ABSTRACT

DUFFY, COLLEEN MARIE. Development of a Novel Paper-based Platform for Large-scale Production of Influenza Virus Vaccine from Adherent MDCK Cells. (Under the direction of Dr. Michael C. Flickinger).

Each year there are between 3 and 5 million severe cases of influenza globally, which results in 250,00 to 500,000 deaths. To protect against influenza virus infection, vaccines are manufactured each year that confer seasonal immunity to the most prevalent 3 to 4 strains on the basis of global surveillance. While flu vaccines are traditionally manufactured by growing influenza virus in chicken eggs, cell-based manufacturing processes have become prevalent today. The most common mammalian cell line used to commercially produce influenza vaccines is the Madin Darby canine kidney (MDCK) cell line. Both egg-based and cell-based manufacturing processes take place in large, centralized facilities that then distribute vaccines. These processes also take a long time to go from strain selection to vaccine distribution and are largely inflexible. A novel approach to continuous vaccine manufacture would be to develop a paper-based, stabilized MDCK cell culture manufacturing process for flu vaccines capable of rapid, high titer, reproducible production of virus from multiple strains for vaccines. In the past decade paper substrates have become of greater interest for cell culture and have been used to create cheap and disposable cell-based assays and life-like disease models. However, paper substrates have never been combined with cell culture manufacturing platforms. The benefits of a paper-based manufacturing platform is that paper is eco-friendly, cheap, readily available, biocompatible, and can be easily physically and chemically modified to create large surface areas for cell adherence. Using paper as a cell-stabilizing substrate could enable roll to roll virus manufacturing as a continuous moving bed plug flow bioreactor. This study screens fourteen common types of paper for properties that would make them suitable for MDCK cell culture. Of the fourteen, seven types

of paper were used as cell culture substrates in batch growth studies. From the seven, Fisher bibulous paper was found to be the best substrate to support the adherence and proliferation of MDCK. A simulated continuous moving bed plug flow bioreactor process was then simulated to show how the MDCK can successfully be grown to a large surface confluence on bibulous paper in preparation for infection with influenza virus. Growth of the MDCK cells on bibulous paper was characterized using confocal microscopy, and it was found that the cells grow predominantly on the surface of the paper. Finally, the pre-inoculation soaking time of sterilized paper was examined as a process improvement to the paper-based cell culture process. This proof-of-concept study successfully demonstrated a feasible roll-to-roll cell culture platform that can be used to make influenza vaccines cheaper, more accessible, and faster to manufacture.

Development of a Novel Paper-based Platform for Large-scale Production of Influenza Virus Vaccine from Adherent MDCK Cells

by
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APPROVED BY:

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DEDICATION

I would like to dedicate this thesis to my grandfather, Dr. James Woelfel, M.D., a self-made man who grew up in poverty in West Virginia during the Great Depression. Through his hard work, all of his grandchildren were able to receive the best education available. Without his example and generosity, I would not be where I am today.

BIOGRAPHY

Colleen Duffy was born in Raleigh, North Carolina. She grew up in Youngsville with her mother, Mary Duffy, her father, Gregg Duffy, and her little brother, Patrick Duffy. She attended Franklin Academy in Wake Forest from elementary school through high school and graduated third in her class in 2010. During her time at Franklin Academy, she participated in National Honor Society, was the captain and MVP of her school's girls' soccer team, and was the captain of the cross country team. Upon graduation, she was awarded her school's Luddy Scholarship, established by Franklin Academy's founder, Bob Luddy.

Colleen attended the University of Notre Dame in Indiana for four years of undergraduate education, where she earned a B.S. in Biological Sciences. She even pursued a minor in studio art due to her love of ceramic and drawing. She graduated *magna cum laude* in 2014.

Colleen then attended North Carolina State University to pursue a M.S. in Biomanufacturing at the Biomanufacturing Training and Education Center (BTEC). She concentrated in both Upstream and Downstream manufacturing. Colleen was also a Teaching Assistant while in grad school, TAing Industry Practicum in Biomanufacturing and Mammalian Cell Culture Engineering laboratory. She will earn her M.S. in Biomanufacturing from the Biomanufacturing Training and Education Center at North Carolina State University in December of 2018.

Colleen did a summer internship at Fujifilm Diosynth Biotechnology in 2018 and is still working as an intern in the Process Sciences group as a Process Engineer in training. She hopes to be hired full-time after her graduation and continue to apply the lessons she learned at BTEC throughout her career.

