow from the two ends of the device (Figure 2.3. a-c) flushed the progeny through the auxiliary channels to the outlet connected to a waste container. The filtration process was continued until all eggs and larvae were completely removed from the sample, as evaluated by visual inspection. Worms were then either transferred to the imaging section or back to the culture flasks depending on the upcoming task.
Figure 2.3 Operational loop of the platform. a) There are 2 operational regimes carried out, depending on the desired task. Daily progeny evacuation was carried out once a day while nematodes were reproducitively active. Nematodes were flown into the main chamber, eggs and larvae were evacuated, and the population was evacuated from the platform and transferred to the culture media. Complete imaging cycles were performed on day 3, 6, 9, and 12 when periodic microscopy was scheduled. b) Population in the main chamber during progeny evacuation. Larvae and eggs are evacuated from platform. c) Progeny evacuation (red arrow shows progeny getting evacuated). d) Nematodes were transferred to the imaging section and trapped in each imaging compartment. e) Worms loaded in each trap. Scale bars are 2, 1, 2, and 0.5 mm, respectively.
2.3.2 Loading and trapping nematodes for high-resolution imaging

High-resolution microscopy of subcellular structures such as synaptic puncta, requires minimal movement of the sample. Worm locomotion complicates the process of high-resolution imaging, since small displacement results in poor quality, unreliable images. To decrease their motility, animals were individually directed to separate compartments that restrict their movement. The size of the nematodes plays a crucial role in designing these channels, since loading efficiency (number of worms loaded/ number of channels) decreases drastically if the channels are either larger or smaller than the worms. As previously mentioned (Figure 2.2.b), three different designs were fabricated to address
the size increase of nematodes as they age. Microscopy performed at days 3 and 6 were performed in Device 1, while Devices 2 and 3 were used at days 9 and 12 respectively.

Animal orientation during imaging is crucial due to the anatomical location of the structure of interest. Nematodes are usually on their lateral side, particularly while crawling on solid media. However, as shown in Figure 2.1.d, the optimum orientation to image synapses located at the dorsal nerve cord is with the dorsal side close to the glass slide. In the case of wrong orientation (ventral side down), the light must travel through the tissue to reach the synapses at the dorsal side, making imaging unfeasible. To address this issue, we incorporated a height restriction (Figure 2.2.b) at the beginning of each imaging channel which increases the probability of positioning the animals at the desired orientation and helps push animals towards the coverslip.

Loading occurs as a result of a pressure gradient across the two ends of the imaging channels. Continuous flow through the imaging section and each channel created the hydrodynamic force needed to maintain the animals in the compartments as microscopy took place (Figure 2.3.a, d, e). Different designs and iterations were tested to optimize the loading parameters which include: loading efficiency, orientation, and escape prevention. The platform with optimized specifications achieved a consistent ~90-95% loading efficiency (Table 2). In addition, the height restriction at the beginning of each channel and the width reduction (8 µm) at the end of the imaging compartments, led to a better orientation and fewer worms leaving the imaging compartments during microscopy compared to channels with wider ends and no height restriction.
Table 2. Loading efficiency of imaging section in different runs.

<table>
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<tr>
<th>Trial</th>
<th>Loading efficiency (%)</th>
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<tr>
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2.3.3 Temperature induced immobilization

As previously mentioned, the tapered imaging channels restricted the motility of the nematodes to a certain extent. However, to perform high-resolution microscopy, complete immobilization is required. C. elegans can be immobilized by reducing the surrounding temperature. To decrease the temperature in the imaging channels, a dry ice pellet was placed on the top of the platform as a heat sink after loading and trapping the nematodes. To ensure that immobilization has taken place as result of temperature reduction and not carbon dioxide diffusion, the PDMS surface was covered with a glass coverslip, thus preventing carbon dioxide permeation (Supplemental Figure 2.1.a). The temperature in the imaging section can be manipulated by the flowrate of room-temperature media introduced to the channels, since it counteracts the effect of dry ice. Higher flowrates lead to shorter fluid residence time, reducing the amount of heat removed from a certain control volume. On the other hand, media with a lower flowrate has more time to be in contact with the cooled PDMS surfaces, allowing more heat to be removed and lowering the temperature. As shown in Supplemental Figure 2.1.b, the flow rate was initially high, and reduced step-wise until immobilization occurred. Higher flowrates were used in the initial steps to avoid freezing. After each flow reduction step, the nematodes’ motility was
assessed, and flow was reduced further if necessary. The flowrate was decreased until full immobilization of nematodes was observed. In this way, we achieved simultaneous drug-free immobilization of up to 72 nematodes.

2.3.4 Evacuation

After immobilization and microscopy, nematodes can be retrieved and evacuated from the imaging channels as well as the platform. Dry ice was removed from the platform after imaging was completed to raise the temperature. Nematodes were kept in the imaging channels for a few minutes until they regained motility. To evacuate worms, a backward flow was introduced (Figure 2.3.a) to direct the nematodes out of the imaging channels to the main chamber and then out of the device to a collection vial. The population was then resuspended in the liquid culture flask and incubated at 25 °C.

2.3.5 Analysis of drug-free synaptic aging

To track how aging alters synaptic sites, 4 microscopy sessions were conducted at days 3, 6, 9, and 12. The strain used in this experiment (XA7810), has a transgene that expresses RAB-3 tagged with GFP. The rab-3 gene encodes a synaptic vesicle membrane protein that localizes to presynaptic sites. Hence, this fusion protein allows for characterization of the morphology of presynaptic sites, and in this case, to assess the effects of aging on presynaptic patterning. Some of these age-induced phenotypes can be identified by visual inspection. However, a large amount of changes exhibited by such small (~1 μm) structures are expected to be subtle, making in-depth quantitative analysis of multiple descriptive metrics necessary. Computer vision made identifying and tracking subtle age-induced changes that the synaptic domain undergoes possible by detecting synapses, extracting quantitative metrics, and comparing these measurements at various
time points. The relatively higher sample population accompanied by the unbiased analysis algorithm used in this study resulted in high accuracy and confidence in recognizing subtle phenotypes that arise as the population ages. Different aspects of synaptic aging were studied by extracting over 60 metrics from the images (Supplemental information). These metrics mainly unveil how the size, intensity, density, and distance of synapses change as nematodes age. Here, both qualitative and quantitative aspect of presynaptic aging have been studied.

2.3.6 Qualitative analysis of synaptic changes

The images acquired of the synaptic domain revealed several changes in synapse morphology as nematodes age. In young individuals, the DA9 synapses are located at a specific segment of the axon at the dorsal side of the tail. Li et al. have previously revealed the migration of presynaptic sites (puncta) toward the dorsal anterior asynaptic region, the commissure, and even to the dendrite in aged individuals. The images taken from 12-day old nematodes in the device (Figure 2.4.a) also showed this phenotype. Sequential analysis of synaptic aging revealed an increase in puncta irregularities, such as mis-localization of puncta and less uniform puncta orientation. As highlighted in Figure 2.4.b (red boxes), small unorganized synaptic sites tend to form in the vicinity of other synapses, which is not observed earlier in the nematode’s life-span. In addition, the localization of synapses in the axon also underwent changes. In some cases, less uniform distribution and uneven orientation of synaptic vesicles (Figure 2.4.b, cyan boxes) was observed later in life, which suggests that the mechanism responsible for maintaining synaptic puncta location and morphology is impaired. In addition to arbitrary distribution of the vesicles along the axon, a segment of the axon itself tends to bend and form a curve rather than a line in a fraction
of the aged population (Figure 2.4.b, green line). None of the images acquired at earlier stages showed such a phenotype. These irregular axon deflections started in day 6 in a subtler form and progressively aggravated.

![Figure 2.4](image)

**Figure 2.4** High-resolution microscopy of synapses located at the DA9 axon. a) Puncta formation along dorsal asynaptic region, comissure, and dendrites is exhibited in aged individuals (Lateral view, yellow dashed boxes). Migration of synaptic sites toward the cell body and dendrite initiated at day 9 and aggravated by day 12. b) Major morphological changes occur throughout the aging process (dorsal view). Small synaptic sites tend to form in the vicinity of larger sites (Red dashed boxes). Uneven and irregular distribution of sites along axonal cord (cyan dashed box). The axonal cord seems to bend and form a curve in aged nematodes (green line). Scale bar is 50 µm.

### 2.3.7 Image processing and quantitative analysis of the data

Neuronal aging can result in drastic morphological changes in neurons, as well as drastic and subtle synaptic patterning and morphology changes. The more evident phenotypes can be identified by qualitatively assessing the data acquired, as previously shown. However, qualitative image analysis and assessment is subject to binarized and biased decision-making, which is inadequate for subtler phenotypes. Most of the age-induced changes in synapses studied in this paper are expected to be very subtle and thus
difficult to identify by visual and qualitative inspection. In addition, phenotypes such as synaptic distribution, localization, and size need unbiased quantitative measurement in order to be compared at different time points. To address these issues, an automated algorithm was exploited to detect synapses and extract descriptive metrics, such as average puncta intensity, puncta size, interpunctal distance, etc.

Detection of synapses was carried out by a machine learning model (Support Vector Machine) developed by San Miguel et al\textsuperscript{49}. This algorithm is a trained model to distinguish synaptic patterns from auto-fluorescence and background noise (Supplemental Figure 2.2). In addition to the raw image, additional input information required for the model to distinguish the synapses includes 25 different filtered images (such as a low pass Gaussian, Laplacian, average, etc.). These filters are used to enhance different aspects of the image, enabling the model to determine whether a pixel is part of a synapse or not. The segmented images were then used to evaluate the metrics discussed earlier. More than 60 metrics were calculated that describe how synaptic size, intensity, density etc., change over time.

2.3.8 Average puncta size, mean of average puncta intensity, and puncta density

The average puncta size is an indirect measurement of the synaptic sites available at presynaptic domain and is measured by the number of pixels it occupies. The average puncta size of the population imaged exhibited an overall increase through time (Figure 2.5.a, b). The puncta enlargement started between days 6 to 9 and was stabilized with minor change from day 9 to day 12. There is a significant increase of mean size between the days 3 and 12 (P<0.001), suggesting an accumulation of synaptic vesicles. In parallel with puncta size growth, puncta fluorescence intensity changed in a similar fashion as puncta size, showing an evident increase between days 6 and 9 (Figure 2.5.a, b). The increase in
intensity of synaptic puncta can be an indication of more vesicles available at presynaptic sites, which hypothetically could lead to more neurotransmitter available. However, studies have shown that both the locomotion rate and synaptic transmission declines in aged worms due to a reduction in kinesin-driven vesicle trafficking (reduced unc-104 expression)\textsuperscript{14,16}. This is counter-intuitive with a potential increased concentration of the vesicles at the domain. The accumulation of vesicles can be as result of a reduction in the rate of vesicle recycling to the cell body as nematodes age, which would lead to vesicle build up in presynaptic compartments. In this scenario, the reduction in locomotion could be caused by fewer neurotransmitter-filled vesicles delivered to the presynaptic sites, or a decline in postsynaptic receptor function. However, it is also possible that the observed intensity increase is a result of transgene over-expression. Further tests with endogenously labelled strains will enable testing this hypothesis.
Our data suggests that the synaptic density (number of puncta / total distance of synaptic domain) is reduced in the aged population (Figure 2.5.a, b). This data recapitulates previous results where an aging-induced decrease in synaptic density was concluded to be a result of lower unc-104 neuronal expression16. The lower density could
also be caused by the animals’ size increase, assuming this results in an extension of the axon greater than the addition of synaptic sites.

### 2.3.9 Overall synaptic degradation

The algorithm used in this study enabled us to quantify and compare over 60 metrics (listed in supplemental information) during the population’s aging process. These metrics describe changes in intensity, size, density, and distance of synaptic sites. To analyze and track the changes throughout the lifespan, a multiple comparison (Tukey-Kramer) one-way ANOVA of each measurement was conducted to extract p-values associated with each day compared to day 3 (Figure 2.6.a). As discussed earlier, average puncta size (metric 2) and mean of average puncta intensity (metric 20) undergo significant change starting from day 9 of adulthood. Puncta density (metric 33) and mean of interpunctal distance (metric 30) on the other hand, remain intact until day 9 and the significant changes start to occur from day 12. Overall, the probability values for the first 3 days of adulthood (days 3-6) imply no major change in synapse morphology. Only 2 metrics showed a significant change (p-value< 0.05) suggesting that the overall synaptic structure in dorsal nerve cord is maintained during that period. The most drastic degeneration occurred between days 6-9, where the probability values of more metrics start to decrease. The shift of probability values to lower values is indicative of a higher chance of exhibiting a structurally different synaptic domain later in life.
Deep phenotyping of subtle changes of the DA9 dorsal axon synaptic sites. a) p-Values calculated from one-way ANOVA comparison to day 3. p-Values were corrected using the multiple comparison method (Tukey–Kramer). b) Heatmap of Z = scores calculated at each time point, using day 3 as the baseline (purple boxes represent metrics discussed in Fig. 5) (brown box represents the metric for mean of the interpunctal distance) (black box represents the metric for maximum integrated intensity).

The Z-score, which is a standardized measurement for mean difference, at day 9 versus day 3 also confirms a major change in average values in some metrics (Figure 2.6.b). Consistent with prior results, a few more significant changes occurred from day 9 to 12, implying that more age-induced phenotypes continued to appear, although at a smaller rate. To identify the metrics with drastic changes, we counted the number of metrics with p>0.05(NS), 0.01<p<0.05(*), 0.001<p<0.01(**), and p<0.001(***) at each time point. As anticipated, day 12 has the most metrics with significant changes. In addition, the number of metrics with drastic changes (p<0.001) is higher at day 12 compared to day 9, which implies even the phenotypes formed by day 9 become bolder (Figure 2.7). For instance, the maximum integrated intensity (metric 28) had already exhibited a significant change at day 9 with a P value of ~ 0.025. The difference became even more drastic at day 12 where the p-value reached 7E-07.
Figure 2.6 Characterization of synaptic aging. Binning of p-values calculated at each time point. p>.05 (NS), 0.01<p<0.05 (*), 0.001<p<0.01 (**), and p<0.001 (***)

2.4 Conclusion

Performing high-throughput high-resolution aging studies on *C. elegans* with a large sample size poses various technical difficulties. In this study, we designed and fabricated a microfluidic platform capable of acquiring high-resolution images of subcellular features in a drug free environment. We were able to accomplish 3 main goals with this platform. First, we kept a large population (~1000 worms) age-synchronized throughout their whole lifespan without the need to expose the population to FUdR, which has been known to result in artificial lifespan extension \(^{20,21}\). In addition, the use of antibiotics to prevent the growth of any external bacteria or fungi was replaced with a 3-step sterilizations technique. Second, ~60-70 nematodes were able to be trapped and immobilized simultaneously per run allowing for the use of microscopy to acquire images of synaptic domain located at dorsal nerve cord. Immobilization was accomplished by
establishing a temperature gradient instead of anesthetic drugs such as Levamisole, which may have a side effect at the synaptic levels\textsuperscript{22,23}. Third, subtle aging-induced phenotypes in the synaptic domain were quantitatively characterized using computer-assisted unbiased image processing tools. This platform will thus enable studying age-induced phenotypes at cellular and subcellular levels in \textit{C. elegans} in a quantitative, drug-free, and high-throughput manner. The throughput of progeny evacuation, trapping, and immobilization can be even improved by automating device scanning for imaging, and by parallelizing multiple devices.

In this work, we focused on high-content characterization of DA9 synapses. This platform will enable future studies to deepen our understanding of neuronal aging. In particular, the use this device can be extended to studying the cause of impaired locomotion and reduced synaptic signaling thoroughly by tagging different proteins involved in synaptic transmission, such as proteins in the pre and post synaptic region. For instance, we expect that trans-synaptic labeling\textsuperscript{8} will enable us to determine whether reduced locomotion is a result of loss neuronal connectivity, which could also explain the increase in synaptic sites we observe. Nonetheless, this platform has wide applicability, as it enables aging studies at cellular and subcellular resolution in a wide variety of cell types. Some examples include age-dependent neuronal degeneration, protein aggregation, muscle deterioration, fat accumulation, and germline health. The results acquired using this platform can be coupled with behavioral analysis to establish a correlation between cellular and subcellular phenotypes with functional outputs, such as locomotion.
2.5 Materials and methods

2.5.1 Device fabrication

To accommodate the change in size as worms age, three different designs with different specifications were created. Two separate molds were fabricated with different feature heights. To fabricate the first layer, SU-8 2025 was spun using a Laurell Spin Coater at 1750 rpm to obtain 40 μm tall features or at 1000 rpm for 80 μm features. To add the second 20 μm tall layer, the alignment marks of the first layer were developed before coating with SU-8 2010 spun at 1350 rpm for both designs. Following photo-lithography, soft-lithography was performed by pouring SYLGARD 184 silicone with 10:1 elastomer to crosslinker ratio on the silanized mold and allowed to cure overnight at 80°C.41,42

2.5.2 Strain, media, culture

The strain used in these experiments (XA7810), kindly provided by Kang Shen, has fluorescent tags in the AWC neuron located in the head and in synaptic sites located at the dorsal cord of the DA9 neuron16,38. The AWC neurons are tagged transcriptionally in red with mCherry (Podr-1::mCherry) and DA9 synapses are tagged translationally in green with GFP (Pitr-1 B::gfp::rab-3). To obtain an aged-synchronized population, eggs were extracted from gravid hermaphrodites using a bleach solution (1% NaOCl and 0.1 M NaOH)43. The eggs were placed on seeded NGM plates upon extraction. Worms were cultured on plate for 2 days (until reaching young adulthood) at 25 °C and then transferred to liquid culture media for the rest of their life44. The liquid culture media was made of E. coli OP50 suspended in S-medium. The bacteria were grown and incubated overnight in LB broth at 37°C. The suspension was centrifuged and LB was removed to acquire bacterial
pellets. The pellets were washed thoroughly by S-medium, resuspended in S-medium, and stored at 4 °C. The culture concentration was diluted to $8 \times 10^8$ cells/ml (OD$_{600}$=1) by adding S-medium. A sterilized 0.01% solution of Triton X-100 (TX-100) in M9 was the media used for device operation. TX-100 was added to M9 as a surfactant to prevent adhesion of nematodes to tubing and the device.

2.5.3 Sterilization

For this device, an effort was made to avoid any drugs that are commonly used in conventional aging studies, such as antibiotics and antifungals used to prevent contamination$^{26,45}$. To exclude drugs from the experiment, the entire process was performed in a sterile environment. To keep the process sterile, the media flown to the device was autoclaved prior to use. In addition, a 3-step sterilization of the tubing, connections, and the device was carried out. The first step was comprised of rinsing the inner parts of the tubing, connections, and the device by flowing 70% ethanol. In the second step, the tubing and device were packed unassembled in an autoclavable pouch$^{46}$. Autoclaving parts separated increased the likelihood of steam penetrating through to decontaminate. The pouch was autoclaved for 30 minutes followed by a 15-minute drying cycle. The pouch was then transferred to Thermo Scientific 1300 Series A2 biosafety cabinet and was UV irradiated for 2 hours$^{47}$. To minimize the chance of contaminating the setup, the pouches were opened prior to the experiments in a biosafety cabinet, and assembly was carried out under the hood. The entire platform was always kept in the biosafety cabinet and was only transferred for experiments. The air flown into the conical tubes was filtered by 0.22 µm syringe filters as an extra precaution.
2.5.4 Microscopy

Daily progeny evacuation was performed in brightfield using a Leica DMI8 inverted scope with a 5x objective. Prior to high-resolution microscopy sessions, worm loading was performed and inspected under a 5x objective to assure proper loading. High-resolution microscopy was carried out using 40x/1.30 oil objective. Confocal fluorescence microscopy was performed with an LDI Laser Launch coupled with a CrestOptics X-Light V2 confocal imager and an Orca-Flash 4.0 digital CMOS camera. The exposure time and laser intensity were kept constant through all experiments at 60 ms and 40% respectively. For each individual nematode, images with 31 slices (Z stacks with 1μm spacing) were acquired with Micro Manager and the maximum projection of these slices was further processed with MATLAB.