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CHAPTER 1: Introduction

1.1. The Influenza Virus

Each year there are between 3 and 5 million severe cases of influenza globally, which result in 250,00 to 500,000 deaths.[1] Influenza is caused by infection by an influenza virus, single-stranded RNA viruses of the Orthomyxoviridae family.[2] Only Influenza types A and B are pathogenic in humans.[2] Influenza viruses have 8 segments of negative-sense, single-stranded RNA genetic material.[2] The two major surface proteins on the exterior of the viral envelope are Hemagglutinin (HA) and Neuraminidase (NA), as depicted in Figure 1.1.1.[2] These two glycoproteins, especially HA, are highly visible to the body's immune system, and are therefore the two major antigens of influenza viruses. HA mediates the virus' entry into the body's cells, as it has an affinity for sialic acid present in human glycosylation structures on cell surfaces.[3] The virus binds to the cell surface and is brought into the cell via endocytosis, after which it hijacks the cell's transcription and translation machinery to produce more viral particles.[3]

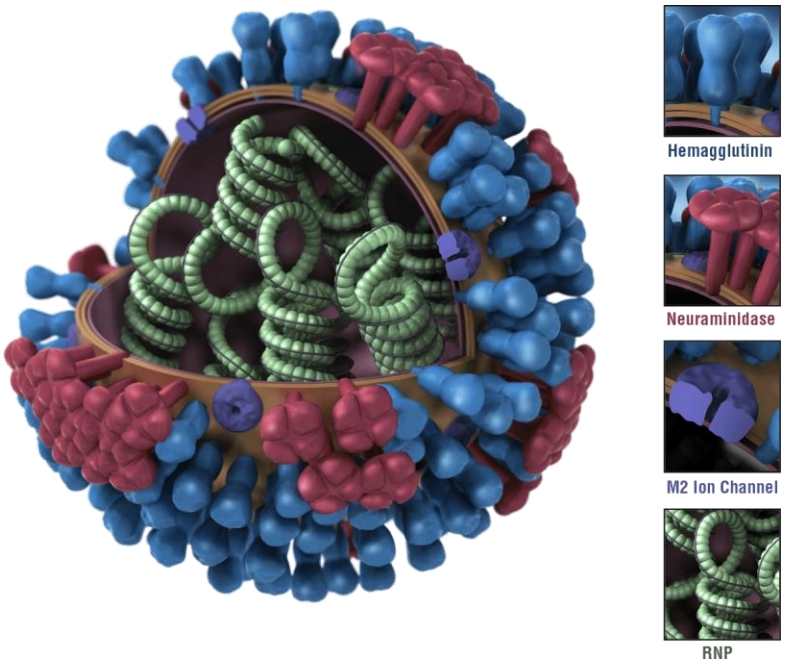


Figure 1.1.1. Structure of the influenza virus. Image from the CDC.[15]

To protect against infection from the influenza virus, millions of doses of vaccines are manufactured annually; during the 2017-2018 flu season, over 155 million doses of flu vaccine were distributed to the U.S. market alone by private manufacturers.[4] Vaccines are either trivalent or quadrivalent, vaccinating against either three or four Influenza A and B strains, respectively. Types of flu vaccines include live attenuated influenza virus (LAIV), inactivated influenza virus (IIV), or recombinant virus or antigens.

Influenza changes from season to season, allowing it to evade the body's immune system and necessitating new vaccines each year to provide immunity. Influenza virus is able to do this by a continuous evolution process driven by antigenic drift and antigenic shift of the HA and NA antigens (Figure 1.1.2). Antigenic drift is caused by point mutations, insertions, and deletions during virus replication that change the sequence of HA and NA enough to go unrecognized by the immune system.[2] Influenza A also undergoes antigenic shift, or a more drastic re-assortment of the viral genes in the 8 RNA segments.[2] Antigenic shift occurs when one cell is infected by more than one strain of Influenza A, allowing the RNA segments to re-assort and produce possibly unseen antigens.[2]

Antigenic shift is often responsible for influenza pandemics that result in many deaths due to low population immunity and insufficient vaccine-conferred immunity.[2] Influenza pandemics, unforeseen and often occurring mid-flu season, result in large numbers of deaths. Examples of influenza pandemics include the 1918 pandemic caused by an avian derivative of H1N1, the 1957 Asian Flu pandemic caused by an avian H2N2 virus, the 1968 pandemic caused by an avian H3N2 virus, and the 2009 H1N1 pandemic.[17] These pandemics caused 500 million, 1.1 million, 1 million, and 575,000 worldwide deaths, respectively.[17] Although the vaccines available today save

thousands of people, they are unequipped to defend a population against pandemics caused by unpredicted strains, as shown in the 2009 H1N1 pandemic.

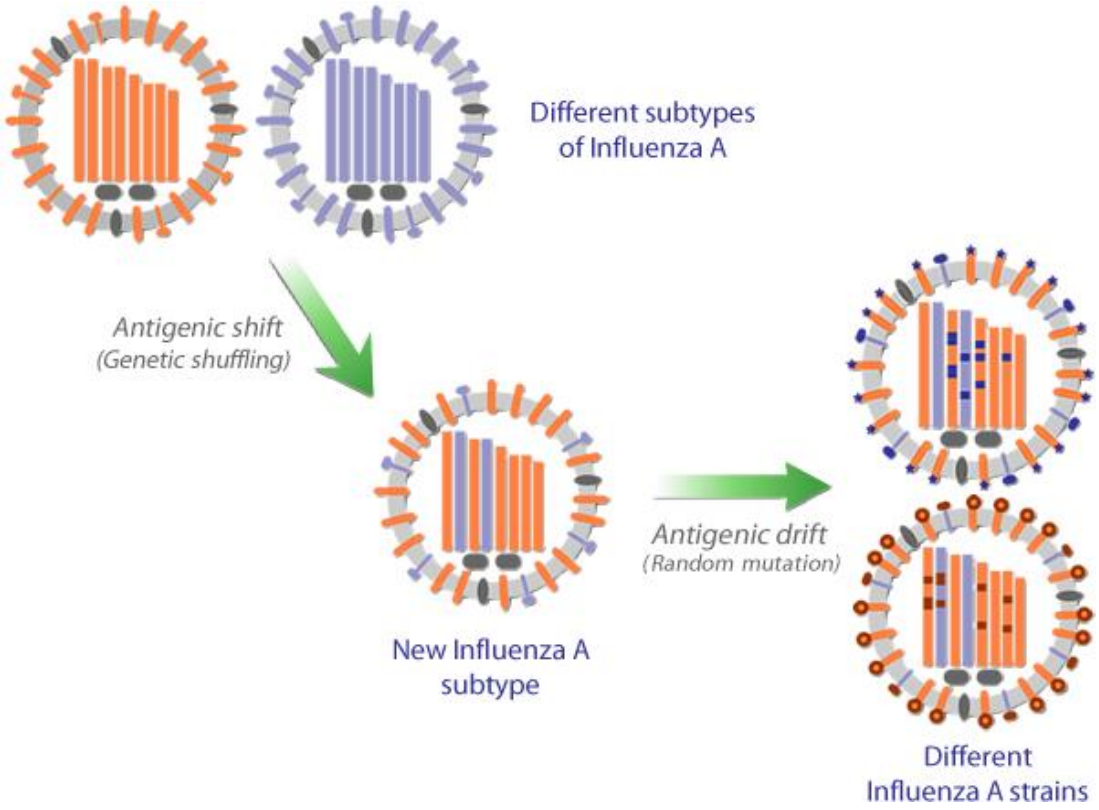


Figure 1.1.2. Influenza A antigenic drift and antigenic shift. Image from the WHO.[16]

1.2. Influenza Vaccine Manufacturing

Seasonal influenza vaccine manufacturing is a lengthy process. Due to the continual evolution of new virus strains, the World Health Organization (WHO) conducts year-round surveillance of the circulating influenza strains.[5] In mid-February, the WHO recommends the most prevalent influenza strains that are most likely to impact the Northern hemisphere during the next influenza season, and the FDA then selects three to four strains to vaccinate against in the U.S.[5] The selected strains are adapted to growth in eggs, and the virus seeds are distributed to vaccine manufacturers, who then have several months to optimize virus growth conditions, bulk

manufacture the virus, and perform downstream processes like inactivation, splitting, purification, and formulation.[5,6] After regulatory release testing, the season's influenza vaccines are distributed to the public as early as July or August, making the process from strain selection to distribution a five to six month process.[5,6]

Since the 1940's, flu vaccines were traditionally LAIVs or IIVs manufactured by growing influenza virus in chicken eggs.[7] In the egg-based manufacturing process, fertilized chicken eggs are injected with virus and allowed to incubate for several days for the virus to replicate. The allantoic fluid is then harvested, and virus particles are isolated for further processing.[7] However, the egg-based manufacturing process has many disadvantages, including its reliance on large quantities of Specific Pathogen Free (SPF) eggs, the six-month window between strain selection and vaccine availability, and the variability of strain adaptation to growth in eggs.[7] The egg-based process is particularly incapable of responding to sudden pandemics. As such, faster manufacturing processes have been developed for influenza vaccines.