2.5.5 On-chip culture

In the first attempt to perform life-long culture of single population in a drug free environment, we sought to culture the worms continuously on-chip where food is being introduced while progeny get evacuated simultaneously from the auxiliary channels. While on-chip culture offers numerous advantages, maintaining a population for ~14-21 days in a microchip is challenging. The major challenges to be overcome during the long-term culture are 1- Maintaining the nematodes well-fed in the main chamber, 2- bacterial aggregation within tubing, and microchannels, and 3- keeping the culture temperature constant. The key to successful long-term on-chip culture is to address these 3 requirements.

2.5.6 Maintaining the nematodes in well-fed in the main chamber

Based on the design of the device, the nematodes were to be cultured in the main chamber and being fed by infusion of bacterial enriched buffer. Successful nutrient delivery greatly depends
on nematodes being maintained only in the main chamber. However, the main chamber is connected to the imaging section where the flow of bacterial enriched media from one of the inlets will scatter the nematodes all through the device. With a dispersed population throughout the device, efficient homogenous food delivery to the individual nematode becomes impossible. In addition, a considerable number of nematodes tend to swim to the outlets if not enforced by flow to stay in the main chamber. In this circumstance, nematodes travel through the tubing and lose their life because of the lack of oxygen and food supply. To remedy this issue and keep the nematodes in the main chamber, we sought to implement two balanced flows entering the main chamber from two ends of it. To ensure that almost equal flowrate enters the chamber from each end, both inlets were connected to a single feed media flask where both are regulated by a same pressure head. In order to maintain equal flowrates in both ends, it is critical to have similar pressure drops within both lines connected to the ends. Thus, we constructed these lines with identical tubing and fittings since a slight difference in each path leads to pressure drop differences that easily shift the nematodes to one side of the chamber and increase the chance of losing them. With this approach, we were able to successfully prevent nematodes from leaving the main chamber and maintain them. In addition, in this work, we implemented overhead air pressure to regulate the flowrate entering the chamber which is unable to deliver consistent and constant flow rate which could be problematic for life-long assays. To further improve this approach, precise liquid handling tools should be integrated to this platform where the dispensing flowrate can be directly measured and regulated.

2.5.7 Bacterial aggregation

The second challenge to overcome while performing life-long nematode cultivation on-chip is bacterial aggregation which can occur in various forms within the tubing, fittings, and the
microfluidic device. The mechanism of creating colonial groups elevates the survival probability of this organism as it faces harsh conditions. Food and pharmaceutical industries suffer from bacterial aggregation formation within their process line\textsuperscript{50}. Despite strict measurements implemented to keep pharmaceutical equipment pathogen free, biofilm is formed in the process lines of these industries. Biofilms and aggregates are formed within the process lines despite the fact that the bacterial concentration present in the media is extremely low. This phenomenon is further exacerbated in our system since the media delivered to the nematodes in the experiments contains orders of magnitude higher OP50 \textit{E. coli} concentration which significantly increases the probability of bacterial aggregation and biofilm formation. Our preliminary tests and observations suggested that the bacterial aggregation can be formed within three different steps of device operation; 1- Bacterial aggregation in the media flask, 2- Bacterial aggregation in the tubing and fittings, and 3- Bacterial aggregation on-chip. It is critical to minimize or eliminate the chance of biofilm and aggregation formation by controlling the factors contributing to this phenomenon. Thus, we sought to identify these factors in each step and develop methods and techniques to optimize them to obtain minimum aggregation formed during culture.

\textbf{2.5.8 Bacterial aggregation in the media flask}

Bacteria tends to aggregate and create clumps in the media flask containing the nutrients required for a population to survive. Our preliminary tests unveiled that the bacterial aggregation gets aggravated in medias possessing higher bacterial concentration. At room temperature where the microfluidic device is to be operated, bacterial aggregation starts to form after 1-2 days of sample preparation. In addition, the bacterial concentration is a function of time where it grows until reaching the saturation point. The increased frequency of aggregate formation in the media as sample gests older suggests that the chance of clump formation increases as the concentration
of bacteria elevates. One of the major reasons of aggregates forming more in concentrated suspensions could be the higher probability of bacterial cells colliding with each other and form clumps while suspended in high concentration media. Based on literature review and preliminary assays, we sought to find the optimum conditions where the bacterial aggregation is minimal throughout the culture by modifying following parameters: 1- Flask storage temperature, 2- Effect of various surfactants and their concentration, 3- Stirring, 4- Bacterial strain implemented, and 5- Dead bacteria.

The first factor we tested was the effect of surfactant on the bacterial aggregation. Two samples one with 0.01% of Triton X-100 and the other one without Triton X-100 were prepared. The results suggested after 2 days incubation of both samples at 20 °C, the media with surfactant exhibited lower frequency of bacterial formation compared to control. This finding was aligned with our hypothesis that presence of surfactant molecules contributes to minimizing clump formation. Next, we sought to implement different surfactants with various chemical properties to investigate the potential distinct effect they could have. Triton X-100, Tween 20, and Nonidet-P40 were three surfactant candidates used for this test. Samples containing these 3 surfactants were incubated at 20 °C for 2 days and results suggested that the one containing Triton X-100 exhibited smaller and less concentrated clumps compared to the other surfactants (Figure 2.8). Considering the positive effect of surfactants on clump size shrinkage, we sought to test higher concentrations of them. Increasing the concentration of the surfactant helped with reducing the aggregation levels. However, the rate at which reduction occurred seemed to reach to a saturation and not change significantly after certain point. In addition, it should be considered that surfactants are external chemical stimulus which in high concentrations may be harmful for nematodes that are continuously up taking the media they swim in. Taking this fact into account, 0.1% (W/V)
surfactant was selected to be the maximum concentration used for various assays.

**Figure 2.8** Flask bacterial aggregation in the presence of different surfactants.

One of the most important factors in bacterial growth rate and aggregation formation is the temperature where the bacterial suspension is stored at. The growth rate of bacteria dwindles by decreasing the temperature where sample is incubated at. To test whether the frequency of bacterial aggregates formation also reduces in flask at lower temperatures, 4 bacterial samples were prepared, and each were stored at 4, 15, 20, and 25 °C and incubated for 2 days. As anticipated, the bacterial aggregation in the batch incubated at 4 °C was considerably lower. The reduced frequency can be due to decreased bacteria growth rate at lower temperature leading to fewer bacterial cells after two days of incubation to form clumps. In addition to environmental factors, the genetics of the bacterial strain used also affects the level of aggregation and biofilm formation. Laganenka et al has demonstrated that the bacterial aggregation can be alleviated by mutating specific genes in *E. Coli*. They have demonstrated that mutating (deleting) specific genes (*flic* or *flu*) decreases the tendency of bacterial cells to form aggregates. These genes are responsible for producing flagellum which regulates locomotion in bacteria. The ability of bacterial cells to migrate toward clumps and aggregate is directly affected by their locomotory capabilities. Thus, the probability of bacterial cells hitting each other and form clumps while they are locomotion deficient is significantly lower. In order to integrate a locomotion deficient line to our assay,
JW1908 which is a strain of *E. Coli* lacking the *flic* gene was purchased from CGSC (Coli Genetic Stock Center) and grown on kanamycin LB plate. Four samples of JW1908 were prepared and incubated at 4, 15, 20, and 25 °C and the results were compared with the OP50 strain as the control. Interestingly, no aggregation was observed after 2 days in the JW1908 sample incubated at 4 °C suggesting that implementing the mutant with locomotory abnormality reduces the chance of aggregation formation in the flask. To put all the factors studied in perspective and identify a comprehensive approach to minimize bacterial aggregation over a certain time-span, we decided to implement the JW1908 strain with 0.1% (W/V) of Triton X-100 incubated at 4 °C which exhibited the least aggregation (Figure 2.9).

![Images showing aggregation formation](image1.png)

**Figure 2.9** delflic defective strain incubated at 4 °C showed lowest aggregation after 3 days. The OP50 strain sample incubated at 20 °C started to have aggregations from day 2 and the aggregates tend to get larger on day 3.
2.5.9 Bacterial aggregation in tubing, fittings, and on chip

Minimizing the bacterial aggregation and clump formation in the food media was the first step toward achieving aggregate free culture. Our preliminary tests suggested that bacteria tends to form biofilms along tubing walls while passing through. Literature suggests that the mechanism of biofilm formation is comprised of several steps. 1- Reversible bacterial attachment 2- Irreversible bacterial attachment and growth 3- Growth and division of bacteria for biofilm formation. 4- Mature biofilm formation 5- Part of biofilm gets dispersed to form new biofilms\textsuperscript{52}. We sought to implement different approaches and techniques to minimize and prevent biofilms and aggregates from forming along the tube based on the mechanism of biofilm generation. The first treatment to minimize bacterial adhesion to surfaces in contact with nutrient media was to prime the tubing, fittings, and the device with Pluronic F-127 (a PEG based co-polymer) to prevent cells from attaching. Our results indicated that surface priming with Pluronic F-127 significantly decreases the on-chip cell adhesion. It also helped with minimizing aggregate formation along the tubing. However, the effect of priming in the tubing was not as significant as it was within microchannels of the device which can be due to the material difference. Aggregation free system was sustainable for about 24 hours with the aid of pre surface priming. However, as time passed, auxiliary channels connected to the main chamber started to get blocked due to bacterial aggregation formation. Small aggregates formed in the tubing were washed into the device and adhere to the auxiliary channels which were designed to be tapered and are \textasciitilde 30-50 microns wide. Observations suggested that the clumps tend to grow as single bacterial cells join the aggregation to form larger aggregates. To circumvent this issue, the flowrate of bacterial media was increased periodically (every 12 hours) to serve as flush regime pushing the aggregates out of the chamber and evacuate them. The increased flowrate partially removed the bacterial aggregates attached to
the auxiliary channels. However, the high flowrates scraped the biofilms formed in the tubing and transferred them to the device causing more drastic clumps forming in the device. Results obtained from different runs of the assays suggested that often the clumps scraped from the tubing entering the device are significantly larger than the width of auxiliary channel which makes proper evacuation impossible. In addition to clogging the auxiliary channels which prevents proper egg evacuation, these clumps can also block the imaging channels during the loading process leading to a significant decrease in loading efficiency. An automated periodic flush system was proposed to be integrated and regularly wash the loose bacterial cells and evacuate them. The media used for this automated flush was S media and different patterns of automated flush were tested to find the optimum program. For instance, the proposed flushing patterns were: 1- 2.5 seconds on /5 minutes off, 2- 5 seconds on /10 minutes off, 3- 5 seconds on / 20 minutes off, 4- 10 seconds on / 20 minutes off, and 5- 10 seconds on /40 minutes off. In addition to various flushing patterns, different flowrates were also tested to find the optimal one leading to minimal aggregation left in the chamber and within the channels.

Our results from number of experiments indicated that the periodic flush helps with keeping the device clean and evacuates the loose aggregations formed in it. However, identifying an optimized set of parameters leading to an aggregation free culture was not achieved. As shown in Figure 2.10 biofilms and bacterial chunks formed in the tubing were washed into the device at different time points clogging the auxiliary channels and blocking the main chamber suggesting that the operational factors studied were not the only driving forces regarding bacterial aggregation. For instance, shear stress is also known to be a factor contributing to bacterial aggregation. The type of aggregation formed in the tubing depends on the shear stress that the bacterial cells experience in the tubing\textsuperscript{53-55}. Rochex et al has reported that bacterial cells under low
shear stress form loose bonds with the tubing\textsuperscript{53}. On the under hand, more durable biofilms are formed while bacterial cells flow under high shear stress. In addition to shear stress, aggregation formation in the tubing seems to reach steady state condition after a while. However, the test conducted in this device have shown that this steady state situation can be perturbed by the flush cycle performed to clean the chamber. To remedy this issue, different designs were considered and tested to minimize the effect of flush perturbation on bacterial aggregation. However, none of them solved the issues associated with bacterial biofilms detach from the tubing wall and flow through the device and microchannels. To fully understand the mechanism behind the bacterial aggregation, more tests should be conducted. Considering the time required for each test and unknown estimate of overall time needed to solve this issue, it was decided to perform life-long off-chip culture where nematodes are cultured at liquid media flasks and transferred to the device on the daily base to evacuate the progeny and keep the population age-synchronized.

\textbf{Figure 2.10} Time points in on-chip culture. The bacterial cells tend to aggregate in the tubing as well as in the device. The bacterial aggregation in tubing is washed to the device and caused major clogging.
2.6 References


49. San-Miguel, A. et al. Deep phenotyping unveils hidden traits and genetic relations in subtle


2.7 Supplementary information

Supplemental Figure 2.1 Immobilization schematic. a) The setup for immobilization process where dry ice is placed on the top of the platform. b) The immobilization process flow.
Supplemental Figure 2.2 Image segmentation and synaptic detection. Maximum projection images taken for each worm are fed to the segmentation algorithm. SVM segmentation algorithm performs unsupervised image processing by exploiting machine learning techniques. The overlay of the original image and the segmented image is shown in the right column. The red segments are the regions that the algorithm eliminated as background or unwanted parts. The yellow regions are the points that algorithm detected as synapses.

2.7.1 Experimental setup

Prior to daily progeny evacuation or periodic microscopy, the platform was transferred from the safety cabinet to the inverted microscope. A custom-made pressure box provided and regulated the pressurized air required to drive the flow through the device. Before introducing the worms to the device, all channels and chambers were completely degassed by flowing M9 with 0.01% of TX-100 at high-flow rates (Supplemental Figure 2.). The bubbles trapped in the auxiliary channels and imaging channels could impact the progeny evacuation and loading efficiency. Prior to transferring the egg-laying population to the device for filtering the progeny,
the clumps formed as a result of eggs attaching to each other were pipetted out to facilitate progeny evacuation by preventing any clogging. To eliminate the probability of carbon dioxide diffusion through the PDMS as dry ice sublimates during microscopy, the top surface of the microfluidic device was completely covered with glass slides. The presence of the coverslips proved that immobilization was temperature induced and not caused by the nematodes’ exposure to carbon dioxide which also causes immobilization but has synaptic level side effects\(^1\). After each run, the containers with worms in them were disconnected from the system and replaced with new conical tubes for the following day’s test. The setup was then transferred back to the biosafety cabinet and UV irradiated for 2 hours to eliminate any contamination on the outer surfaces that could be transferred into the process.

**Supplemental Figure 2.3** Platform degassing. The device is degassed by flowing pressurized air through it. The high-flowrate will be constantly flown until all air bubbles are evacuated.

### 2.7.2 List of metrics extracted for quantitative analysis

1) Number of puncta larger than one pixel. (Number)

2) Average size of all the puncta. (Size)

3) Average size of the puncta larger than one pixel. (Size)

4) \[
\frac{\text{Second central moment of the size of the puncta larger than one pixel}}{\text{Mean of the size of puncta size larger than one pixel}^2}
\] (Size)

5) Number of puncta larger than 8 pixels. (Size)
6) Standard deviation of the puncta size
   Mean of the puncta size 
   (Size)

7) First quartile of puncta size. (Size)

8) Median of puncta size. (Size)

9) Third quartile of puncta size. (Size)

10) 90\textsuperscript{th} percentile of puncta size. (Size)

11) Maximum puncta size. (Size)

12) Mean size of the smallest half of the puncta. (Size)

13) Standard deviation of the size of the smallest half of the puncta
    Mean of the size of the smallest half of the puncta
    . (Size)

14) Mean size of the largest half of the puncta. (Size)

15) Standard deviation of the size of the largest half of the puncta
    Mean of the size of the largest half of the puncta
    . (Size)

16) 90th percentile of the puncta size
    First quartile of the puncta size
    . (Size)

17) Mean size of the largest half of the puncta
    Mean size of the smallest half of the puncta
    . (Size)
18) Mean of average puncta intensity (average puncta intensity refers to the mean pixel intensity value for each puncta). (Size)

\[ \text{Mean of average puncta intensity} \]

19) \( \frac{\text{Standard deviation of average puncta intensity}}{\text{Mean of average puncta intensity}} \). (Size)

20) Mean of integrated puncta intensity (integrated intensity refers to the sum of all pixel intensity values for each puncta). (Intensity)

\[ \text{Mean of integrated puncta intensity} \]

21) \( \frac{\text{Standard deviation of integrated puncta intensity}}{\text{Mean of integrated puncta intensity}} \). (Intensity)

22) \( \frac{\text{Second central moment of integrated puncta intensity}}{(\text{Mean of integrated puncta intensity})^2} \). (Intensity)

23) Minimum of integrated puncta intensity. (Intensity)

24) First quartile of integrated puncta intensity. (Intensity)

25) Median quartile of integrated puncta intensity. (Intensity)

26) Third quartile of integrated puncta intensity. (Intensity)

27) 90th percentile of integrated puncta intensity. (Intensity)

28) Maximum of integrated intensity. (Intensity)

29) Total distance of synaptic domain (computed by adding distance of individual interpunctal segments larger than 3 pixels). (Distance)

30) Mean interpunctal distance (ignoring segments smaller than 3 pixels). (Distance)
31) Standard deviation of interpunctal distance (ignoring segments smaller than 3 pixels) 
   - Mean of interpunctal distance (ignoring segments smaller than 3 pixels) 
   . (Distance)

32) 90\textsuperscript{th} percentile of interpunctal distance (including all segments). (Distance)

33) Density, computed by: 
   \[
   \text{Density} = \frac{\text{Number of puncta larger than 1 pixel}}{\text{Total distance of synaptic domain (ignoring segments smaller than 3 pixels)}} 
   \]
   . (Density)

34) Percentage of puncta smaller than 15 pixels and larger or equal than 10 pixels. (Size)

35) 95\textsuperscript{th} percentile of interpunctal distance (including all segments). (Distance)

36) Third quartile of interpunctal distance (including all segments). (Distance)

37) Mean of average interpunctal intensity \textit{(interpunctal intensity refers to the mean pixel intensity value for each interpunctal segment)}. (Intensity)

38) \[
   \text{Standard deviation of interpunctal intensity} \quad \frac{\text{Mean of interpunctal intensity}}{} 
   \]
   . (Intensity)

39) 10\textsuperscript{th} percentile of puncta size. (Size)

40) 10\textsuperscript{th} percentile of integrated intensity. (Intensity)

41) Fraction of puncta pixels with intensity larger or equal than 500 and smaller than 1000. (Intensity)

42) Fraction of puncta pixels with intensity larger or equal than 1000 and smaller than
1500. (Intensity)

43) Fraction of puncta pixels with intensity larger or equal than 1500 and smaller than 2000. (Intensity)

44) Fraction of puncta pixels with intensity larger or equal than 2000 and smaller than 2500. (Intensity)

45) Fraction of puncta pixels with intensity larger or equal than 2500 and smaller than 3000. (Intensity)

46) Fraction of puncta pixels with intensity larger or equal than 3000 and smaller than 3500. (Intensity)

47) Fraction of puncta pixels with intensity larger or equal than 3500 and smaller than 4000. (Intensity)

48) Range of puncta pixel intensity values (computed by subtracting the dimmest pixel value from the brightest pixel value). (Intensity)

49) \[
\frac{\text{Standard deviation of puncta pixel values}}{\text{Mean of puncta pixel values}}.
\] (Intensity)

50) First quartile of standardized pixel values (standardized pixel values refers to the intensity pixel values where the value of the dimmest pixel has been subtracted). (Intensity)

51) Median of standardized pixel values. (Intensity)

52) Third quartile of standardized pixel values. (Intensity)
53) 90\textsuperscript{th} percentile of standardized pixel values. (Intensity)

54) Fraction of pixels with standardized intensity values smaller than 0.1(Range of pixel values). (Intensity)

55) Fraction of pixels with standardized intensity values smaller than 0.25(Range of pixel values) and larger than 0.1(Range of pixel values). (Intensity)

56) Fraction of pixels with standardized intensity values smaller than 0.5(Range of pixel values) and larger than 0.25(Range of pixel values). (Intensity)

57) Fraction of pixels with standardized intensity values smaller than 0.75(Range of pixel values) and larger than 0.5(Range of pixel values). (Intensity)

58) Fraction of pixels with standardized intensity values smaller than 0.9(Range of pixel values) and larger than 0.75(Range of pixel values). (Intensity)

59) Fraction of pixels with standardized intensity values smaller than 0.95(Range of pixel values) and larger than 0.9(Range of pixel values). (Intensity)

60) Number of puncta larger than 0.25(Range of puncta size); where range of puncta size is computed by subtracting the smallest puncta size from the largest puncta size. (Number)

61) Total integrated intensity: sum of all puncta pixel intensity values. (Intensity)
2.7.3 Reference

Chapter 3: Deep learning-enabled phenotyping reveals distinct patterns of neurodegeneration induced by aging and cold-shock

3.1 Abstract

Background: Access to quantitative information is crucial to obtain a deeper understanding of biological systems. In addition to being low-throughput, traditional image-based analysis is mostly limited to error-prone qualitative or semi-quantitative assessment of phenotypes, particularly for complex subcellular morphologies. PVD neuron in *C. elegans*, which is responsible for harsh touch and thermosensation, undergoes structural degeneration as nematodes age. Traditionally, investigating the bubble-like neurodegenerative protrusions forming along PVD is labor-intensive, time-consuming and limited to qualitative assessments.

Results: In this work, we apply deep learning to perform quantitative image-based analysis of complex neurodegeneration patterns exhibited by the PVD neuron in *C. elegans*. We apply a Convolutional Neural Network algorithm (Mask R-CNN) to identify neurodegenerative subcellular protrusions that appear after cold-shock or as a result of aging. A multiparametric phenotypic profile captures the unique morphological changes induced by each perturbation. We identify that acute cold-shock-induced neurodegeneration is reversible and depends on rearing temperature, and importantly, that aging and cold-shock induce distinct neuronal beading patterns.

Conclusion: The results of this work indicate that the deep learning technique implemented is capable of tracking subtle morphological and structural variations occurring at the PVD neuron in an accurate high-throughput and unbiased manner. This method can be used to further elucidate and study the parameters involved in orchestrating the neurodegeneration as well as identifying new potential stressors that degenerate PVD.
Keywords: Deep Learning, Convolutional Neural Network, Neurodegeneration, High-throughput quantitative data analysis, Machine Learning

3.2 Background

Aging, environmental stressors, and injury can induce reversible or irreversible changes at the subcellular, cellular, and tissue levels of an organism\textsuperscript{1,2,11,3–10}. The \textit{Caenorhabditis elegans} nervous system is not an exception and undergoes morphological and functional deterioration under these conditions. Morphological phenotypes indicative of neurodegeneration in this roundworm include somatic outgrowth, distorted soma, branched and wavy dendrites, and dendritic beading\textsuperscript{2,7,12–18}. The ability of neurons to recover from degeneration has also been studied. For instance, Oren-Suissa et al. found that primary dendrites in the PVD neuron reconnect via branch fusion following laser surgery\textsuperscript{19}. PVD is a widely studied multi-dendritic nociceptor neuron that responds to harsh touch (mechanosensor) and cold temperatures (thermosensor) (Figure 3.1a)\textsuperscript{20–27}. Prior work has identified genetic pathways important for organization of dendritic branches and dendritic self-avoidance\textsuperscript{28–33}. Dendritic organization in PVD is also affected by aging; while young animals have well-organized menorah-like dendritic structures, these tend to be replaced by non-uniform and chaotic outgrowth of dendritic branches\textsuperscript{32}. Recently, Lezi et al. identified the formation of protrusions (or beading) along the dendrites of PVD during aging, through a process driven by the expression of an antimicrobial peptide\textsuperscript{34}.

Characterization of PVD beading has thus far been performed by visual inspection and manual counting of fluorescent images, which is labor-intensive, time-consuming, and does not provide additional information about the observed morphological changes, aside from number of beads. Traditional image processing approaches typically rely on intensity difference for image segmentation\textsuperscript{35–37}. The protrusions that appear in PVD have fluorescence intensities similar to the
rest of neuron and autofluorescent lipid droplets. Thus, traditional image processing approaches are unable to perform the challenging segmentation of PVD protrusions. Quantitative analysis of PVD neurodegeneration morphology is important to understand the root causes of neurodegeneration. Machine learning has proven useful for analysis of biological systems and deep phenotyping. In this work, we sought to integrate cutting-edge deep learning approaches to segment beads in PVD fluorescence images from live animals (Figure 3.1b). Convolutional Neural Networks (CNNs) have recently shown state-of-the-art performance in image segmentation tasks across a wide range of biological and biomedical images datasets. Here, we utilize Mask R-CNN, a CNN model that is designed to predict binary instance masks (one mask per predicted bead object) from an image to detect PVD beads. We follow this user-free segmentation approach with multiparametric phenotyping of PVD by extracting 46 quantitative features that describe beading patterns. These metrics include number of beads, cumulative area occupied by beads, average bead size, average pair-wise inter-bead distance, etc. We take advantage of the quantitative data provided by this pipeline to track subtle neurodegenerative phenotypes caused by different physiological stressors (Figure 3.1c). We validate our pipeline by assessing the effects of aging on PVD beading, and recapitulate previously observed changes. In addition, we identify a previously unknown degenerative effect of exposure to acute cold-shock on neuronal structure. Finally, we show that this deep phenotyping approach enables predicting the biological status of a nematode (young, aged, cold-shocked) based on the quantitative metrics generated by the pipeline with over 85% accuracy. This analysis reveals that different stressors (aging and cold-shock) induce distinct neurodegenerative phenotypes hinting at potentially different underlying neurodegeneration mechanisms. This approach enabled automating image analysis of PVD neurodegeneration thus increasing throughput, eliminating the human bias and error introduced by
manual assessment, and facilitated high content quantification of the subtle neurodegenerative changes in PVD, unfeasible in conventional methods.

Figure 3.1 Quantitative analysis of PVD neurodegeneration by deep learning. a) Schematic of PVD neuron with menorah-like dendritic branches. Fluorescence images of PVD anterior and posterior to the cell body. b) Schematic of quantitative analysis pipeline to study PVD neurodegeneration. c) Aging and acute cold-shock induce neurodegeneration on PVD dendrites. These two stressors increase the formation of bubble-like protrusions along the dendritic arbors of PVD.

3.3 Results and discussion

3.3.1 Training the Mask R-CNN algorithm to perform complex image segmentation

We adapted the convolutional neural network (CNN) model Mask R-CNN\(^{48}\) to automatically detect bead protrusions in high resolution images of nematode dendrites (Figure 3.2a). The input to Mask R-CNN is a 1-channel grayscale microscopy image (1024 × 1024 × 1) and the output is a set of predicted bead regions consisting of one binary instance mask (1024 × 1024 × 1) per bead, i.e., a pixel has a value of 1 in the mask when it is part of a bead and 0
otherwise. A tiling procedure was employed to adapt Mask R-CNN for use with 2048 × 2048 × 1 microscopy images (see Methods and Materials), since this image size was sufficient to resolve the smallest bead protrusions. The Mask R-CNN architecture first generates regions of interest (ROIs) using a Faster R-CNN model, composed of a residual network (ResNet-101) and a feature pyramid network. ROIs are then processed with region proposal and ROI align neural network layers to produce an instance segmentation mask for each detected object. In contrast to thresholding based methods, which only rely on image intensity for predicting segmentations, CNNs automatically learn and then use hierarchical sets of image features directly from the training data without requiring manual feature engineering. Learning features enables relevant local context to be used in making segmentation predictions, e.g., the shape and size of the bead, what a dendrite looks like, and the proximity of beads to dendrites. We leveraged a transfer learning approach in which Mask R-CNN is pre-trained on a large annotated dataset (ImageNet), and then fine tuned on a data set of nematode images that we manually annotated.

The Mask R-CNN algorithm requires a training data set comprised of raw images of PVD and their corresponding ground truth masks that label the protrusions. The masks were created from raw images using a custom MATLAB code that allows the user to draw around each bead location. A total of 19 images (each with ~50-150 beads with an average size of ~150 pixels) were manually segmented to compile the training set. In addition, an independent validation set was generated with 12 raw images and their associated binary masks. The validation set includes diverse images with ~30 to ~150 beads. These were equally split into images with a low (<100) and a high (≥ 100) number of beads, to test segmentation consistency. To assess segmentation performance, we quantified precision and recall, described as:
Precision = \frac{True Positive}{True Positive + False Positive}
Recall = \frac{True Positive}{True Positive + False Negative}

In these expressions, true positives are correctly identified beads, false positives are non-bead objects identified as beads, and false negatives are non-identified beads (Figure 3.2b). As shown in Figure 3.2c, the segmentation precision for the validation data set was 85% and 91% for images with low and high bead numbers, respectively. Similarly, a recall of 90% and 93% was obtained for low and high bead number images, respectively (Figure 3.2d). These slight differences could stem from the low number of beads while retaining the same level of objects that can be falsely identified as beads in the first group. The optimized Mask R-CNN algorithm successfully scored 88% in precision and 91% in recall for the entire validation set. Thus, this machine-learning approach offers consistent unbiased segmentation with high accuracy.

Importantly, precision and recall do not provide information to assess the performance of the model in ignoring objects that can easily be identified as beads (true negatives). In this particular phenotyping problem, this type of objects are prevalent. Autofluorescent lipid droplets can be easily mistaken for neurite protrusions, due to their round shape and location, which can overlap with PVD dendrites in maximum projections. Distinguishing round objects with comparable intensity levels and with similar locations and sizes is a significant challenge. To assess the power of the algorithm to distinguish between the two, we chose 3 images from animals with an abundance of fat-droplets that overlapped with dendrites, as part of our training set. As shown in Figure 3.2e, the algorithm is successful in discerning fat droplets from beads, despite their similarities. Prior approaches have addressed this problem by performing dual color microscopy to compare images that show only lipid droplets with images that show the fluorescent
This deep learning approach eliminates the need to perform alternative analyses or dual color microscopy to subtract autofluorescent objects.

Figure 3.2 Deep learning approach successfully identifies beads in the PVD neuron. a) Schematic of segmentation pipeline. Raw 2048×2048 images are fed to the trained Mask R-CNN model to perform instance segmentation. Yellow arrows point to neuronal beads. b) Illustration for true positive, true negative, false positive, and false negative cases used to quantify the performance of instance segmentation. c-d) The performance of the algorithm was examined by defining precision and recall of segmentation where 12 validation images were used. Error bars are Standard Error of Mean (SEM). e) Images showing the algorithm successfully distinguishes bubble-like protrusions (beads) from fat droplets.

3.3.2 Deep phenotyping of age induced PVD neurodegeneration

The nervous system in C. elegans undergoes morphological and functional decline due to aging\cite{14,18}. Morphological changes in PVD include dendritic outgrowth and beading, which become more common as animals age, as evidenced in Figure 3.3a. As previously mentioned,
quantitatively investigating beading is difficult as animals can exhibit tens to hundreds of beads with fluorescence intensity levels similar to those of labeled neurons and autofluorescent lipid droplets. Moreover, beading is a highly variable process, and quantification thus requires analysis of large animal populations. We first aimed to quantitatively analyze aging-induced beading in PVD using the deep learning pipeline. Our results (Figure 3.3b) show that the average bead count increases from days 2 to 4 and 6 of adulthood. Interestingly, the average number of protrusions does not appear to change significantly afterwards. These results suggest that there may be a saturation point for the beading process, which animals reach at mid-age.

One of the advantages of computer-based image segmentation is that quantification of beading neurodegeneration is not limited to the number of beads. Our post-segmentation MATLAB pipeline enabled extracting additional metrics (a total of 46, Supplemental information) to comprehensively describe the morphological neurodegeneration phenotypes. The average bead size (Figure 3.3c) seems to decrease slightly as animals age (days 6-12 vs. days 2-4), which can be explained by an increase in percentage of small beads (area < 100 pixels) (Figure S3.1b). While the size is slightly reduced, the total area occupied by beads increases as nematodes age (Figure S3.1a). These results suggest that the main morphological change induced by aging is an increase in total beading (as measured by number or total bead area), rather than in bead size. The average inter-bead distance (i.e., average of all pairwise distances) describes how dispersed the beads are, and decreases in older populations as expected due to an increase in total number of beads (Figure 3.3d). Other metrics that describe bead size and spatial bead distribution (such as 90th percentile of bead size, and percentage of pairwise inter-bead distances < 300 pixels, Figures S3.1d-e) confirmed an overall trend towards accumulation of smaller beads with increased density throughout the neuron in older animals.
To deepen our understanding of aging-induced beading, we compared the patterns exhibited anterior (towards the head) and posterior (towards the tail) to the PVD cell body, since separate images were acquired (Figure 3.3a). While both regions exhibit an increase in number of beads (Figure 3.3e), this change was more drastic in the anterior section. This difference could be explained by either a higher susceptibility to beading or by the fact that the anterior region occupies larger area, since the posterior is closer to the animal’s tail and is thus more tapered. The average inter-bead distance in the posterior region tends to be larger than in the anterior side (Figure 3.3f), as would be expected for a reduced number of beads. As shown in Figure S3.1f, bead morphology appears to be homogeneous, as there is no significant difference in anterior vs. posterior average bead size. Metrics such as the percentage of small beads (< 100 pixels) or the percentage of beads with close neighbors (pairwise inter-bead distances <300 pixels) did not show any significant differences along the two different sections of PVD (Figure S3.1g-h). This deep learning – based analysis corroborates the neuronal beading reported by Lezi et al., while deepening our understanding of the subtle neurodegenerative patterns that result from aging.
Figure 3.3 Deep learning allows quantitative analysis of aging induced morphological changes in PVD. a) Qualitative inspection of PVD at 3 time points of their life-span shows an increase in number of beads throughout the dendrites. Protrusion formation was identified in both anterior and posterior parts of the PVD neuron. Yellow arrows point to neuronal beads. b-d) Average number of beads, average of mean bead size, and average inter-bead distance of both anterior and posterior regions of PVD throughout aging. Lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. e-f) Average number of beads, and average inter-bead distance of anterior versus posterior regions of PVD throughout the aging process. Error bar is SEM.
3.3.3 Acute cold-shock induces neurodegeneration in PVD neuron

In addition to sensing harsh touch, PVD acts as a thermosensor activated by cold temperatures\textsuperscript{53}. Cold-shock has been previously studied as a stressor for \textit{C. elegans}\textsuperscript{53,54,63,64,55–62}. Robinson et al. identified that animals can survive short (4 hrs.) exposures to acute cold-shock (2 °C), but longer exposures (24 hrs.) result in death for a fraction of the population\textsuperscript{65}. Furthermore, Ohta et al. showed that the pre cold-shock culture temperature is inversely correlated with survival rate (more animals survive cold-shock if previously cultured at lower temperatures)\textsuperscript{66}. While the detrimental effects of cold-shock on nematodes’ survival and PVD’s involvement in responding to cold temperatures have been independently studied, the impact of cold-shock exposure on PVD health has not been investigated. To answer this question, we first tested the effects of exposure to cold-shock on PVD morphology, where we identified the appearance of PVD neurite beading. Thus, we sought to examine the neurodegenerative effects of acute cold-shock at 4 °C through our deep learning phenotyping pipeline.

To characterize the relation between cold-shock and beading, we first exposed different \textit{C. elegans} populations to cold-shock for various durations. As shown in Figure 3.4a, eggs extracted from gravid hermaphrodites were transferred to NGM plates and cultured at 20 °C until day 2 of adulthood, when pre-cold-shock microscopy was performed. Nematodes were then split into four separate plates and transferred to 4 °C for either 4, 8, 16, or 24 hrs. Visual inspection of raw images suggested beading increases with longer cold-shock, but is especially evident in populations that were exposed for 16 hrs. or more. Quantitative analysis performed using the trained Mask R-CNN and post segmentation feature extraction pipeline shows that the number of beads gradually increases with longer periods of cold-shock (Figure 3.4b), and is almost doubled after 16 hrs., as compared to non-exposed animals. Similar to the aging process, beading reaches a saturation point,
where no significant change in the number of beads is observed after 16 hrs. Interestingly, the percentage of small beads (area < 100 pixels) increases after 4 and 8 hrs. of cold-shock, but this effect is not observed after 16 and 24 hrs. (Figure S3.2b). This suggests that new small beads are generated in the first 8 hours, resulting in a higher percentage of smaller beads. The drop in percentage of small beads after 16 and 24 hrs. could be due to existing protrusions becoming larger once the number of beads saturate. This fluctuation in percentage of small beads is also reflected in the average size (Figure 3.4c), which slightly decreases during the first 8 hours of cold-shock and grows after 16 and 24 hrs. One potential explanation for these observations is that initially new small beads form, but eventually the beading mechanism switches to bead growth rather than bead generation.