The first cell-based influenza vaccine in the U.S. was approved by the FDA in 2012.[7] Cell-based manufacturing processes for influenza utilize mammalian cell lines, most commonly Madin Darby canine kidney (MDCK) cells, in large-scale suspension or microcarrier cultures to propagate virus particles. Most common in industry are suspension cell cultures, which have been adapted to grow in a single-cell suspension within common vessels like stirred tank reactors or wave bags. Mammalian cells also grow adherently, meaning they attach to a surface. Adherent cells in the industry are often grown in roller bottles or on microcarrier beads in a simulated suspension culture. Cell-based processes have many advantages over the egg-based alternative. It is quicker and more flexible, allowing it to supply vaccines much faster, particularly in pandemics.[7] It also is a more controlled process, meaning that the final product has less variability.[7] However, the

cell-based process still requires a lengthy seed train scale-up before reaching production volume, where virus infection occurs.

A novel approach to further optimize cell-based influenza vaccine manufacturing and to create a continuous plug flow virus manufacturing process is to create a paper-based MDCK platform capable of immediate, high titer production of influenza virus for vaccines from cells stabilized and stored on paper. By growing and stabilizing adherent cells on paper, a high viable cell density can be achieved prior to virus infection. When combined with long-term storage of cells grown to a high cell density on paper, the paper-based manufacturing scheme becomes a ready-to-use, customizable, and fast solution to producing influenza virus globally for vaccines.

1.3. Growth of Mammalian Cells on Paper

In the past several years, there has been interest in exploring paper as a platform for growing mammalian cells. Paper is of interest as a cell growth substrate due to its many advantageous qualities over traditional substrates like glass or surface-modified plastics. Paper is environmentally friendly, biocompatible, and cost-effective to manufacture in large roll-to-roll quantities.[8,9] In addition, paper is very easily chemically and physically modified to promote cell attachment, spreading, and proliferation.[8,9] The porous structure of paper allows cells to grow in a 3D environment that more closely mimics the in vivo cellular environment as compared to 2D growth on polystyrene substrates.[8,9] The pores also allow mass transfer.[8]

The vast majority of mammalian cells on paper technology falls into the category of cells-in-gels-in paper (CiGiP).[10,11] In this technology, cells do not grow directly on paper, but instead paper is used as a structural scaffold to layer cells growing within hydrogels. By stacking multiple layers of paper, gel, and cells, researchers can create a simulation of *in vivo* tissues.[10,11] CiGiP is

also often combined with the printing of hydrophobic waxes or inks onto the paper, which prevent cell adherence.[9,12] This allows the control of the cell growth area.[9,12]

Many studies have demonstrated the growth of mammalian cells on paper for three major purposes – tissue engineering, diagnostics, and disease modeling.[8,9] Paper is an exceptional substrate for tissue engineering technologies because it can be used to implant stem cells *in vivo* for tissue regeneration.[8,9] The paper then degrades and leaves only the newly generated tissue. Paper is also utilized as a diagnostic tool to perform cell-based assays. This is particularly useful for cheap and disposable high-throughput screening of new drug candidates or disease diagnosis.[8,9] Finally, many researchers are utilizing paper to model tissues and better understand diseases. These studies all leverage CiGiP techniques to create life-like representations of tissues.[8,9]

While most mammalian cells grown on paper also rely on hydrogel as a substrate, three studies have grown mammalian cells directly on paper. These three studies are detailed in Table 1.3.1.

Table 1.3.1. Studies demonstrating the growth of mammalian cells on paper substrates.

Application	**Paper Type**	**Cell Type**	**Findings**
Cell-based assays	Proprietary mix of paper, pigments, and latex binder	ARPE-19	Of 4 screened paper types, cells grew best on smoothest paper with the highest surface free energy. Printing of hydrophilic PDMS was successfully used to control the cell growth area.[12]
Stem cell scaffolds	Weigh paper, chromatography paper, wiping tissue	hADSC	Stem cells preferred the smooth weigh paper. Stacks layered with cells and paper were implanted into mice and successfully used to regenerate bone.[13]
Cell-based assays	Chinese rice paper, Whatman filter paper, lens paper	DU145	Cells grew best on Chinese rice paper, and proved that cells on paper can be used as cheap platforms for cell-based assays.[14]

1.4. Paper-Based Mammalian Cell Manufacturing Platform

Mammalian cells capable of generating flu virus when infected have never been grown on paper, to the best of the author's knowledge, with the purpose of creating a cell-based virus manufacturing platform. This proof-of-concept study attempts to demonstrate the growth of MDCK cells on paper surfaces for the purpose of expedient, cost effective, and flexible manufacturing of influenza vaccine. The benefits of this paper-based manufacturing platform over traditional adherent platforms include the increase of surface area provided by the paper, the ease of modifying paper to promote the best cell growth, and cost effectiveness and availability of paper.