Computer-based image processing and quantitative analysis also enabled identifying subtle differences between aging and cold-shock beading patterns. While an increase in bead number was observed in both cases, cold-shock resulted in an increase in average inter-bead distance (Figure 3.4d), in contrast to aging. This counterintuitive result can potentially be explained by the tendency of cold-induced protrusions to form in more distant dendrites (such as 3rd or 4th order branches) of healthy menorahs. With aging, beads are generated evenly throughout the entire neuron, likely as a result of the aging-induced disorganized branching that increases the density of dendrites (where beads are formed) throughout the worm’s body (Figure 3.4e). The information extracted from anterior and posterior regions of PVD for nematodes exposed to acute cold-shock shows very similar patterns to aging-induced neurodegeneration. The number of beads in the anterior part is greater than in the posterior side (Figure 3.4f) and the beads are on average farther apart in areas closer to the tail (Figure 3.4g). Bead size appears to be homogeneous in both sides (Figure S3.2f), while the anterior region has a slightly higher percentage of small beads (area < 100 pixels) (Figure
Utilizing this deep learning quantitative phenotyping enabled the identification of a previously unknown effect of acute cold-shock on PVD degeneration, which is exacerbated with longer exposures. Moreover, this analysis suggests that beading patterns differ for aging and acute cold-shock, suggesting potentially different mechanisms of protrusion formation.
Figure 3.4 PVD neuronal structure undergoes morphological changes upon exposure to acute cold-shock. **a)** Schematic of acute cold-shock assay. Nematodes were cultured at 20°C until day two of adulthood, split into four plates, and cold-shocked for 4, 8, 16, or 24 hours. Fluorescence microscopy was conducted before exposure to cold-shock and after specific periods of shock. **b-d)** Average number of beads, average of mean bead size, and average inter-bead distance of anterior and posterior regions of PVD as nematodes experienced various duration of cold-shock. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. **e)** Illustration of distinct beading patterns in aging and acute cold-shock based on the inter-bead distance. Inter-bead distance decreases with aging while it increases with cold-shock. **f-g)** Average number of beads and average inter-bead distance of anterior versus posterior parts of PVD upon cold-shock. Error bar is SEM.
3.3.4 Post cold-shock recovery can eliminate PVD dendritic protrusions

Given the significant increase in number of dendritic protrusions in PVD upon exposure to acute cold-shock, we next sought to determine its potential for regeneration. To test this hypothesis, we designed experiments to characterize PVD beading patterns after acute cold exposure and following a subsequent period under normal culture conditions (referred to as rehabilitation or recovery). As shown in Figure 3.5a, we performed 3 one-day rehabilitation regimes at 3 different temperatures, selected to cover the entire physiological range (15, 20, and 25 °C). Given that nematodes growth rate and life-span depend on culture temperature, we expected the population cultured at 25 °C to show a faster recovery rate than those grown at 15 °C. After exposure to 16 hrs. of acute cold-shock, the average number of beads increased by 100% as compared to pre cold-shock conditions. After one day of rehabilitation we observed a decrease in the number of dendritic protrusions in all three rehabilitation temperatures (Figure 3.5b). As expected, populations cultured at 15 °C and 25 °C had the lowest (~30%) and highest (~50%) recovery, respectively, suggesting that recovery rate is correlated with growth rate.

In addition to a reduction in number, the average bead size slightly decreases after rehabilitation (Figure 3.5c and Figure S3.4). This recovery is corroborated by the total area covered by beads (Figure S3.3a), which increases after cold-shock and decreases in all recovery regimes, indicating that bead formation due to cold-shock is reversible. These results suggest that recovery occurs by both bead elimination and a gradual size reduction. To further understand the spatial patterns of cold-shock bead formation, we also explored inter-bead distances. As previously mentioned (Figure 3.4c), the average inter-bead distance increased post cold-shock, suggesting beads are formed in the farthest dendrites. One-day recovery treatment at all three temperatures reduced this metric (Figure 3.5d), suggesting that beads on the farthest dendrites are more prone
to disappear post-recovery. As expected, the percentage of beads with close neighbors (inter-bead distance < 300 pixels) decreases with cold-shock and increases after recovery (Figure S3.3d). Taken together, these quantitative features suggest that cold-shock induces the formation of beads, particularly in distal regions (as the inter-bead distance increases), and that subsequent culture at physiological temperatures reverts these changes.

In line with previous findings, the anterior region of PVD exhibits a higher number of beads than the posterior region, post-rehabilitation. However, recovery does not appear to favor either side, as both areas show a reduction of beading post recovery (Figure 3.5e). Likewise, while the posterior region shows higher inter-bead distances than the anterior region, both exhibit a reduction of inter-bead distance post recovery (Figure 3.5f). The average bead size, percentage of small beads (area < 100 pixels), and percentage of beads with close neighbors (inter-bead distance < 300 pixels) do not show any significant differences between the anterior and posterior regions, either post cold-shock or post recovery (Figure S3.3f-h), for most conditions. This suggests that the propensity of the anterior region to increased beading observed with aging is also observed upon cold-shock and after recovery from cold-shock. Taken together, these results indicate that after acute cold exposure, one day recovery at different temperatures can almost completely alleviate the induced neurodegenerative effects. In addition, this data suggests that a more efficient recovery can be achieved by rehabilitation at higher temperatures. Finally, it appears that cold-shock preferentially induces beading in the farthest dendrites, but these are also preferentially removed during recovery.
Figure 3.5 PVD neurodegenerative phenotypes caused by acute cold-shock are reversible. a) Schematic of post cold-shock rehabilitation treatment assay. Nematodes were cultured at 20 °C until day 2 of adulthood and were exposed to cold-shock for 16 hrs. To perform recovery, the population was split into three plates at either 15 °C, 20 °C, or 25 °C for one day. b-d) Average number of beads, average of mean bead size, and average inter-bead distance of PVD neuron as nematodes experienced cold-shock for 16 hrs. and undergo rehabilitation at 3 different temperatures. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. 

e-f) Average number of beads, and average inter-bead distance of anterior versus posterior regions of PVD as nematodes experienced cold-shock for 16 hrs. and rehabilitation at 3 different temperatures. Error bar is SEM.
3.3.5 Pre cold-shock culture temperature affects neurodegeneration severity

Physiological culture temperature is a key environmental factor that affects development, growth, and life-span in poikilotherms, such as *C. elegans*\(^{55}\). Nematodes habituate to imposed environmental conditions, including temperature\(^{66-70}\). Previous studies have identified that after a 4 °C of cold-shock over 85% of animals cultured at 25 °C die, while most animals cultured at 15 °C survive\(^{66}\). These findings motivated us to investigate whether the pre cold-shock culture temperature plays a role in beading neurodegeneration. To test this, 3 parallel cold-shock/recovery experiments at 3 physiological temperatures were conducted (Figure 3.6a) where populations were cultured at 15, 20, and 25 °C for ~3.5, 2.5, and 1.5 days, respectively. These animals were then exposed to acute cold-shock at 4 °C and subsequently returned for one day to their culture temperature. The difference in culture time prior to cold-shock allowed animals to reach the same developmental stage. Based on prior studies where nematodes cultured at lower temperatures prior to cold-shock have a higher survival rate\(^{66}\), we hypothesized that lower temperatures would result in less severe neurodegeneration than high temperatures.

Post cold-shock behavioral analysis revealed that animals grown at 25 °C were the most affected, as they recovered mobility long after transfer to room temperature (30 – 40 min), while this time was considerably shorter for animals cultured at 15 and 20°C. Once animals started crawling, nematodes cultured at 25 °C moved significantly slower than those cultured at lower temperatures, indicating that worms habituated to a higher temperature undergo a more drastic shock under cold exposure. These observations suggest that a larger temperature gradient between culture and cold-shock results in increased neuronal damage. As shown in Figure 3.6b, the number of beads present after cold-shock and rehabilitation confirm this trend. The average number of beads increases post cold-shock in all samples, with the smallest change for nematodes grown at
15 °C. The mean bead count after cold-shock reaches the same level for samples cultured at 20 and 25 °C, potentially due to beading reaching a saturation point. This upper limit in number of beads was also observed in neurodegeneration caused by aging and in cold-shock exposure for different periods of time. Interestingly, while populations rehabilitated at 15 and 20 °C show a reduction in number of beads, this effect was not present in those recovered at 25 °C. This could be explained by either a delayed or slower regeneration, or an inability to regenerate for animals cultured at 25 °C. Interestingly, in contrast to animals cultured at 15 and 20 °C, the mean bead size slightly decreased after the rehabilitation regime at 25 °C (Figure 3.6c), suggesting that recovery at 25 °C does induce some regenerative effect. The regeneration results observed in animals cultured at 20 °C and recovered at 25 °C (presented in the previous section) support the idea that regeneration at 25 °C is possible, but is likely slower for the population cultured at 25 °C pre cold-shock. Such delayed regeneration could stem from the more drastic difference between the baseline and cold-shock temperature. Finally, these experiments corroborate that cold-shock induced beading occurs in the farthest regions of the neuron, as inter-bead distance increases with cold-shock, and is then reduced after rehabilitation for all culture temperatures (Figure 3.6d). The percentage of small beads (area < 100 pixels) and the percentage of beads with close neighbors (inter-bead distances < 300 pixels) (Figure S3.5b,d) also show a reversal of the cold-shock exposure effect in all three physiological temperatures.
Figure 3.6 Populations cultured at different temperature before being exposed to cold-shock show different susceptibility to neurodegeneration. **a**) Schematic of the experimental setup to study the effect of pre cold-shock cultivation temperature. Animals were cultured at 20 °C until young adulthood and then transferred to 15 °C, 20 °C, or 25 °C for 3.5, 2.5, and 1.5 days, respectively. Cold-shock was then performed for 16 hrs. and rehabilitation was performed for one day at the pre cold-shock temperature. **b-d**) Average number of beads, average of mean bead size, and average inter-bead distance of PVD for populations that undergo cold-shock as described in part a). The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. Statistical analysis was performed with one-way ANOVA followed by Tukey multiple comparison correction. *P<0.05, **P<0.001, and ***P<0.0001. **e-f**) Average number of beads, and average inter-bead distance of anterior versus posterior part of PVD for populations that undergo cold-shock as described in part a). Error bar is SEM.
Consistent with our previous results, the anterior region of PVD showed a higher number of protrusions than the posterior (Figure 3.6e). Both regions, recapitulate the trends observed for pre cold-shock, post cold-shock, and post rehabilitation in the entire animal. The anterior region consistently exhibits ~20-100% higher number of beads than the posterior, with pre cold-shocks showing the largest difference. The average bead size does not show differences between these regions (Figure S3.5f). However, similar to previous experiments, the protrusions are more densely distributed in the anterior part, as is expected for a higher number of beads (Figure 3.6f). The results from this assay support our hypothesis that the culture temperature impacts how nematodes respond to acute cold-shock. Animals cultured at 15 °C exhibited the least neurodegenerative signs and faster recovery, while those grown 25 °C showed more drastic beading and slower rehabilitation rate. This difference in response indicates that the magnitude of the cold-shock (based on the baseline temperature) correlates with the induced neurodegenerative effects through a yet unknown mechanism.

3.3.6 Predicting biological status using deep quantitative classification

The quantitative analysis of beading induced by aging and cold-shock indicate that the induced patterns of PVD degeneration are different. To further investigate the morphological changes observed, we took advantage of the rich information obtained from the Mask R-CNN segmentation and feature extraction pipeline, which includes all 46 metrics. Through visual inspection of the raw images, as well as the quantitative analysis of the beading patterns, it is clear that beading phenotypes cannot be fully described with a single feature, such as number of beads. Furthermore, there is significant variability within a population. As shown in Figure 3.7a, a large fraction of aged animals exhibits less than 70 beads, which is considerably lower than the average of the population and is closer to the number of beads for young individuals. Likewise, some young
animals showed more than 70 beads, which is significantly higher than the average of the population. The same variability was observed in cold-shock experiments, suggesting that the number of beads does not offer a comprehensive description about biological status of a nematode. Combining two metrics such as number of beads and average bead size still does not provide enough information to distinguish between young and aged adults (Figure 3.7a).

Given that beading patterns relay information about the health state of PVD, we reasoned that beading phenotypes could be used to predict the biological state of the animals. To test this hypothesis, we sought to incorporate all 46 metrics extracted from each image in a classification model. In first attempt, as shown in Figure 3.7b, we performed PCA (Principal Component Analysis) on the 46 metrics. Two principal components (PC1 and PC2) explain 46% of the total variance, and are unable to accurately differentiate nematodes from these two stages in their life-span. Thus, we aimed to test the ability of classification models to distinguish young and old nematodes using the metrics extracted from PVD beading patterns. As shown in Figure 3.7c, animals from different groups (e.g. pre and post cold-shock), can exhibit very similar beading patterns. Successful predictive models would prove the presence of subtle neurodegenerative patterns that can only be described using multiple metrics. We first developed a classification model to distinguish young versus old adults. To create a labeled training set, data from the posterior side of PVD for worms younger than 4 days old were grouped together while the second class was comprised of information from nematodes older than 4 days old. An independent validation data set was then generated to test classification accuracy. It should be noted that these two classes are more difficult to distinguish than comparing day 2 vs. day 12 animals (i.e., the youngest vs the oldest samples). We tested four classification algorithms: Subspace Discriminant Ensemble (SDE), Support Vector Machines (SVM), Logistic Regression, and K-Nearest
Neighbors (KNN). Two models, SDE and SVM, achieved both training and validation accuracies above 80%, with the validation accuracy of SDE reaching 90% (Figure 3.7d). For age-based classification, the information acquired from the PVD anterior side was also used to train separate models leading to training and validation accuracies higher than 80% (Figure S3.6a). These results suggest that age-induced PVD neurodegeneration causes subtle morphological changes that can only be captured using quantitative deep phenotyping. Similarly, we tested models for classifying nematodes exposed to cold-shock from those that did not experience this stressor. The training and validation set for this analysis was comprised of data from cold-shock performed at all three pre-cold-shock temperatures and, as shown in Figure 3.7e, ~80% classification accuracy was obtained both in training and validation. Since differences between degenerated (i.e., old or cold-shocked) and healthy (young or non cold-shocked) animals have been shown, it was expected that these populations are distinguishable. However, given the significant variability in each population, the high classification accuracy obtained was surprising and points to consistent phenotypic patterns exhibited upon degeneration that are not evident to visual inspection.

To further test the power of our deep phenotyping pipeline, we next investigated potential differences in PVD neurodegeneration exhibited upon aging and acute cold-shock. We compiled data from the anterior and posterior part of the PVD from aging and cold-shock assays to generate training and validation sets. As shown in Figure 3.7f, the SDE model reaches ~90% training and validation accuracy for the anterior and ~80% for the posterior regions (Figure S3.6c). This difference in classification accuracy could stem from the anterior part of PVD undergoing stronger beading patterns than the posterior. Notably, these results indicate that these two stressors cause distinct neurodegeneration patterns which can be captured by in depth quantitative analysis. As a last test, we sought to establish whether the differences in bead patterning between the anterior
and posterior part of the PVD could be used to classify images of each class. An accuracy of ~85-90% was achieved from different models, confirming underlying beading pattern differences between these two regions of the neuron (Figure S3.6d). This analysis reveals that at least some of the phenotypic changes in beading patterns are specific to each neurodegenerative stressor. The developed classification models are a powerful tool to identify potential differences in neurodegenerative patterns caused by various environmental stressors (cold-shock or aging).
Figure 3.7 Biological status of a nematode can be predicted based on PVD neuron’s health. 

a) Average of mean bead size versus the average number of beads for young and aged nematodes. Age-induced PVD degeneration patterns are complex and two metrics are not sufficient to accurately classify the two populations. 

b) Principle component analysis (PCA) for young and aged adults does not enable distinguishing young and aged groups, based on the two first principal components. 

c) Schematic of the pipeline for computer-based machine learning models to predict the nematode’s biological status based on the morphological structure of PVD. Raw images are fed to Mask R-CNN algorithm to obtain binary mask, which is then used to extract the 46 metrics. Multiple models were trained based on these 46 metrics, and tested on separate data sets. 

d-f) Classification accuracy for young vs. aged, cold-shocked vs control, and cold-shocked vs. aged nematodes. SDE=Subspace discriminant ensemble, KNN=K-nearest neighbor, SVM=Support vector machine.
3.4 Conclusions

Neurons undergo degeneration during the aging process. In *C. elegans*, PVD, a neuron responsible for mechano-sensation and thermo-sensation experiences morphological and functional changes as animals age\textsuperscript{32,34}. Prior studies have identified changes in dendrite morphology characterized by disorganization in the menorah-like dendritic arbors\textsuperscript{28,29}. In addition, Lezi et al. identified that aging results in the formation of protrusions (or beads) along PVD dendrites\textsuperscript{34}. However, analyzing such morphological changes is challenging. Manual inspection to quantify the number of protrusions is time-consuming, labor-intensive, low-throughput, and subject to human bias. In addition, manual counting provides limited information and makes thorough analysis of the complex phenotypes acquired in fluorescence images unfeasible. In order to track morphological changes that PVD undergoes with degeneration, we integrated a cutting-edge deep learning technique to segment the protrusions that form along PVD. This technology decreased the time required to process each image from 3 hours to less than a minute, while eliminating the human bias in analyzing the data. In addition, a secondary algorithm was developed to extract 46 different metrics that make up a comprehensive phenotypic profile that describes the neurodegenerative beading patterns.

We implement a Convolutional Neural Network based algorithm (Mask R-CNN) to carry out challenging image segmentation, unfeasible with traditional image processing approaches. The algorithm segmentation precision and recall achieved 88% and 91% respectively. An important advantage offered by this technology (which cannot be quantified using the metrics above), is its capability to distinguish autofluorescent lipid droplets from actual protrusions, in spite of their remarkable similarities in shape, intensity, and location. The in-depth quantification of PVD morphology enabled by this technology revealed subtle neurodegenerative changes induced by
aging and upon exposure to acute cold-shock. With this approach, we identified an increase in the number of beads formed along PVD as animals aged, recapitulating earlier work by Lezi et al\textsuperscript{34}. In addition, the reduction in average bead size and inter-bead distance quantified in later points of the nematode’s life-span suggested that the protrusions formed due to aging tend to be small and appear close to each other.

Prior work has focused on the effect of acute cold-shock on a population’s survival and on PVD degeneration independently\textsuperscript{32,34,54,66}. However, the neurodegenerative impacts of acute cold-shock on PVD were still unexplored. We sought to test the effect of acute cold-shock on PVD by exposing populations of worms to 4 °C and subsequently quantifying the protrusions generated as a result. We demonstrate that exposure to cold-shock for 16 hrs. or more induces bead formation in PVD. In contrast to the beading patterns induced by aging, the average inter-bead distance increased in animals as a result of cold-shock, a counterintuitive result as an increased bead density is expected with a higher number of beads. This finding, however, can be explained by the formation of beads in the farthest regions of the neuron. These results were the initial signs of aging and cold-shock inducing phenotypically distinct neurodegenerative patterns. We next sought to study the regenerative potential of PVD post cold-shock. Thus, populations of worms exposed to cold-shock were transferred to 3 different temperatures (15, 20, and 25 °C) for a day of recovery. Interestingly, a decrease in the number of beads was observed after the rehabilitation in all 3 temperatures, while the population cultured at 25 °C exhibited the greatest decrease. The increased inter-bead distance induced by cold-shock was reversed in all three temperatures. These results suggest that bead formation due to cold-shock is a reversible process, at least at the earlier stages of adulthood. We also investigated whether culture temperature impacts the severity of bead formation due to cold-shock. Our data suggested that populations cultured at lower temperatures
experience less drastic neurodegeneration, while those cultured at higher temperatures undergo more severe damage.

Finally, we use our deep phenotyping approach to predict the biological status of nematodes based on 46 metrics extracted from the images. We tested multiple algorithms (SVM, KNN, SDE, and Logistic Regression) to classify young and old adults, cold-shocked and non-shocked nematodes, and cold-shocked and aged worms. These models achieved ~85% classification accuracy, indicating distinct beading patterns result from different stressors. Importantly, this classification method, which relies on multiple descriptive metrics of beading patterns, enables deeper exploration of the relevant parameters that describe the biological status of the neuron and its particular degeneration pattern. These promising results suggest that this approach can be used in future studies to characterize beading patterns associated with other conditions or environmental stressors. While the nature of the beads is still unclear, this approach will be crucial in understanding their role, composition, and generation mechanisms, by applying it in genetic or drug screens, and to test the beading patterns formed under other conditions.

In this work, we developed a computer-based comprehensive pipeline to study PVD neurodegeneration in a high-content, automated manner. Our quantitative analysis enabled interrogating the morphological changes that PVD undergoes under different scenarios, leading to deeper understanding of neuronal degeneration. Through this deep phenotyping pipeline, we identify a new environmental stressor (cold-shock) that induces neurodegeneration characterized by beading and reveal distinct neurodegeneration patterns induced by aging. The presented results are evidence that this high-content phenotyping technology can be used to characterize subtle, and noisy degeneration beading patterns with differences amongst stressors unnoticeable to the human eye. This pipeline is a promising approach to further explore the mechanisms underlying of
beading neurodegeneration in these and other contexts (such as oxidative stress, dietary restriction, and neurodegenerative disease models), to understand the differences that lead to distinct aging and cold-shock induced degeneration, and to identify whether beads are a result of loss of neuronal integrity or could act as a protective mechanism.
3.5 Materials and methods

3.5.1 Worm culture

The *C. elegans* strain used in this work is NC1686 (wdls51 [F4H12.4::GFP + unc-119(+)]), which expresses GFP in PVD. All populations were cultured on solid Nematode Growth Media (NGM) plates. For aging experiments, 12 mg of Fluorodeoxyuridine (FUDR) was added to 1L of media (50 µM). Animals exposed to this concentration of FUDR produced non-viable eggs. For cold-shock experiments, plates without FUDR were used since experiments took place in 4 days. Age-synchronized populations were obtained by extracting eggs from gravid hermaphrodites using a bleaching solution (1% NaOCl and 0.1 M NaOH). Eggs were then transferred to NGM plates seeded with *E. coli* OP50. M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, and 1 mL of 1 M MgSO4 in 1 L of water) with 5 µM Triton X-100 was used to transfer worms.

3.5.2 Microscopy

Animals were mounted on 2% agarose pads on glass slide. Agarose pads were placed at room temperature overnight before microscopy. A drop of 10 mM Tetramisole in M9 buffer was added for immobilization. Images were acquired on a Leica DMi8 equipped with a spinning disk confocal head (CrestOptics X-light V2) and a Hamamatsu Orca-Fusion camera using a 63x objective. The illumination source is a Laser Diode Illuminator (89 North LDI). The imaging settings were maintained constant for all images (exposure time of 60ms and laser power at 50%). Due to small field of view provided by the high magnification 63x objective (NA=1.40), two sections of each worm (anterior and posterior of PVD cell body) were imaged separately to cover larger area of the body. Images were acquired as z-stacks of 31 slices taken 1 µm apart. The final raw images used in this study were maximum projections of the z-stacks taken at every one-micron step.
3.5.3 Image segmentation and analysis

The input to the Mask R-CNN machine learning algorithm trained for this study were 2048×2048 maximum projection PNG images. Images were preprocessed before being fed to the algorithm using MATLAB image processing toolbox (imadjust function) to equalize the image contrast throughout the data set. We modified the Mask R-CNN implementation open-sourced by Matterport Inc. under the MIT license\(^1\) using Python3, Keras\(^2\), and Tensorflow\(^3\). During training, each 2048 x 2048 x 1 image and its set of corresponding binary instance masks were split into 9 overlapping tiles of size 1024 x 1024 x 1. Only non-zero instance masks were kept. The trained Mask R-CNN model was used to predict instance masks by similarly tiling the testing images. Predictions were made sequentially on 9 tiles from the top left to the bottom right of each image, and newly predicted instance masks were kept only if they did not overlap with any previously predicted mask by more than 30%. Only objects yielding a predicted probability greater than 0.9 of being in the foreground or ‘‘bead’’ class were kept. The Mask R-CNN head was trained for 20 epochs and the entire model was trained for 400 epochs, starting from pretrained ImageNet weights. The training data were augmented using random combinations of flips, 90 degree rotations, and affine shearing. The model with the lowest validation loss after 400 epochs was used for predicting instance masks. The binary masks acquired by performing image segmentation using the Mask R-CNN were then coupled with raw images and fed to secondary MATLAB based algorithm to extract metrics describing the morphology of neuron.

3.5.4 Aging assay

Eggs extracted from gravid hermaphrodites were transferred to a seeded plate and maintained at 20 °C until the population reached late L4 stage and then transferred to an FUdR plate. FUdR plates were checked daily to ensure no viable eggs or progeny were produced. During
the first 7-8 days of adulthood nematodes were transferred to a fresh FUdR plate on a daily basis to provide worms with sufficient food especially during their early adulthood. Every 2 days, a subset of nematodes was picked to perform high-resolution microscopy.

3.5.5 Cold-shock assay

The cold-shock experiments were designed to be conducted in ~4 days, which included Pre/Post cold-shock microscopy and rehabilitation. For the first two cold-shock assays, eggs extracted from gravid hermaphrodites were transferred to NGM plates and cultured for 4-5 days at 20 °C until they reach day 2 of adulthood, when pre cold-shock microscopy was performed. Cold-shock was performed by transferring plates to a 4 °C refrigerator for the designated amount of time. Plates were then placed at room temperature for 1 hr. before performing post cold-shock microscopy. This hour-long rehabilitation allowed nematodes to regain their mobility. For the tests where rehabilitation was needed, plates were transferred to designated temperature (15 °C, 20 °C, and 25 °C) for one day before post-rehabilitation microscopy was performed. For the pre cold-shock culture temperature effect assay, nematodes were cultured at 20 °C until reaching young adulthood. Subsequently, the three populations were transferred to 15°C, 20 °C, and 25°C incubator and cultured for 3.5, 2.5, and 1.5 days before performing pre cold-shock microscopy on each population (to ensure all three samples reach the same developmental stage). Populations experienced 16 hrs. of cold-shock at 4 °C prior to post cold-shock microscopy. The samples were then transferred back to the temperature they were cultured at before cold-shock for one-day to examine the post shock recovery.

3.5.6 Principal Component Analysis and classification

Principal Component Analysis (PCA) based on correlation was performed using JMP Pro 14 software. For this analysis, a data set comprised of 150 images (half from animals younger than
4 days and half from animals older than 4 days old). All 46 metrics extracted from images were incorporated in the analysis. The first two principal components explained 46% of the variance. Classification of biological status was conducted using MATLAB Classification Learner App. For all training sessions, all 46 metrics extracted from images were incorporated to train the models, and used 5 folds cross-validation was carried out. A separate validation set was used to test performance.
**3.6 References**


43. Ghaffari, M., Sowmya, A. & Oliver, R. Automated Brain Tumor Segmentation Using Multimodal Brain Scans: A Survey Based on Models Submitted to the BraTS 2012–2018


70. Ardiel, E. L. et al. Dopamine receptor DOP-4 modulates habituation to repetitive


3.7 Supplemental information

3.7.1 List of 46 metrics extracted from each image

1. Number of beads
2. Total area occupied by beads
3. Average bead size
4. Standard deviation of bead size
5. Standard deviation of mean of bead size (Standard deviation/Mean)
6. Standard error of mean for bead size
7. 90\textsuperscript{th} percentile of bead size
8. 75\textsuperscript{th} percentile of bead size
9. 50\textsuperscript{th} percentile of bead size
10. 25\textsuperscript{th} percentile of bead size
11. Average bead size for beads larger than 100 pixels
12. Average bead size for beads smaller than 100 pixels
13. Number of beads with area larger than 100 pixels
14. Number of beads with area smaller than 100 pixels
15. Percentage of beads with area larger than 100 pixels
16. Percentage of beads with area smaller than 100 pixels
17. Average inter-bead distance
18. Standard deviation of inter-bead distance
19. Standard deviation of mean inter-bead distance (Standard deviation/Mean)
20. Standard error of mean for inter-bead distance
21. Percentage of beads with inter-bead distance less than 150 pixels
22. Percentage of beads with inter-bead distance less than 300 and greater than 150 pixels
23. Percentage of beads with inter-bead distance less than 450 and greater than 300 pixels
24. Percentage of beads with inter-bead distance greater than 450 pixels
25. Median of bead size
26. Maximum of bead size
27. Average size for beads larger than 100 pixels/ Average size for beads smaller than 100 pixels
28. Mean size for smallest beads (Smallest beads are smaller half of the beads)
29. Mean size for largest beads (Largest beads are larger half of the beads)
30. Mean size of largest beads/ Mean size of smallest beads
31. Average of mean bead intensity
32. Median of mean bead intensity
33. Max of mean bead intensity
34. Standard deviation of mean bead intensity
35. Standard deviation of mean bead intensity (Standard deviation/Mean)
36. Standard error of mean of bead intensity
37. 90th percentile of mean bead intensity
38. 75th percentile of mean bead intensity
39. 50th percentile of mean bead intensity
40. 25th percentile of mean bead intensity
41. Mean intensity of smallest beads
42. Mean intensity of largest beads
43. 90th percentile of inter-bead distance
44. 75\textsuperscript{th} percentile of inter-bead distance

45. 50\textsuperscript{th} percentile of inter-bead distance

46. 25\textsuperscript{th} percentile of inter-bead distance
Supplemental Figure 3.1 Age-induced degeneration causes morphological variation on PVD structure. a) Total area of PVD neuron covered with beads. b) Percentage of beads with size lower than 100 pixels. c) Average of the mean intensity of the beads. d) The percentage of inter-bead distances lower than 300 pixels. e) 90th percentile of beads fluorescence intensity. Lines are 25th percentile, mean, and 75th percentile. Whisker is the standard deviation. f-h) Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.
Supplemental Figure 3.2 PVD neuronal structure undergoes morphological changes upon exposure to acute cold-shock. a) Total area of PVD neuron covered with beads. b) Percentage of beads with size lower than 100 pixels. c) Average of the mean intensity of the beads. This line is a transcriptional line; thus, the change is the representative of the fluctuation in concentration on promoter along beads. d) The percentage of inter-bead distances lower than 300 pixels. e) 90th percentile of beads fluorescence intensity. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. f-h) Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.
Supplemental Figure 3.3 PVD neurodegenerative phenotypes caused by acute cold-shock is reversible and can be alleviated by post shock rehabilitation. a) Total area of PVD neuron covered with beads. b) Percentage of beads with size lower than 100 pixels. c) Average of the mean intensity of the beads. This line is a transcriptional line; thus, the change is the representative of the fluctuation in concentration on promoter along beads. d) The percentage of inter-bead distances lower than 300 pixels. e) 90th percentile of beads fluorescence intensity. The lines are 25th percentile, mean, and 75th percentile. Whisker is the standard deviation. f-h) Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.
Supplemental Figure 3.4 Histogram distribution of individual bead size for recovery assay of a population cultured at 20 °C.
**Supplemental Figure 3.5** Populations cultured at different temperature before being exposed to cold-shock respond in various ways to this external stressor. **a)** Total area of PVD neuron covered with beads. **b)** Percentage of beads with size lower than 100 pixels. **c)** Average of the mean intensity of the beads. This line is a transcriptional line; thus, the change is the representative of the fluctuation in concentration on promoter along beads. **d)** The percentage of inter-bead distances lower than 300 pixels. **e)** 90th percentile of beads fluorescence intensity. The lines are 25th percentile, mean, and 75th percentile. Whisker is standard deviation. **f-h)** Average of mean bead size, percentage of beads with inter-bead distance less than 300 pixels, and percentage of beads with size lower than 100 pixels for anterior versus posterior part of the PVD neuron.
Supplemental Figure 3.6 Biological status of a nematode can be predicted based on PVD neuron’s health. a) Classification accuracy for young vs aged nematodes. The images were acquired from anterior part. b) Classification accuracy for cold-shocked vs control nematodes. The images were acquired from posterior part. c) Classification accuracy for cold-shocked vs aged nematodes. The images were acquired from posterior part. d) Classification accuracy for posterior vs anterior images of nematodes.
Chapter 4: Affordable microfluidic bead-sorting platform for automated selection of porous particles functionalized with bioactive compounds

4.1 Abstract

The ability to rapidly and accurately evaluate bioactive compounds immobilized on porous particles is crucial in the discovery of drugs, diagnostic reagents, ligands, and catalysts. Existing options for solid phase screening of bioactive compounds, while highly effective and well established, can be cost-prohibitive for proof-of-concept and early stage work, limiting its applicability and flexibility in new research areas. Here, we present a low-cost microfluidics-based platform enabling automated screening of small porous beads from solid-phase peptide libraries with high sensitivity and specificity, to identify leads with high binding affinity for a biological target. The integration of unbiased computer assisted image processing and analysis tools, provided the platform with the flexibility of sorting through beads with distinct fluorescence patterns. The customized design of the microfluidic device helped with handling beads with different diameters (~ 100-300 µm). As a microfluidic device, this portable novel platform can be integrated with a variety of analytical instruments to perform screening. In this study, the system utilizes fluorescence microscopy and unsupervised image analysis, and can operate at a sorting speed of up to 125 beads/hr (~ 3.5 times faster than a trained operator) providing > 90% yield and > 90% bead sorting accuracy. Notably, the device has proven successful in screening a model solid-phase peptide library by showing the ability to select beads carrying peptides binding a target protein (human IgG).
4.2 Introduction

The ability to rapidly identify small synthetic molecules capable of binding biological targets (e.g., single proteins, viruses, cells) with high affinity and selectivity is key to the development of novel drugs, diagnostic reagents, biosensing moieties, and ligands for the purification of biotherapeutics. The introduction of solid-phase combinatorial libraries of synthetic compounds has resulted in an exponential growth of bioactive compounds. Peptides and peptide mimetics are among the most utilized class of molecules, owing to their innate affinity for biological targets and the rich variety of strategies and amino acid building blocks available for peptide synthesis. Bioinformatic tools have also emerged that guide the design of peptide libraries in terms of peptide length, structure, and amino acid composition, based on molecular-level information on the biomolecular target and putative binding sites on its surface. Together with library design, the technology for library screening is crucial to identify peptide sequences with high binding affinity. With the growth of basic research utilizing protein-binding ligands and bioactive compounds, a number of high-throughput systems for the screening of combinatorial libraries have been developed and made commercially available. This instrumentation, however, is often cost-prohibitive to most academic groups, and alternative cost-effective solutions are highly sought after.

Solid-phase peptide libraries comprise a large number (from thousands to millions) of small porous beads, wherein every bead carries multiple copies of a unique peptide sequence. Throughout the screening process, beads are (i) incubated with a labeled target, often in presence of other impurities, (ii) sorted using a detector that recognizes beads that have captured the labeled target, and finally (iii) analyzed to identify the peptide sequence they carry. Commercial beads feature a polydispersed distribution of sub-millimeter diameters, and capture an amount of
labeled target that likely depends not only on the binding affinity of the peptide they carry, but also on their particle diameter (~100µm-300µm) and pore size distribution. This inherent variability makes library screening and selection of candidate beads extremely labor intensive and reliant on the operator’s ability and subjective visual inspection. To streamline solid-phase screening and ensure rigorous peptide selection, fluorescence-activated cell sorting (FACS) has been previously used for screening peptide libraries\(^{21}\). However, when using large beads as solid substrates (~ 100-300 µm), sorting using FACS is not feasible. Instruments that address the size incompatibility issue from FACS screening, such as the Union Biometrica COPAS Flow Pilot system for library screening, have been made commercially available\(^{18,19,22}\). Hintersteiner et al. also developed a confocal nanoscan and bead picking (CONA) platform for high-throughput screening of one-bead one compound libraries\(^{23–25}\). Their cost, accessibility, flexibility, and manufacturability, however, renders them prohibitive for academic research groups. Hintersteiner and coworkers cleverly sought to address this need with low-cost instrumentation for rapid picking from a monolayer of beads based on imaging by wide-field fluorescence microscopy\(^{17}\); this technology, however, does not allow for automated sorting and requires operator-directed selection of beads.

Microfluidic platforms have previously been used to sort particles, cells and droplets\(^{26–29}\). We therefore sought to develop a low-cost, accessible, and portable platform for inexpensive, automated sorting of library beads featuring equipment that is either present in academic laboratories or can be inexpensively fabricated, and yet that provides rigorous selection of peptide ligands with high affinity and selectivity for biological targets. (Figure 4.1a, b, c). This system enables (1) simultaneous positive and negative selection by orthogonal labeling to isolate peptide binders with high affinity for a target and low-to-no binding for biological competitors, (2) rapid
library screening with a rate of up to 100-150 beads per hour, (3) handling beads with different diameters (~100-300 µm), (4) ability to integrate library screening with external stimuli to select peptides that feature stimuli-controlled protein binding, and (5) identification of diverse targets of varying sizes by providing image-processing based spatial binding distribution analysis. Our platform is adaptable to a different of detection and sorting modes, providing the flexibility to screen with a variety of targets. To this end, our system comprises a microfluidic bead-sorting chamber, a dual wavelength fluorescent microscope, and customized software for real-time bead monitoring, image processing, and sorting. The microfluidic bead-sorting chamber is manufactured by photo-lithography followed by soft-lithography. The software, based on a MATLAB Graphical User Interface, enables rapid (< 0.75 sec) acquisition of fluorescent bead images, followed by image-processing based analysis and sorting (to either the selection receptacle or to waste). Notably, the quantitative image analysis algorithm takes into account both intensity and radial distribution of fluorescence, which combined improves the sensitivity and specificity of the platform in selecting high-affinity peptides by reducing the risk of false positives and negatives. To ensure identification of peptides with high binding selectivity, the algorithm enables orthogonal dual-color sorting by selecting the beads that only capture the fluorescently-labeled target while excluding beads that carry competitor proteins labeled with a different color. Finally, the device can be operated in either bulk or single bead separation mode. In bulk separation mode all positive beads are directed to a common flask, whereas in single bead separation mode each positive bead is individually directed to a single well in a 96 well-plate. In this work, we validated the integrated bead-sorting device and code using a dual fluorescence library mimetic. This comprises a combination of ChemMatrix beads labeled either by covalent conjugation of fluorescent dyes (Texas Red and Fluorescein) or by adsorption of fluorescently-labeled, streptavidin-conjugated
proteins of different molecular weight to biotinylated beads resulting in either a homogeneous or halo-like radial color distribution, respectively. The fluorescence intensity and radial pattern observed in each class of beads were used as metric to inform sorting parameters. Beads with different fluorescence patterns (homogenous or halo-like) were analyzed to quantify the accuracy of bead-sorting protocol. Our results indicate that the device was capable of sorting beads with a detection yield and accuracy of ~ 92% and 94% respectively. It should be noted that the system performs all sorting and quantitative image analysis without any user supervision, thereby enabling a fully automated library screening process. It is worth mentioning that high sensitivity and specificity were achieved while microscopy was performed in a non-confocal setup demonstrating the robustness of algorithm in handling images with suboptimal quality. The flexibility of quantitative image analysis algorithm in handling images with various qualities, enables the set up to be used with different instruments. In this work, we designed and fabricated an automated, low-

Figure 4.1 Microfluidic device for automated screening of bioactive compounds. a) Schematic of experimental setup. b) Photograph of the microfluidic platform. c) Device schematic. Beads enter the device through the bead inlet and are trapped in the imaging zone by on-chip valves. Beads are then directed to positive or negative outlets depending on their fluorescence profile. d) The width and length of imaging zone is 350 µm while the height is 400 µm. The PDMS membrane separating flow and valve channels is 140 µm. Scale bar is 1 cm.
cost, accessible, and portable microfluidic platform capable of sorting targets with various fluorescence patterns with high precision and accuracy.