Growth of manufacturing cell lines on paper has a range of possible applications. Cells grown to high density on paper can be manufactured roll-to-roll, mimicking the continuous method of paper manufacturing. Cells on paper can then be frozen or possibly dried under controlled conditions (lyopreservation) to create a very large manufacturing cell bank that can be shipped to multiple manufacturing facilities to ensure uniform global flu virus manufacture. Upon thawing the cells on paper, they can be immediately infected for virus production, thereby skipping the lengthy seed train required in traditional cell-based manufacturing. A paper-based manufacturing platform could also be leveraged to create an integrated, continuous plug flow moving bed manufacturing platform by modifying the paper attributes to selectively bind cells and cellular debris but release virus particles, thereby providing a clarification step at the end of virus production.

One proposed use of this manufacturing platform is as a basis for a continuous plug flow reactor with a moving paper bed. A plug flow reactor is a long, thin reactor that has a constant feed into one end and continuous expulsion of product and waste from the other end (Figure 1.4.1).[25]

The plug is a cross-sectional area that moves laterally down the length of the reactor. Plugs do not mix laterally with each other, so there are variable concentrations throughout the length of the reactor.[25] However, plugs do mix axially, thereby mixing with any immobilized catalyst within the reactor.[25] Plug flow reactors are often used with immobilized catalysts, where intermediates are fed into the reactor, interact with the fixed catalyst, and are then released as final product.[27,28] In the context of this study, whole cells are used as catalysts immobilized on paper, and they "catalyze" the formation of new virus particles.

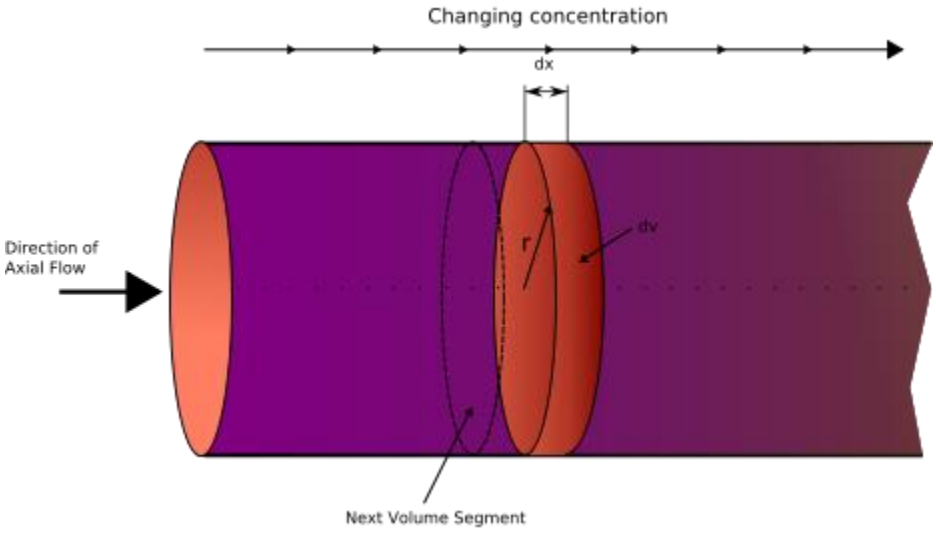


Figure 1.4.1. Plug flow reactor with unidirectional axial flow of separate plugs.[26]

As a first step in the pursuit of using paper as a substrate for cell-based manufacturing, this study examines 14 different types of paper for their suitability for cell culture. The seven most likely papers were used as cell growth substrates, and the paper that yielded the best cell growth was selected for simulated plug flow reactor (SPFR) studies.

CHAPTER 2: Materials and Methods

2.1. Cell Culture

Adherent MDCK (ATCC Cat# CCL-34) was cultured with Eagle's Minimum Essential Medium (ATCC, Cat# 30-2003) supplemented with 5% fetal bovine serum (FBS) (Gibco, Cat# 10099-141), 4 mM L-Glutamine (Gibco, Cat# A2916801), and 3.5 g/L glucose. Cells were cultured in Corning tissue culture-treated T-flasks and were detached using TrypLE Select, a recombinant Trypsin (Gibco, Cat# 12563-011). A growth study was performed with the 10% FBS-adapted cell line, using a Vicell XR to determine viable cell count, to determine the growth rate of the cell line. The cell line was then weaned from 10% FBS to 5% FBS by adapting it first to 7.5% FBS over the course of 3 passages and then to 5% over the course of 3 passages. Another growth study was performed with the 5% FBS-adapted MDCK to assess any changes to growth rate that the reduction of FBS had. The 5% FBS-dependent cell line was banked at $5x10^6$ cells/mL in growth media supplemented with 10% DMSO. More detailed cell culture protocols, including vial thaw, cell passaging, and banking are detailed in Appendixes A through C.