4.3 Results and discussion

Peptides and peptide mimetics with high affinity and selectivity for biological targets will play a critical role in next generation medicinal chemistry, diagnostics, and downstream biomanufacturing technology. Streamlining their identification requires affordable, automated devices capable of sorting solid-phase peptide library beads with accurate and rigorous orthogonal selection logics. To meet these needs, we have developed a low-cost accessible automated microfluidic platform to handle, capture, image, analyze, and sort beads based on fluorescence patterns that directly translate into the binding affinity of the peptides carried by the selected library beads. We applied this technology for the isolation of positive (i.e., protein binding) beads from a library mimetic consisting of a mixture of labeled ChemMatrix beads with different fluorescent color, intensity, and radial distribution.

4.3.1 Diverse fluorescence patterns for protein binding detection

In prior studies, PEG-based ChemMatrix resin has been successfully utilized as a substrate for the synthesis and screening of solid-phase peptide libraries against target proteins for the selection of peptide ligands. In this context, however, the small pore diameter of ChemMatrix resin has been shown to limit protein diffusion through the bead, resulting in a protein-rich corona, or “halo” whose width decreases with the molecular weight of the protein. When fluorescently labeled targets are utilized, this translates in a halo-like fluorescence pattern; in theory, smaller proteins produce a diffuse halo, while larger ones produced a narrow halo. To replicate this effect, a number of fluorescent bead classes were prepared, either by conjugating different amine-reactive fluorophores (i.e., Texas Red NHS and FITC) onto aminomethyl ChemMatrix beads or by
adsorbing fluorescently labeled streptavidin or streptavidin-conjugated proteins of different molecular weight onto biotin-ChemMatrix beads (Figure 4.2). Specifically, seven classes of beads were prepared, namely: Class 1, homogeneous only-red fluorescence, prepared by conjugating amine-reactive Texas Red to aminomethyl ChemMatrix beads; Class 2, homogeneous green-only fluorescence, prepared by reacting Fluorescein isothiocyanate (FITC) with aminomethyl ChemMatrix beads; Class 3, homogeneous dual red-green fluorescence, prepared using both Texas Red and Fluorescein; Class 4, broad halo only-red fluorescence, prepared by adsorbing red-Streptavidin (MW ~ 56 kDa) onto biotin-ChemMatrix beads; Class 5, medium halo only-red fluorescence, prepared by adsorbing Texas Red-labeled Streptavidin-BSA conjugates (MW ~ 118 kDa) onto biotin-ChemMatrix beads; Class 6, narrow halo only-red fluorescence, prepared by adsorbing Texas Red-labeled Streptavidin-AD (MW ~ 202 kDa) conjugates onto biotin-ChemMatrix beads; and Class 7, broad halo only-green fluorescence, prepared by adsorbing green-Streptavidin (MW ~ 56 kDa) onto biotin-ChemMatrix beads.
Figure 4.2 Various classes of beads with different fluorescence profiles. Beads in classes 1-3 exhibit homogeneous fluorescence patterns in green, red, and green/red. Class 4 and 7 were prepared by adsorbing red and green Streptavidin onto biotin beads producing broad halo pattern in red and green channel respectively. Classes 4-5 were prepared by adsorbing red-Streptavidin-BSA and red-Streptavidin-AD onto biotin beads producing medium and narrow halo pattern respectively. The scale bar is 200 µm. Contrast has bee modified in the Unlabeled class image for visibility.

4.3.2 Flexible automated detection, analysis, and sorting of beads

To enable automated bead selection and sorting, we developed customized algorithms to detect the presence of a bead, to extract image-based descriptive metrics required for classification, and to sort the bead according to the criteria provided by the operator into a positive line to the collection receptacle or a negative line to be discarded. Bead selection was based on (i) radial distribution of fluorescence, (ii) fluorescence intensity, and (iii) difference of color 1 vs. color 2 intensities. Radial distribution of fluorescence depends on the diffusion of the target biomolecule through the bead, and thus on its hydrodynamic radius; “halo”-like radial distribution can be utilized as a criterion of choice when screening against large protein targets, so that beads showing a homogeneous fluorescence distribution are discarded as false positives. Fluorescence intensity correlates to the ability of the peptides carried by the bead to effectively capture the target biomolecule; thus, selection of beads carrying high intensity promotes the selection of peptides
with high binding strength. Finally, the difference of intensities in two colors, is utilized for selection of ligands with high binding selectivity. Library selection is performed in “competitive conditions”, that is, by co-incubating the labeled target protein with a myriad of other protein impurities communally labeled orthogonally to the target (e.g., using fluorescence dyes with non-overlapping emission wavelengths); accordingly, the selection of beads carrying only the desired label ensures capture specificity. To demonstrate the full potential of our technology, we sorted the beads from the library mimetic according to various fluorescence patterns and intensities, showing that the device is capable of unsupervised bead sorting with high accuracy in response to criteria set by the user.

Screening the peptide library using the microfluidic platform comprises a series of tasks performed in a loop. Throughout the process, on/off-chip valves are utilized to trap single beads in the imaging zone and sort them as positive or negative based on the selection criteria. The first step involves loading an individual bead to the imaging zone (Figure 4.3, Top left); in this step, the beads are withdrawn from the suspension and flown through the device, while keeping the loading and positive outlets valves closed, and the negative outlet and imaging valve open. Importantly, the valves used in this system are only closed partially, thus trapping beads while allowing fluid to flow through closed valves. As the fluid flows through the imaging zone, the algorithm is constantly acquiring and analyzing frames from the camera to detect the presence of a bead. Detecting a bead triggers the second step, where the imaging, loading and positive outlet valves are closed, and the bead is retained in the imaging zone (Figure 4.3, Top middle). A second image is then acquired to ensure the presence of a bead in the imaging zone. This image is fed to an image processing algorithm that segments the bead and extracts various descriptive metrics. The system then determines whether the bead is positive by comparing these metrics with the
thresholds input by the operator. If the bead is assessed as negative, the loading valve opens and allows the bead to be expelled through negative outlet, while flow through positive outlet is stopped by both on and off chip valves (Figure 4.3, Top right). Off-chip pinch valves are used in both positive and negative outlets to ensure the flow is completely stopped. As mentioned earlier, collection of positive beads can be performed in two different modes, either by transferring individual beads to single wells in a 96 well plate or by collecting them in bulk in a common flask (Figure 4.3, Bottom right and left). When operating in bulk collection mode, once a positive bead is detected, the negative outlet valves (on and off-chip) are closed while the positive outlet valves and the loading valve open, allowing the bead to flow toward the flask. The incoming fluid from the reservoir containing the bead suspension is sufficient to direct the bead to the collection flask. When operating in single bead collection mode, once a positive bead is detected, the system is programmed to pause the operation and ask the operator for permission to continue. As the positive outlet tube is placed in the designated well, upon receiving permission to continue by the operator, the system opens the positive outlets and the loading valves, allowing the bead to travel to the collection well with the flow provided by the flush stream. With this set up, were able to perform sorting at a speed of up to 125 beads/hr (~3.5 times faster than a trained operator) (Supplemental Figure 4.2).
Figure 4.3 Screening process flowchart. Beads are flown through the device while the loading valve is closed and the imaging valve open. Once a bead is detected in the imaging zone, the imaging valve closes trapping the bead. If the bead is assessed to be negative, the loading valve will open up and allow the bead to flow through the negative outlet. If the bead is assessed to be positive, the negative outlet valve closes and the positive outlet valve opens while the loading valve opens and allows the bead to flow through the positive outlet.

Several tests were performed to evaluate the bead sorting accuracy and precision of the system using fluorescently labeled beads of Class 1 – 7. Library mimetics depicting different screening scenarios were prepared by mixing beads of one class, considered as positive, with a combination of beads from other classes. In every experiment, a different library mimetic was suspended in PBS at a density of ~2 beads/mL and maintained under gentle agitation to prevent aggregation. Beads were fed at a rate of ~2.5 beads/min. The beads conforming to the set selection criteria were sorted as positive, and subsequently analyzed to calculate the yield and accuracy of
the sorting process, respectively defined as ratio of positive beads collected vs. positive beads fed and ratio of positive beads collected vs. total beads collected in the positive flask.

4.3.3 Sorting beads with homogenous fluorescence patterns

We first processed beads with uniform high-intensity fluorescence patterns in either a single (red-only or green-only) or dual (red and green) color (Figure 4.2). In the first test, beads with uniform red-only fluorescence (Class 1) were used as the positive set, and mixed with untreated ChemMatrix beads and green-only fluorescent beads (Class 2) as negatives. We established the positive selection criterion based on the 90\textsuperscript{th} percentile of the bead’s intensity in the red channel and the 90\textsuperscript{th} percentile of the bead’s intensity in the green channel. Beads exhibiting values above 0.5 in normalized red channel 90\textsuperscript{th} percentile and below 0.2 in normalized green channel 90\textsuperscript{th} percentile were considered positive (Figure 4.4.a, b). These threshold values were established based on preliminary images acquired from each bead class and analyzed to identify distinctive features. Using these thresholds, we were able to retrieve 17 out of the 18 positive beads initially present in the reservoir flask (~ 95% yield); while all 17 beads were confirmed as positive, indicating 100% accuracy.
**Figure 4.4** Detection and sorting of beads with homogeneous fluorescence patterns. 
a,c,e) Dot plot of samples screened with the goal of sorting class 1, 2, and 3 beads respectively. The values for decision hyperplanes (lines) were established based on preliminary data acquired for each class. The purple point in figure (c) occurred due to having class 1 and class 2 beads entering the imaging zone together. Both beads were sorted as negative. 
b,d,f) Bar plots of the platform performance in sorting class 1, 2, and 3 beads respectively. “Positive added” is the known number of positive beads added to the flask. “Positive Retrieved” represents the true positives collected at the outlet. “False Positive” and “False Negative” are the beads sorted incorrectly.

The second test aimed to sort beads with green-only homogeneous fluorescence (Class 2) from a mixture of Class 2, Class 1, and unlabeled beads. For this test, beads with normalized 90\textsuperscript{th} percentile pixel intensity higher than 0.5 in the green channel and normalized 90\textsuperscript{th} percentile pixel intensity lower than 0.2 in red channel were considered positive (**Figure 4.4.c, d**). Using these thresholds, we retrieved 53 out of 57 initial positive beads (~ 93% yield); all 53 beads were confirmed as positive (100% accuracy). The third test aimed to isolate beads carrying dual red-
green fluorescence (Class 3) from a mixture of Class 3, Class 2, and unlabeled beads. Beads were considered positive when the 90\(^{th}\) percentile of pixel intensity in the red and green channels was above 0.5 and 0.4, respectively (Figure 4.4.e, f). As a result, 48 positive beads and 1 false positive were retrieved out of 51 positive beads fed to the sorting device (~ 94% yield rate and ~ 98% accuracy).

4.3.4 Sorting beads with “halo”-like fluorescence pattern

We then sought to evaluate the capability of this platform to sort beads with more complex fluorescence patterns. The homogeneous fluorescence distribution initially utilized is representative of library screening against small protein targets, which can easily diffuse through the pores of the library beads and be captured by the ligands displayed throughout the entire radius of the bead. In contrast, prior work by the Camperi group \(^1^8\) on screening ChemMatrix-based peptide libraries indicates that, due to diffusion limitations, larger target proteins effectively penetrate only the outer corona of the beads. This translates into a “halo”-like fluorescence pattern, potentially accompanied by fluorescing spots randomly distributed throughout the bead (Figure 4.2). Accordingly, additional tests were designed to assess the sorting of beads with beads with halo-like fluorescence pattern.

We first sought to separate Class 6 (narrow halo) beads from a mixture of Class 6, Class 4 (broad halo), Class 7 (broad halo green-only), and unlabeled beads. To distinguish beads with subtler fluorescence patterns, a more complex set of parameters was defined. Specifically, to be accepted as positive, a bead ought to meet three criteria:

\[
\alpha_{er} > 0.15, \alpha_{er} - \alpha_{eg} > 0, 90^{th}_{eg} < 0.1
\]

\[
\alpha_{eg} = \frac{M_{eg} - 90^{th}_{eg}}{M_{eg}}
\]
\[ \alpha_{er} = \frac{M_{er} - 90^{th}}{M_{er}} \]

\[ 90^{th}_{eg} = 90^{th} \text{ Percentile pixel intensity of entire bead in green channel} \]

\[ 90^{th}_{er} = 90^{th} \text{ Percentile pixel intensity of entire bead in red channel} \]

\[ M_{eg} = \text{Maximum pixel intensity of entire bead in green} \]

\[ M_{er} = \text{Maximum pixel intensity of entire bead in red} \]

Where \( \alpha_{er} \) and \( \alpha_{eg} \) measure the fluorescence homogeneity through the bead. Smaller \( \alpha_{er} \) and \( \alpha_{eg} \) numbers indicate homogenous distribution of fluorescence within the bead, whereas larger values of \( \alpha_{er} \) and \( \alpha_{eg} \) indicate a wider range between the maximum pixel value and the 90th percentile. These threshold values were chosen based on preliminary analyses conducted on images acquired from beads of Class 4, 5, 6, and 7.

As a result, 21 positive beads and 3 false positives were retrieved out of 24 positive beads fed to the sorting device (~87.5% yield). Considering that 3 beads sorted as false positive, we obtained a ~87.5% accuracy (Figure 4.5.a, b). The presence of false positive and false negative beads is imputed to the variability inherent to protein-peptide binding. In some instances, beads of Class 6 did not exhibit a halo-like pattern, likely due to heterogeneous pore size distribution through the bead. False positives and false negatives can also occur if two beads enter the imaging zone together, a result of aggregation or simple proximity in the flow. When two or more beads of different classes enter the imaging zone, the algorithm inevitably sorts them as either positive or negative, correspondingly resulting in false positive or false negative sorting. However, our data suggests the error caused by these phenomena is only a minor occurrence.
Figure 4.5 Capability of platform in sorting halo fluorescence patterns. a,c) The 3D plot of samples screened to sort class 6 and 5 beads respectively. The values for decision hyperplanes (surfaces) were established based on preliminary data acquired for each class. b,d) the bar plot of the platform performance in sorting class 6 and 5 beads respectively. “Positive Added” is the known number of positive beads added to the flask. “Positive Retrieved” represents the true positives collected at the outlet. “False Positive” and “False Negative” are the beads sorted incorrectly. e) Unsupervised K-mean clustering of data extracted from images taken from class 5 and 6 beads. ~30 beads from each class were imaged and processed. f) The ground truth labels for points used in the unsupervised K-mean clustering. These labeled data were used to calculate the accuracy of unsupervised clustering of data points acquired from class 5 and 6 beads.

As a final experiment, we aimed to sort Class 5 beads (medium halo red-only fluorescence) from a mixture of Class 5, Class 4 (broad halo red-only), Class 7 (broad halo green-only), and unlabeled beads. Similar to the previous test, three criteria were defined to identify positive beads with non-uniform patterns:
\[ \alpha_{or} > 0.15, \alpha_{or} - \alpha_{og} > 0, 90^{th}_{eg} < 0.1 \]

\[ \alpha_{og} = \frac{M_{og} - 90^{th}_{og}}{M_{og}} \]

\[ \alpha_{or} = \frac{M_{or} - 90^{th}_{or}}{M_{or}} \]

90^{th}_{eg} = 90^{th} Percentile pixel intensity of entire bead in green channel

90^{th}_{og} = 90^{th} Percentile pixel intensity bead’s outer ring in green channel

90^{th}_{or} = 90^{th} Percentile pixel intensity bead’s outer ring in red channel

\[ M_{og} = \text{Maximum pixel intensity of bead’s outer ring in green} \]

\[ M_{or} = \text{Maximum pixel intensity of bead’s outer ring in red} \]

By applying these thresholds, we were able to retrieve 30 out of 33 positive beads fed to the sorting device (~91% yield), with 4 false positives and 2 false negatives (~88% accuracy) (Figure 4.5.c, d).

We performed a detailed statistical analysis aiming to find metrics that would enable sorting of beads from Classes 5 and 6, which exhibit comparable halo and non-uniform fluorescence patterns. Specifically, Class 5 beads are labeled with a red Streptavidin-Albumin conjugate and exhibit non-uniform fluorescence pattern through the bead’s core as well as a partial halo on the corona; Class 6 beads are labeled with a red Streptavidin-Alcohol Dehydrogenase conjugate and exhibit a strong fluorescent halo and low-to-no fluorescence in the bead’s core.

Initially, 34 descriptive metrics were extracted from 61 images acquired from Class 5 and 6 beads, and a K-means clustering was performed using the two metrics that showed the largest difference between Class 5 and 6 beads, namely the normalized 99^{th} percentile pixel in the red channel and
the normalized 90th percentile pixel of non-bright segment of bead in the red channel (Figure 4.5.e, f). To extract these metrics, the bead was segmented in regions of high and low brightness. To detect regions of high brightness, a local first order statistic threshold was used with decreased sensitivity toward the bright foreground (in comparison to that used for entire bead detection). Regions of low brightness were detected by subtracting the mask for bright segments from the mask of the whole bead. Based on results of the K-means clustering analysis, a hyperplane was specified to differentiate between the Class 5 and 6 beads. The clusters obtained from unsupervised sorting were compared with the labeled data to assess the accuracy (Number of beads correctly classified/Total number of beads) of the K-means-based clustering. This comparison indicated that these groups can be discerned with a ~ 87% accuracy, which accounted for the non-homogeneities associated with fluorescence patterns emerging within the samples prepared. There were instances where distinguishing these two classes was not possible even by visual inspection performed by a trained operator. Collectively, these tests demonstrated the flexible capabilities of this platform in detecting and sorting beads based in various fluorescence intensities and patterns. By discerning populations with either uniform or more complex fluorescence patterns, this system proved fit for automated, unbiased screening of peptide libraries against different protein targets for the identification of synthetic bioactive compounds.