2.2. Paper Characterization

Fourteen papers were selected to test based on their ease of procurement. Those papers are listed in Table 2.2.1 with the manufacturer/distributor and the catalogue number.

Table 2.2.1. Paper type, manufacturer/distributor, and catalogue number.

Manufacturer/Distributor	Paper Type	Catalogue Number
Staples	Multipurpose Paper	Staples 651659
Staples	Loose Leaf Paper	Staples 849398
Oxford	Stone Paper	Staples 2739887
Legion	YUPO® Translucent	Legion L21-YPT153WH912
Strathmore	300 Series Bristol Smooth	Strathmore 342-9
Kirkland's	Parchment Paper	Kirkland's 773084
Pacon Corporation	Poster	Pacon 5460-5
Whatman	3MM Chromatography	Sigma-Aldrich WHA3030917
Fisherbrand	Autoclave Pouch	Fisher Scientific 01-812-54
Texwipe	VersaWipe	Texwipe TX622
Whatman	Weigh Paper	GE Healthcare 10347671
Fisherbrand	Lens Paper	Fisher Scientific 11-996
Forest Biomaterials Department	Blotter Paper	N/A
Fisherbrand	Bibulous Paper	Fisher Scientific 11-998

2.2.1. Sterilization and Incubation of Paper

Discs with a 32mm diameter were punched from each of the fourteen papers. Discs were put into autoclave pouches and were autoclaved at 122˚C for 60 minutes. Autoclaved paper discs were aseptically transferred into Corning 6-well plates using autoclaved forceps. Paper discs were incubated in cell culture media at 37˚C and 5% CO_2 for 5 days, after which wells were examined for signs of contamination or turbidity. Autoclaved forceps were then used to transfer paper into fresh cell culture plates with fresh media and incubated a further 24 hours. Wells were again examined for any sign of contamination.

After the 5-day incubation in cell culture media, paper discs were also examined for their ability to withstand incubation. Paper discs were removed from wells using sterile forceps, and the discs were then imaged alongside the original dry paper and autoclaved paper to draw conclusions about the wet durability of the paper.

2.2.3. Cold Water pH Extraction

The pH of each type of paper was characterized via the cold water extraction pH determination method, adapted from a 1939 study standardizing the measurement of paper pH.[18] Each type of paper was cut into 5-10 mm squares using at least 5 different sheets of paper. 1.00±0.1 g of cut paper was weighed out and then added to a clean Erlenmyer flask. 10 mL of MilliQ water was then added to the each flask, and a metal stir rod was used to macerate the paper discs in the water. A starting pH of the MilliQ water, before addition to paper, was recorded. The paper was incubated in water for one hour, statically, after which the pH of the water was taken.

2.2.4. Surface Roughness

Surface roughness was measured according to the Technical Association of Pulp and Paper Industry (TAPPI) standard test method T 555 om-99, "Roughness of Paper and Paperboard (Print-Surf Method)."[23] This method uses a Parker Print Surf testing machine (Lorentzen & Wettre, L&W PPS Tester) to measure the surface roughness of the paper. The machine clamps the paper's top and bottom surface and measures the resistance of airflow past the clamp to determine an average surface roughness measurement in µm.[24] 5 pieces of each paper were tested, and the results were averaged.

2.2.5. Paper Thickness

Paper thickness was measured with a Mitutoyo Absolute digital micrometer with a 12.5 mm flat foot probe attached. Three measurements were taken of each type of paper.

2.3. Batch Growth Study of Adherent MDCK on Paper

Paper was cut into 32 mm diameter disks and were sterilized via autoclaving at 121˚C for 60 minutes. Sterile paper was transferred into Corning 6-well ultra-low attachment plates using

sterile forceps, and 2 mL of complete EMEM was aliquoted into each well. Plates with paper and media were incubated for at least 2 hours to allow complete wetting of the paper, but were most commonly incubated for 24 hours before inoculation. MDCK were seeded onto paper at 30,000 cells/cm^2, and plates were incubated statically at 37˚C and 5% CO_2. Negative and positive controls were run alongside cells seeded on paper. The negative control consisted of paper discs incubated in media in 6-well plates, but with no cells seeded onto the paper. The positive control consisted of cells seeded at 30,000 cells/cm^2 in T-25 tissue culture-treated T-flasks. At 12- to 24-hour intervals, staining and imaging was performed to view cells on paper and compare this to the negative and positive controls. At each time point, 3 separate paper disks/flasks were imaged, and 2 images were taken from each disk/flask for a population size of 6. Calcein AM (Invitrogen, Cat# C3099), a fluorescent viability stain, was used to stain live cells on paper and in T-flasks. At the time of imaging, cells were incubated with 1 μM of a Calcein AM solution in complete EMEM for 20 minutes. Cells were then imaged with a Nikon inverted light microscope with a fluorescence illuminator (Nikon Intensilight C-HGFI) and a fluorescence filter cube (Nikon FITC-HYQ) filtering light wavelengths for an excitation of 460-500 nm. Fluorescent images were captured at a 4X objective with a Dino-Eye digital eye-piece microscope camera. Images were analyzed using Olympus CKX-CCSW Cell Confluence Checker software to quantify the confluence, or percent surface area, that the cells encompass. Surface confluence was compared between cells grown on paper and cells grown in T-flasks. The method of seeding MDCK on paper is depicted in Figure 2.3.1., and the full protocol is detailed in Appendix D.

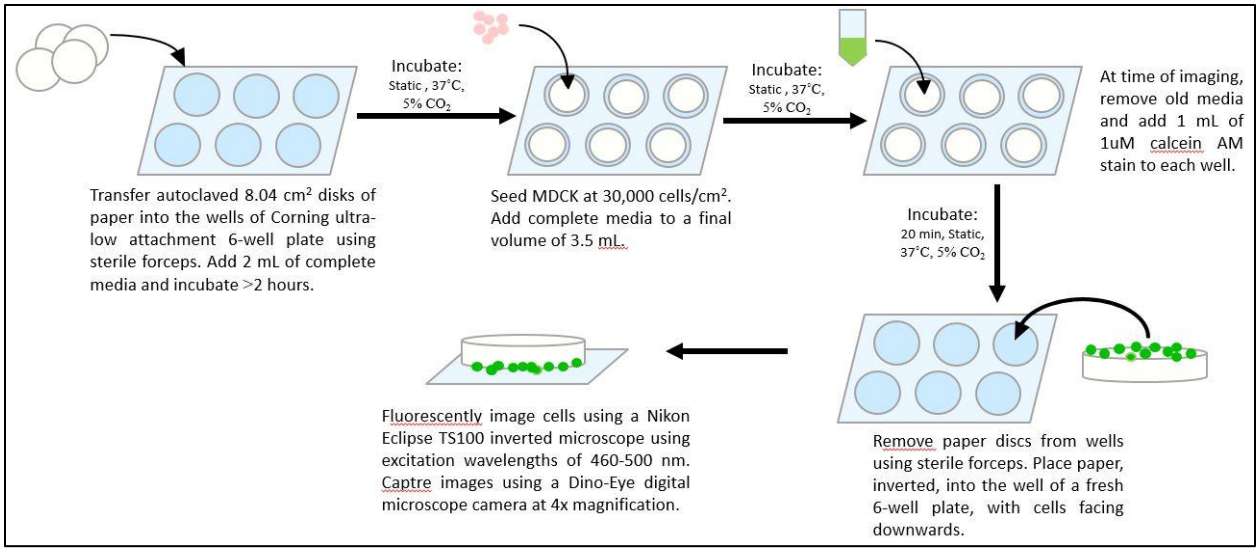


Figure 2.3.1. Batch growth study of MDCK on paper method

2.4. Simulated Plug Flow Reactor (SPFR) Growth of MDCK on Bibulous Paper

Bibulous paper was cut into 32 mm diameter disks and were sterilized via autoclaving at 121˚C for 60 minutes. Sterile paper was transferred into Corning 6-well ultra-low attachment plates using sterile forceps, and 2 mL of complete EMEM was aliquoted into each well. Plates with paper and media were incubated overnight to allow complete wetting of the paper. MDCK were seeded onto paper at 30,000 cells/cm^2, and plates were incubated statically at 37˚C and 5% CO_2. Negative and positive controls were run alongside cells seeded on paper. The negative control consisted of paper discs incubated in media in 6-well plates, but with no cells seeded onto the paper. The positive control consisted of cells seeded at 30,000 cells/cm^2 in T-25 tissue culture-treated T-flasks.