4.3.5 Screening through a ChemMatrix-peptide library to sort “halo”-like fluorescence pattern.

Screening through a ChemMatrix-peptide library to sort “halo”-like fluorescence pattern after demonstrating the ability of the proposed device to sort complex halo-type fluorescence patterns, we proceeded with the selection of protein-binding ligands from a library of combinatorial peptides under competitive conditions (target protein mixed with protein
impurities). In particular, we focused on the identification of immunoglobulin G (IgG)-binding ligands as a case study. Initially, a one-bead-one-component (OBOC) octameric peptide library $X_1X_2X_3X_4X_5X_6X_7X_8$-GSG was synthesized, wherein $X_1X_2X_3X_4X_5X_6X_7X_8$ represents the variable region and GSG is a glycine-serine-glycine spacer arm. The library was produced on solid phase (ChemMatrix resin) using an equal ratio of alanine, aspartatic acid, tyrosine, arginine, glycine, glutamate, histidine, leucine, glutamine, and serine. The library was then spiked with $\sim 5\%$ in volume of ChemMatrix beads functionalized with the IgG-binding control peptide HWRGWV-GSG. This peptide ligand has been previously shown to selectively bind IgG in complex fluids, including human plasma and Chinese hamster ovary (CHO) cell culture fluids.\textsuperscript{20,36,37} The procedures for the synthesis of the library and HWRGWV-GSG on ChemMatrix beads are detailed in the Supplemental Information. The HWRGWVGSG-spiked library was incubated with red-labeled IgG (tagged with Texas Red NHS ester) mixed with green-labeled CHO host cell proteins (HCPs, tagged with Alexa Fluor 488 NHS Ester) and sorted using the proposed device to identify beads with “high” IgG binding and “low” HCP-binding (red only beads). In this context, we aimed to sort and sequence beads displaying selective halo-patterned binding of IgG to verify that the platform is capable of extracting IgG-binding sequences, including HWRGWVGSG. Nonetheless, positive sequences other than HWRGWVGSG were expected due to limited compositional bias in the combinatorial library. Prior to library screening, optimized hyperplanes for sorting positive beads were determined. In order to identify these hyperplanes, $\sim 30$-$40$ positive control beads were flown through the device and quantitative data was extracted. Based on the results acquired by running these beads through the device, the following thresholds were set for a bead to be considered positive: $(i)$ $\alpha_{\text{or}} - \alpha_{\text{og}} > 0$, $(ii)$ $\alpha_{\text{or}} > 0.25$, $(iii)$ 90$^{\text{th}}$ percentile pixel intensity of entire bead in green channel $< 0.1$, and $(iv)$ 95$^{\text{th}}$ percentile pixel intensity of entire bead in red channel $> 0.08$. 


Using the platform, we screened ~ 200 beads of this library and identified 12 beads as positive. To further verify the existence of fluorescence pattern of interest, the selected positive beads were imaged again individually in a single well. All 12 beads exhibited halo pattern in post-sorting microscopy, which is indicative of platform’s ability in identifying true positive. Finally, the peptides carried by the selected beads were sequenced by Edman degradation using a Shimadzu PPSQ 33A Protein Sequencer to verify the presence of the control sequence HWRGWV-GSG (Supplemental Figures 4.3-4.4). Prior to sequencing, the beads were treated at low pH (0.2 M acetate buffer, pH 3.5) and washed to remove all bound proteins. Finally, the peptides were sequenced directly from the collected beads. Nine of the 12 positive beads were sequenced, resulting in 2 beads carrying HWRGWVGSG.

4.4 Conclusions

Screening combinatorial peptide libraries using fluorescence-based readouts is a powerful approach for the identification of protein-binding peptides. With solid-phase libraries, in particular, which feature peptides conjugated on porous beads, fluorescence detection of the beads following capture of the labeled protein target is a successful approach for high-throughput screening of combinatorial solid-phase libraries. Despite its success, manual screening is extremely labor-intensive and commercial devices for automated screening are likely unaffordable to academic labs. In this work, we developed a low-cost accessible platform for automated screening of solid-phase peptide libraries that integrates lab-scale microfluidics and microscopy with user-friendly software that enables unsupervised bead imaging and sorting. The device, which can process 100-150 beads per hour, was tested to evaluate yield and accuracy of automated bead sorting. This setup was successfully able to handle beads of various size (~100-300) and flexible enough to detect and sort beads with different fluorescence pattern. To this end, we utilized seven
classes of beads featuring different patterns of fluorescence labeling that mimic the appearance of library beads screened against protein targets with different size. The average yield and accuracy of positive beads recovered by the device from mixtures of different classes was found to be 92% and 94% respectively. Particularly encouraging was the recovery of beads with complex fluorescence patterns, which afforded ~ 88% yield and ~ 88% accuracy. Notably, the acquisition of the metrics needed to perform the bead sorting was unsupervised; specifically, two bead patterns (i.e., non-homogeneous and halo-like) were produced using labeled proteins of different molecular weight, automatically acquired, and successfully utilized to recover positive beads from both classes. This demonstrates that the device provides a cost-efficient, accessible alternative for the automated sorting of bead-based combinatorial libraries with high sensitivity and specificity. As a demonstrative case study, we employed the device for selecting IgG-binding peptides by screening a combinatorial library spiked with IgG-binding HWRGWV-ChemMatrix beads upon incubation with IgG spiked in a complex protein mixture (CHO cell culture fluid). We finally and confirmed the presence of IgG-capturing beads by Edman sequencing of the positive beads. This platform achieved high accuracy and yield in sorting beads via incorporation of fluorescence microscopy. In addition, as a microfluidic platform, multiple devices and setups can be assembled in a relatively short period of time.

The proposed device platform can also be integrated with other analytical instruments as well as systems that enhance the decision-making algorithms. Additionally, image processing and pattern recognition can be carried out using machine-learning algorithms that would improve the accuracy of the decisions made during sorting over the currently utilized statistical algorithm. Integrating these supervised machine-learning algorithms would lead to detecting more subtle and complicated patterns occurring throughout screening a library of peptides. The throughput of this
platform can also be improved by implementing parallel devices simultaneously or by increasing
the concentration of beads in the suspension flown through the device.

4.5 Materials and methods

4.5.1 Device fabrication

The microfluidic platform was fabricated by traditional photolithography followed by soft
lithography. Negative photoresist SU-8 2150 was spun at 1460 rpm to achieve a feature height of
400 μm (Figure 1.d). Soft bake was carried out for 10 and 90 minutes at 65°C and 95°C,
respectively. The wafer was then exposed to UV light for 14 seconds using a UV-KUB 3 mask
aligner. The mold was then further baked at 65°C and 95°C for 5 and 30 minutes respectively to
ensure complete cross-linking of exposed regions. Soft lithography was performed in two steps
since on-chip valves require a more flexible material for proper operation. First, a thin PDMS layer
at a 20:1 ratio of polymer to cross-linker was poured and cured for 20 minutes at 80°C. A second
thick PDMS layer at a 10:1 ratio of polymer to cross-linker was then poured on the mold and cured
for 2 hours at 80°C.

4.5.2 Preparation of fluorescently labeled beads

Beads were prepared to feature two different fluorescence profiles, namely homogeneous
and “halo”-like distributions, as described by Marani et al 18. Beads with homogeneous
fluorescence were prepared by labeling ChemMatrix aminomethyl beads with Texas Red NHS
ester, fluorescein isothiocyanate (FITC), or both; to this end, 50 mg ChemMatrix dry resin was
swollen in 1 ml 0.1 M sodium bicarbonate, pH 8.3, for 1 hour at room temperature and incubated
with 50 μl of 2 mg/mL fluorescent dye solution in dimethyl sulfoxide (DMSO) in dark, for 1 hour
at room temperature under gentle agitation. After incubation, the dye solution was removed and
the beads were washed thoroughly with 0.1% Tween 20 in phosphate buffered saline (PBS), pH
7.4 (PBS-T) for storage and to remove unreacted fluorescent dye. Beads featuring halo-like beads were produced using proteins with a range of molecular weights. To this end, biotin was initially conjugated onto ChemMatrix aminomethyl beads by incubating the resin with a 1 mg/mL solution of biotin and 2 mg/mL 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 0.1 M MES, pH 4.6, to achieve a ratio of 1 mg biotin:2 mg EDC:100 mg resin, for 2 hours under gentle agitation at room temperature. Meanwhile, high purity bovine serum albumin (BSA, 66 kDa) and alcohol dehydrogenase (AD, 150 kDa) were dissolved at 2 mg/ml in phosphate buffered saline and conjugated to streptavidin (53kDa) using a LYNX Rapid Streptavidin conjugation kit. The streptavidin-conjugated proteins were then fluorescently labeled with Texas Red NHS ester. Briefly, 10 µL of streptavidin-conjugated protein was dissolved at 2 mg/ml in 0.1 M sodium bicarbonate, pH 8.3, and mixed with 1 µL of 10 mg/mL solution of Texas Red NHS dye in DMSO under gentle agitation for 1 hour, light-protected at room temperature. The unreacted dye was removed by diafiltration against PBS-T. For labeled protein-bead interactions, 20 µL of the labeled streptavidin and protein conjugates was incubated with 2-5 µl settled volume of biotinylated beads for at least 1 hour at 2-8°C.

4.5.3 Experimental setup

In this study, beads are sorted based on their fluorescence intensity or pattern in both red and green channels. Simultaneous dual color fluorescence microscopy was performed using a LEICA DMi8 inverted microscope connected to a Hamamatsu Orca-D² camera equipped with two charge-coupled devices (CCDs) enabling simultaneous microscopy in wavelengths of interest. The ChemMatrix beads used in this study tend to aggregate in solution, leading to clogging of the microfluidic platform and sorting errors (false positives/negatives). To prevent aggregation, beads were maintained in a diluted suspension in PBS buffer (150 beads per 80 ml) and gently stirred on
an orbital shaker throughout the duration of the sorting cycle. On-chip valves were filled and degassed with a 50% glycerol solution with a similar refractive index as PDMS, which improves image quality in the vicinity of the valves. A custom-built pressure box equipped with pressure regulators was used to drive fluid flow in the tubing and device. Valve operations was controlled by a custom-developed MATLAB Graphical User Interface (GUI).

4.5.4 Image processing

To automate the sorting process in an unbiased and quantitative manner, computer-vision algorithms were implemented to detect bead presence in the imaging zone and classify it as negative or positive based on the criteria provided by the operator. Bead detection was carried out by a custom developed MATLAB algorithm that identifies the presence of beads in the channel independently of bead size or fluorescence intensity. As shown in Supplemental Figure 1, bead detection begins by converting the raw grayscale image to a binarized image using a local first order statistic threshold. The background noise detected in the binarized image is eliminated by removing objects smaller than 100 pixels. The image is then dilated and filled to re-construct the bead structure. To further refine the mask, the image is opened, eroded, and dilated to smooth the bead shape. We integrated this algorithm with the live image acquisition setup where the presence of each incoming bead was detected. Once the bead is detected and isolated in the imaging zone, a second image processing algorithm extracts the image intensity profile using the mask previously generated. Various metrics such as mean intensity, max intensity, intensity N\textsuperscript{th}-percentile, and different combinations of these metrics are extracted. The values extracted using this algorithm are normalized by converting the 12-bit pixel value range from 0 (black)-4095(white) to 0 (black)-1 (white). Positive beads are detected and isolated based on the values of these metrics. In this study, more than 20 metrics were extracted for each bead. Depending on the pattern of interest, a
combination of two or three metrics is used as criteria for the identification and sorting of positive beads.
4.6 References


Scand. 4, 283–293 (1950).
4.7 Supplemental information

Supplemental Figure 4.1 Flowchart of image segmentation.
Supplemental Figure 4.2 Comparison of speed of bead sorting between trained operators and the microfluidic device.

The operators were asked to plate 96 well-plates with single beads and perform fluorescence microscopy of each well.
Supplemental Figure 4.3 Edman sequencing of positive control bead collected in well F3 from library screening. All amino acid residues were correctly assigned by the Edman data analysis, except for the third and last residues. The Arginine (Arg) peak is the only peak whose intensity does not decrease from the 2nd chromatogram to the 3rd chromatogram, thereby indicating that Arginine is actually located in position 3, as expected. Since the last three amino acids in all sequences of the library and in the spiked IgG-binding peptide HWRGWV-GSG are Gly-Ser-Gly, we can assume that the last amino acid is Glycine.
**Supplemental Figure 4.4** Edman sequencing of a positive control bead collected in well F12 from library screening.
4.7.1 Library synthesis

The combinatorial octamer peptide X₁-X₂-X₃-X₄-X₅-X₆-X₇-X₈ was synthesized by Fmoc/tBu strategy onto 2 g of HMBA-ChemMatrix resin (HMBA: hydroxymethylbenzoic acid) preloaded with the tripeptide GSG (G: Glycine, S: Serine). Ten protected amino acids Fmoc-Ala-OH, Fmoc-Asp(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Glu(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Leu-OH, Fmoc-Gln(Trt)-OH, and Fmoc-Ser(tBu)-OH were used as building blocks to produce the combinatorial segment of the library (X₁-X₈) via “split-couple-recombine” technique. First, every amino acid was conjugated to a 0.2 g aliquot of resin using 3 equivalents (eq.) of amino acid in DMF, 3 eq. of 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) in DMF, and 6 eq. of diisopropylethylamine (DIPEA) in NMP at 45°C for 20 min. After amino acid conjugation and removal of the Fmoc protecting group using 20% piperidine in DMF for 20 minutes at room temperature, the ten aliquots were combined, mixed, and re-divided into 10 aliquots. The steps of amino acid conjugation, Fmoc removal, aliquot mixing, and splitting were repeated seven more times, resulting in a pool of 10⁸ peptide combinations. Finally, the peptides were collectively deprotected using a cleavage cocktail TFA/Thioanisole/EDT/anisole (90/5/3/2) for 2 h. The resulting library was finally washed thoroughly with DMF and stored in 20% aqueous ethanol at 4°C.

4.7.2 Conjugation of fluorescent dyes to protein

Human Immunoglobulin G (IgG) and Chinese Hamster Ovary Host Cell Proteins (CHO HCPs) were labelled with Texas Red and Alexa Fluor 488 respectively, following the manufacturer’s protocol. CHO HCPs were dialyzed against 0.1 M Sodium Bicarbonate Buffer pH 8.3 at 10 mg/mL using a Thermo Scientific Pierce 9K MWCO Protein Concentrator. IgG was also
dialyzed against 0.1 M Sodium Bicarbonate Buffer pH 8.3 at 10 mg/mL. Texas Red and Alexa Fluor 488 were each dissolved in dry dimethyl sulfoxide (DMSO) at 10 mg/mL and immediately added to the corresponding protein solution at a 1:10 mass ratio of label to protein. The reaction was incubated for 1 hour at room temperature on a rotator, and then diafiltered into 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, 0.1% Tween 20 (PBS-T) using Amicon Ultra-0.5 ml 10 kDa MWCO filters to remove any unreacted dye.

4.7.3 Incubation of beads against IgG and CHO HCP

To calibrate the instrument with positive control beads, 20 µL of Texas Red-IgG protein in PBS-T was added to 7 µL of settled HWRGWVGSG beads. For the library incubation, 12 µL of settled combinatorial octamer peptide beads were added with 0.5 µL of settled HWRGWVGSG-ChemMatrix beads. Subsequently, 66 µL of 2 mg/mL IgG and 33 µL of 2mg/mL CHO HCP in PBS-T were added to 12.5 µL of the spiked peptide library, achieving a total protein incubation solution of 100 µl of 1.3 mg/mL IgG and 0.7 mg/mL CHO HCP in PBS-T. The positive control and the spiked library were incubated with their respective protein solutions overnight at 4°C in the dark. The beads were then washed three times with PBS-T and screened.

4.7.4 Sequencing of selected beads

After incubation and screening, each selected bead was placed in an individual well of a 96-well plate and washed to remove the bound protein. Specifically, every bead was treated with (i) 100 µL of 0.2 M acetate buffer pH 3.5 for three times, at room temperature, for 30 minutes; (ii) 100 µL of 50 mM PBS pH 7.4 for three times; (iii) copiously rinsed with water and acetonitrile for one minute each. They were then stored in acetonitrile at 4°C until sequencing.

On-bead sequencing of selected peptides was conducted via Edman degradation using a PPSQ 33A protein sequencer equipped with an LC-20AT solvent delivery module and an SPD-
20A UV-VIS detector (Shimadzu). Following a phenylthiohydantoin (PTH)-amino acid standard, single beads were placed onto a polyvinylidene difluoride (PVDF) membrane and loaded into a PPSQ 33A reactor according to the instrument protocols. The bead is sequenced using the Edman degradation reagents and separated with a Wakopak® Wakosil PTH-II φ4.6mm × 250mm (S-PSQ) column. The sequence was identified by matching amino acid retention times to the calibrated PTH-amino acid standard.
Chapter 5: Conclusion

5.1 Summary and overview of the projects

In this dissertation, we sought to further elucidate unknowns and answer some elusive questions regarding biological systems by integrating hardware and software to gather large sets of fluorescent images and convert them into quantitative meaningful data describing various phenotypes. The biological phenotypes with subtle changes and complex patterns can be imperceptible to human vision while observing the fluorescent images. However, fluorescent images acquired do contain rich dynamic information about the phenomena under investigation. In the absence of appropriate tool to quantify the images and extract the information stored, scientists are compelled to limit the interpretations of images to qualitative human observations. In order to study complex subtle biological phenotypes accurately while reducing misinterpretation of phenomena and minimizing the errors, big quantitative data sets are required to be generated. Thus, in this work, we developed microfluidic devices to facilitate and automate high-throughput image acquisition. The high-throughput hardware was then coupled with machine learning and computer-based software to access hidden layers of images and extract information. The quantitative data extracted provided us the topological information needed about synaptic and neuronal morphological alterations to identify very subtle alterations which was not feasible by qualitative human observation-based approaches.

In the first project, we sought to elucidate age-induced synaptic degeneration by deep phenotyping the presynaptic sites located at DA9 neuron. To acquire high-quality fluorescent images while nematodes are at correct orientation during the aging process, a microfluidic platform was designed and fabricated to perform life-long high-resolution microscopy. The platform was comprised of two main sections, main chamber and imaging arrays. The main chamber was
designed to eliminate the need for FUdR to age-synchronize a population by mechanically evacuating the progeny through custom-designed auxiliary channels. We were able to age-synchronize a population of up to 600 worms in single round of device operation. The imaging section was comprised of arrays of tapered channels where worms enter and get trapped individually. The height restriction at the beginning of each channel increased the probability of nematodes having desirable orientation and served as an obstacle to prevent worms from leaving the channels easily. The imaging section of this platform accommodated up to 76 nematodes at each round of microscopy. With this platform, we were able to achieve drug free long-term culture and high-resolution microscopy of nematodes which was not possible in the absence of it.

To fully extract the information stored in fluorescent images of synaptic domain, we integrated an in house developed SVM machine learning technique followed by feature extraction and quantitative analysis to obtain a better understanding of presynaptic sites’ localization, distribution, and morphology. The custom developed SVM algorithm segmented the images to generate binary masks of the presynaptic sites which were then fed to a secondary algorithm created in MATLAB to extract more than 60 metrics characterizing the morphology of presynaptic domain. The quantitative measurements obtained by integrating this technique led to unveiling subtle and previously undiscovered phenotypes. For instance, the results suggested that the density of presynaptic sites along the domain decreases which was compatible with previous findings reported. The reduction in synaptic density while number of sites remain constant suggests that aging may be interrupting accurate delivery of vesicles to the designated destination. In addition, an increase was observed in average size and fluorescent intensity of synaptic sites suggesting a potential accumulation of vesicles within presynaptic domain. The accumulation of vesicles can be due to impaired docking and vesicle fusion in presynaptic cleft leading to vesicles being
processed in a slower rate and pile up. The results also indicated a gradual significant overall change in more metrics as the population aged. Only 2 metrics were significantly altered on day 6 compared to control worms while this number was elevated to more than 30 metrics for 12-day old nematodes as an indication of morphological changes occurring to synaptic domain. These significant changes in many metrics suggests that the morphological structure of presynaptic domain undergoes alteration and deviates from it baseline as a population age. The identification of these subtle neurodegenerative patterns was the direct result of extracting quantitative features from images and access deep layers of data which is hidden from human observation.

The second project was mainly focused on deep phenotyping PVD neurodegeneration where a cutting-edge Convolutional Neural Network machine learning technique was coupled with quantitative analysis. In neurons like PVD with complicated structure, the degenerative variations are extremely subtle and non-trackable by human vision. Thus, there is a need to extract as many quantitative metrics possible to describe the topology of the system. In this work, the segmentation of the protrusions forming along PVD neuron was fully automated and performed by a MaskRCNN algorithm developed in Python. The trained algorithm achieved 88% and 91% in precision and recall in unbiased segmentation of the degenerative beads generated. The high precision was achieved as the process had been automated and the throughput of it was improved. The implementation of this pipeline decreased the time required to create the binary masks from 3 hours per image on average to less than a minute. Thus, processing ~2000 images and extracting quantitative measurements to describe the subtle degenerative phenotypes was only possible through high-throughput segmentation offered by this technique.

To satisfy the need for quantitative metrics to track the neurodegenerative pattern, 46 features describing the distribution, localization, and morphology of protrusions were extracted.
The features unveiled subtle complex neurodegenerative phenotypes while eliminating human interpretation based on qualitative observation. Our results indicated an increase in number of beads as nematodes age which is compatible with previous findings. In addition, we discovered that the average bead size and average interbead distance decrease as a population undergoes aging process suggesting that the new beads generated are smaller ones and more densely packed. The results from this assay was a proof of concept that the pipeline can be implemented to investigate various stressors. Thus, this pipeline was implemented for the first time to quantitatively track the neurodegenerative impacts of acute-cold shock. The changes occurring due to cold-shock were very subtle and challenging to track solely based on human vision and observation. Thus, an in-depth characterization of fluorescent images was required to secure a reliable visualization of the phenomena. Interestingly, our results indicated that populations experiencing long enough cold-shock exposure (shock for 16 and 24 hrs) showed higher frequency of bead formation. Interbead distance in cold-shock assay exhibited an unexpected trend where it elevated as the number of beads grew which suggested that the beads are formed in a more dispersed fashion and along remote areas of the neuron which was a distinct pattern from age-induced degeneration. The availability of quantitative information enabled us to discover this distinct beading pattern and deviation between aging and cold-shock which was the proof of capability of our pipeline in identifying subtle differences between various degenerative pathways that could not have been recognized in the absence of it.

To further exploit the rich information extracted from each image, we sought to train secondary machine learning-based classifiers to predict the biological status of a nematode based on the degenerative profile of PVD. Training the second classifier based on all 46 metrics enables us to study combination of the metrics simultaneously and use the full capacity of the quantitative
pipeline to search for complicated degenerative patterns. Our aim was to discover whether the degenerative profile of PVD neuron could lead to predict the biological status of each nematode. With this approach, we classified young vs aged, control vs cold-shocked, and cold-shocked vs aged nematodes based on beading pattern of PVD neuron. Interestingly, we were able to achieve ~85% accuracy on average classifying these groups of nematodes which suggested the presence of subtle differences within these groups that can only be identified and tracked by incorporating larger number of metrics. Successful prediction of nematodes’ biological status unveiled the presence of subtle PVD degenerative pattern differences generated under certain conditions. In addition, it proved the ability of coupled MaskRCNN and feature extraction technology to quantitatively analyze the data and track subtle phenotypes which are imperceivably with human vision.

In our third and final project, we sought to increase the throughput and accuracy of protein-peptide binding library screening by integrating a fully automated microfluidic-based system. The key to an increased accuracy and sensitivity in target identification and isolation is high-content image analysis leading to extraction of quantitative metrics which could be then used to distinguish hits accurately eliminating human interpretation based on observation. Thus, a fully automated microfluidic-based set up was developed where solid particles are imaged and based on their fluorescent pattern the beads meeting the criteria defined by the operator will be sorted as positive while the rest are discarded as negative. To achieve full automation, the microfluidic platform was coupled with a custom developed MATLAB algorithm where all flow handlings, valve operations, image processing, and target assessments were conducted simultaneously. This automated platform achieved throughput of 150 beads/hr screening and isolating targets. In addition to throughput, our fully automated platform was able to achieve 92% yield and 94% accuracy in
competitive sorting of beads with homogenous fluorescent pattern and 88% for the ones with more complex halo-like pattern. The quantitative features extracted from each image generated high sensitivity and specificity that subtle fluorescent pattern difference between different classes of halo beads were detected. In this work, we also generated an actual library of beads and spiked it with limited number of beads with halo pattern with the aim to successfully select and collect them in positive outlet. We were able to successfully retrieve number of targets with halo patterns possessing the sequence of interest suggesting the capability of platform in isolating beads with complicated fluorescent pattern. Coupling these high-content approaches with customizable microfluidic systems and computer-controlled systems, provided very powerful tool to solve elusive problems in biology.

5.2 Future work/ Applications beyond scope of this dissertation

5.2.1 Project 1

The applications of tools and techniques developed in this work are not limited to the studies mentioned in this dissertation. The platforms with same procedure or with minor modification can be flexible to conduct other assays. In addition to platforms, the biological fields and questions investigated in this work can be further elucidated and expanded. For instance, the platform in first project was implemented to study age-induced degenerative phenotypes occurring to presynaptic sites located at dorsal side of the DA9 motoneuron. However, this platform can be further used in other assays as a whole or individual sections can be implemented for specific needs. In a separate study conducted in San Miguel Lab, the main chamber has been implemented to age-synchronize a population to perform forward genetic screens on 3-day old adults. One of the key components of forward genetic screens is capability of isolated mutant to lay viable eggs. Thus, majority of the forward genetic screens performed on C. elegans are carried out at early
stages of egg-laying phase to increase the chance of target mutants having viable progeny. In the study carried out in San Miguel Lab, the forward genetic was required to be performed at day 3 of adulthood (almost the end of their reproduction period) which entailed keeping the population age-synchronized up to that time point. Population exposure to FUdR completely synchronizes the population. However, it contradicts with the nature of the assay which requires viable progeny post screen. To circumvent this issue and maintain the population age-synchronized, the main chamber of the device equipped with tapered auxiliary channels was used to filter out the progeny while the parent population remained intact within the chamber. In addition to synchronization, nematodes can be exposed to various chemical stimuli within the main chamber. For instance, assays where exposure to certain chemical stimulus prior to microscopy is intended, the main chamber can serve as the station where the population can experience exposure to homogenous substance. The drug and food mixture can be flown through the device for circumstances where several hours of exposure to stimulus at lower concentration is intended prior to microscopy.

The imaging section of platform can also be implemented separately to perform high-resolution microscopy on-chip. For instance, we tested the possibility of anesthetic perfusion while nematodes are trapped individually in imaging channel to fully immobilize worms. For assays where sub-cellular features are not being studied and using Tetramisole and Levamisole does induce major side-effects, these anesthetics can be injected through imaging channels as immobilization reagent. Within several trials, we were able to successfully immobilize the nematodes using this technique and recover them post microscopy. In addition to expanding fields where the device can be utilized for, fundamentals of synaptic plasticity studied in this work can be further elucidated by follow up assays. For instance, various assays and platforms has been developed to record nematodes locomotive behavior and quantify the responses acquired. The
number of bends nematodes perform in unit of time or the crawling/swimming speed are two metrics used to determine a worm’s locomotive performance. Coupling the quantitative results obtained from high-resolution microscopy of synaptic sites with behavioral analysis can unveil how subtle age-induced changes in sub-cellular levels could have an impact on macro phenotypes such as locomotive variations. To further investigate the correlation between synaptic plasticity and locomotive behavior, nematodes can be subjected to artificial workout regimes where training sessions will be created by exposing specific strain of nematodes to blue light to trigger movement\(^1\). The results obtained from quantitative analysis of synaptic plasticity coupled with locomotion behavior data obtained post exercise could lead to a better understanding of the impact of physical activity on health in sub-cellular and behavioral level.

The findings from performing high-resolution microscopy of synaptic domain unveiled an increase in size and fluorescent intensity of presynaptic sites suggesting the possibility of vesicle accumulation. The potential accumulation of synaptic vesicles could be as a result of failure in various mechanisms within synaptic machinery such as unsuccessful vesicle recycling or defective vesicle transportation along the domain. The pathways leading to degenerative phenotypes of presynaptic sites can be further elucidated by tagging various players in synaptic machinery and study the age-induced effect on them. For instance, strains of worms with fluorescent markers tagging the proteins involved in vesicle recycling could be implemented to investigate whether accumulation is due to age-induced flaws in recycling. The magnitude of degeneration observed in vesicle recycling and its rate would help us gain better understanding of its impact on accumulation of synaptic vesicles along presynaptic domain. In addition, the age-induced degeneration of post-synaptic sites that are responsible for receiving the neurotransmitters released from presynaptic sites can be studied to acquire bettering understanding of the connectivity of pre
and post synaptic sites. The locomotive deficiencies could be as a result of malfunction in receiving mechanism of neurotransmitters which could be further elucidated by investigating post-synaptic sites. The connectivity of pre and post synaptic partners could also be investigated using GFP Reconstitution Across Synaptic Partners (GRASP) technique\textsuperscript{2-4}. This approach will help us obtain a better understanding of how well connected pre and post synaptic partners remain as the organism undergoes aging process. These assays and experiments are example of many future goals which could be set and achieved based on findings in the first project and the microfluidic platform developed.

5.2.2 Project 2

In the second project, we integrated a cutting-edge novel Convolutional Neural Network-based machine learning technique to investigate PVD neurodegeneration as a result of aging and exposure to acute cold-shock. The horizon of these assays can be further broadened by including other stressors to the survey to obtain a better understanding of elements causing bubble-like beading along PVD dendrites. For instance, oxidative stress and dietary restriction are two stressors well-known for having impact on neuronal health. Previous studies have shown that oxidative stress has neurodegenerative impacts on different classes of neurons at different rate with distinct phenotypes\textsuperscript{5}. However, the neurodegenerative effect of oxidative stress has not been studied on bead formation mechanism along PVD. Thus, exposing populations of worms to various drugs such as Paraquat, Juglone, and Hydrogen Peroxide to induce oxidative stress through distinct pathways could be the first step toward understanding degenerative effect of this stressor on PVD. The integration of MaskRCNN technology coupled with feature extraction algorithm will help track subtle phenotypes caused by oxidative stress quantitatively. In addition, the sensitivity provided by this pipeline enables us to discover existence of any potential different
neurodegenerative phenotypes or patterns induced by each of these substances. Based on preliminary data acquired from oxidative stress-induced neurodegeneration, the morphological variation occurring to PVD structure is subtle and qualitative approaches may not be accurate and sensitive enough to capture the changes. Thus, our quantitative pipeline would offer the ability to discover new beading patterns generated as a result of exposure to each of these drugs. In addition to surveying various drugs inducing neurodegeneration, concentration and exposure time of these substances are two other critical factors contributing to the magnitude of degeneration. Conducting experiments where nematodes are exposed to drugs at different concentration and exposure time would enable us to create models to predict the neurodegenerative impacts caused by specific substance causing oxidative stress at certain concentration and exposure time.

One of the fields that the computer-based pipeline developed can be implemented in is forward genetic screen where mutants with subtle morphological variations compared to control nematodes can be targeted and isolated. By performing forward genetic screens, mutant candidates with abnormalities in PVD morphology can be identified and isolated accurately and quantitatively by this pipeline. Deep phenotyping the mutants can lead to pathways and genes causing degeneration or the ones enhancing neuronal health. Forward genetic screens in C. elegans is typically performed by exposing population of control worms to EMS mutagenesis to create random genetic mutations among worms. The nature of random mutagenesis generates hundreds of worms with unique macro and micro traits. Investigations with the goal of targeting mutants with macro phenotypic variances such as uncoordinated locomotion have been performed extensively since online high-resolution microscopy and sorting is not required\textsuperscript{6,7}. An operator could easily screen through plates of worms and seek for nematodes with abnormality in locomotion. However, even the subtle locomotory behavior changes occurring due to minor
mutations can not be identified and tracked manually by observation. Thus, the forward genetic screens requiring high-resolution microscopy become more complicated due to the need for complete immobilization of each individual worm during image acquisition. In addition, the phenotypes that are required to be captured using high-resolution microscopy are subtle and obscure. Thus, approaches that entail qualitative analysis or human based assessments fail to deliver the accuracy and sensitivity desired.

To circumvent these issues and improve the accuracy and sensitivity of genetic screens for subtle phenotypes, the pipeline developed in this work could be modified to facilitate forward genetic screens to identify genes and pathways involved in formation of bubble-like protrusions along PVD. This technique with the access to rich information embedded in each image enables screens with higher accuracy and sensitivity. Extracting quantitative metrics will incorporate more comprehensive angles of PVD morphological structure and its degeneration which leads to more reliable decisions with regard to target isolation. To fully automate the screen, this pipeline could be coupled with an in house developed microfluidic device specialized for high-throughput nematode sorting. The worms enter the imaging section of the device individually and get trapped there. High-resolution image is acquired and is sent to the pipeline where the mask of the protrusions is created and forwarded to the feature extraction algorithm to obtain the metrics required for decision making process. Based on preliminary results and the requirement of screen, up to 46 metrics can be incorporated in the process of making decision of whether a mutant should be isolated or not. Pretrained machine learning classifiers can also serve the role of decision making based on the phenotype of interest. The same pipeline can be used at the verification step where the mutants isolated from the screen are grown and imaged to certify and confirm the presence of abnormality in the beading pattern of PVD neuron. Thus, the machine learning pipeline
coupled with the automated microfluidic platform could help identifying the genes and genetic pathways associated with PVD dendritic degeneration under various conditions. These findings would further elucidate the nature of these protrusion which has not been discovered yet.

In this project we only focused on automating the image segmentation and quantification of protrusions formation along the PVD neuron. However, the other main degenerative phenotype observed in PVD is dendritic disorganization, branching, and outgrowth which has been previously studied extensively. The major challenge associated with studying dendritic degeneration traditionally was the manual qualitative analysis conducted by an operator. In these studies, the disorganization and branching of dendrites were recorded qualitatively by and operator surveying through hundreds to thousands of images acquired and assessing the presence of the phenotype of interest manually. In addition, comprehensive understanding of morphological variations occurring to the structure of PVD dendrites would not be possible due to insufficient information provided by traditional qualitative approaches. Thus, a computer-based approach where the dendrites are traced and segmented can add new perspectives and angles in studying the degeneration. The segmentation should be followed by topological analysis and quantitative feature extraction to track the changes occurring to the structure of the neuron with measurable metrics.

In order to acquire preliminary results, we sought to perform dendritic segmentation and obtain quantitative measurements by training a machine learning-based algorithm to trace the dendrites and create the binary mask of them. In our first attempt, eight 2048×2048 images and the binary ground truth masks of the dendrites were used as training data set. Two images were implemented to validate and test the accuracy and efficiency of this method in segmenting images. Interestingly, considering limited number of images used for training and validation, the algorithm
demonstrated promising capability in segmenting the dendrites. However, the algorithm’s performance in distinguishing bright fat-droplets from dendrites needed to be improved. Thus, we sought to add four more training images with large number of bright fat-droplets present to train the algorithm to discern these particles from dendritic branches. Our goal was to increase the accuracy of segmentation by introducing more examples of fat-droplets during training so that the algorithm’s weights get optimized toward identifying these objects as negative. The machine learning-based image segmentation coupled with topological analysis and feature extraction would facilitate investigations focused on age-induced degenerative phenotypes. This novel pipeline would enable us to score the morphological variations of PVD neuron throughout a population’s lifespan quantitatively to provide a comprehensive understanding of fundamentals of degeneration and the pathways associated with it. In addition to age-induced degeneration, nematodes can be exposed to acute cold-shock, oxidative stress, heat-shock, dietary restriction, and chemical drugs to quantify and track the neurodegenerative effects of these environmental stressors.

This novel technology coupled with the bead segmentation and quantification pipeline would help obtain more comprehensive understanding of fundamentals of neurodegeneration in PVD. By combining the results acquired from these two pipelines, we can improve the classification accuracies which was obtained by only incorporating the data from beading process. Visual inspection did not suggest any significant morphological alteration of dendrites for nematodes experiencing acute cold-shock while aged worms exhibit serious degenerative phenotypes. On the other hand, the different beading patterns generated as a result of aging process compared to acute cold-shock were subtle and not distinguishable visually. Thus, these differences further suggest that combining bead and dendrite segmentation and quantification pipelines could
elevate the accuracy, reliability, and robustness of models trained to predict the biological status of worms.

5.2.3 Project 3

In the third project, we developed an automated high-throughput microfluidic-based system to screen through library of bioactive compounds and select the targets with the desirable fluorescent patterns. In this work, the bulk screening was conducted in a fully automated fashion. However, in order to isolate each target identified individually, there was a need for an operator to handle a well-plate and direct the solid particles once detected into each well by moving the micro-tubing manually. This minor issue prevented the platform from operating fully automated and required an operator to be present during the screen to move the well-plate. To circumvent this obstacle, we proposed to integrate a programmable stage where the well-plate can be placed on during the screen. Considering the constant spacing between the wells within a well-plate, the algorithm can be programmed to dispense the liquid into first well and deliver the solid particle. Once the delivery is completed and screening is in progress, the programmable stage moves based on the gap present between two wells to align the empty well with the micro-tubing to be filled once a target is identified. The addition of this automated step eliminates the need for post bulk screen pipetting of individual particles into wells to separate them. In addition, the full automation of the platform is a robust alternative eliminating the need for an operator to follow the screen and isolate the targets once acquired.

In the second attempt to expand the capabilities of this platform to meet the requirements for more complicated diverse assays, we sought to modify the operational procedure to study light sensitive ligands. Our collaborators were interested in studying the impact of exposing specific ligands to lights with designated wavelengths. The assays designed required the potential target to
be exposed to the light while being washed by fresh media for few minutes. The traditional approaches fail to deliver desirable outcome since simultaneous light exposure and wash with fresh media is not feasible in well-plates. Our automated platform with minor modification in its operational procedure could be an ideal solution for this challenge. The imaging zone where beads get trapped individually could serve as a chamber to isolate the particles, shine light, and perfuse the washing agent. This controlled environment provides the assay with the capability to perfuse various buffers while exposing the ligands to the light with desired wavelength. Online image acquisition and processing will be coupled with the hardware set up to quantify the fluorescent pattern change throughout the test. Consistent, reliable, and unbiased quantitative information extracted online while the solid particle is isolated in the imaging zone increases the accuracy and sensitivity of sorting by lowering the probability of generating false positive and negatives. Once the light and buffer treatment is over, the algorithm makes the decision to accept or reject the potential target and the particle gets sorted accordingly. This automated set up also can be further modified to encompass more applications and conduct variety of assays. For instance, PH sensitive ligands are a separate field which can be studied using this platform. Potential candidates identified in imaging zone can be washed with buffers with different PHs and the dynamic changes of fluorescent patterns be recorded, and assessments be made. In addition, temperature sensitive ligands can also be investigated by implementing this technology. Integrating a platform to control the temperature within the microfluidic channels could facilitate sorting ligands sensitive to temperature gradient.
5.3 References