Starting at 48 hours post-inoculation, plug flow was simulated by completely replacing spent media with fresh complete EMEM. This re-feeding was performed every 24 hours after the first re-feeding. At 24 hour intervals starting 24 hours post-inoculation, spent media was analyzed for viable cells and metabolites, and staining and imaging was performed to view cells on paper

and compare this to the negative and positive controls. At each time point, 3 separate paper disks/flasks were imaged, and 2 images were taken from each disk/flask for a population size of six. Calcein AM (Invitrogen, Cat# C3099), a fluorescent viability stain, was used to stain live cells on paper and in T-flasks. At the time of imaging, cells were incubated with 1 μM of a Calcein AM solution in complete EMEM for 20 minutes. Cells were then imaged with a Nikon inverted light microscope with a fluorescence illuminator (Nikon Intensilight C-HGFI) and a fluorescence filter cube (Nikon FITC-HYQ) filtering light wavelengths for an excitation of 460-500 nm. Fluorescent images were captured at a 4X objective with a Dino-Eye digital eye-piece microscope camera. Images were analyzed using Olympus CKX-CCSW Cell Confluence Checker software to quantify the confluence, or percent surface area, that the cells encompass. Surface confluence for this simulated plug flow reactor (SPFR) study was compared between cells grown on paper and cells grown in T-flasks. The full protocol is detailed in Appendix E.

2.5. Confocal Microscopy of MDCK on Bibulous Paper

A confocal microscope LSM 880 at the Cellular and Molecular Imaging Facility was used to image MDCK on bibulous paper. During microscope user training, samples of batch-growth MDCK at 24 hours, 48 hours, and 72 hours post-inoculation were imaged. The 40X immersion objective was used to image. A green channel (AF488) and blue channel (DAPI) were used to image cells and paper. The best image quality was determined to be 1024x1024, and the optimal slice thickness was determined to be 2μm.

A time-course of MDCK grown with simulated plug flow conditions was generated, with two samples for each 24-hour interval, starting at 72 hours and going through 196 hours. Each sample was imaged two to three times. The z-stack of the images were examined to determine how deeply the cells penetrated within the structure of the paper. Images from the bottom surface of

the paper were also taken to determine if cells had grown through the paper or had adhered to the bottom surface of the paper. A more detailed protocol for imaging using the LSM 880 microscope can be found in Appendix F.

2.6. Investigation of the Effect of Soak Time on MDCK Growth on Bibulous Paper

Due to variability in growth curve data for SPFR MDCK on bibulous paper, the effect of the soak time of autoclaved paper in media before inoculation was investigated. Soak times of 24 hours and 0 hours were studied. 32 mm discs of bibulous paper were autoclaved at 121˚C for 60 minutes. Sterile paper was transferred into Corning 6-well ultra-low attachment plates using sterile forceps. The 24-hour soak time samples were incubated with 2 mL of complete EMEM overnight. After 24 hours, the media was tested for pH and glutamine concentration. At the time of inoculation, 2 mL of complete EMEM was added to the 0-hour soak time samples. All paper was then seeded with 30,000 cells/cm^2. The discs were then stained, imaged, and analyzed for surface confluence at 24-hour intervals as previously described. The surface confluence of the 24-hour soak time and 0-hour soak time samples were compared.

CHAPTER 3: Results

3.1. Paper Characterization

Each of fourteen papers was characterized for (1) its ability to be sterilized via autoclaving, (2) its ability to withstand incubation in cell culture media under cell culture conditions, (3) pH, (4) surface roughness, and (5) thickness.

All fourteen papers were able to be sterilized via autoclaving at 122˚C for 60 minutes. Autoclaved paper discs were incubated in cell culture media at 37˚C and 5% CO_2 for 5 days, after which no signs of contamination or turbidity were observed. Autoclaved forceps were then used to transfer paper into fresh cell culture plates with fresh media and incubated a further 24 hours. No contamination was observed for any type of paper.

Each paper was also examined for its ability to be incubated in media for extended periods of time at 37˚C and 5% CO_2. Autoclaved paper was incubated in media for 5 days, and then discs were removed with sterile forceps and imaged (Table 3.1.1). Staples multipurpose photocopy paper and loose leaf paper were particularly delicate after incubation, and tore upon removal from wells with forceps. Lightweight papers like stone paper, parchment paper, autoclave pouch, and weigh paper floated on top of the media and did not fully submerge in the wells. Heavier, more rigid papers like YUPO®, multimedia art paper, poster, and chromatography paper sat flat on the bottom of the well, fully saturated with media.

The pH of each type of paper was characterized via the cold water extraction method. The starting pH of the distilled water was 6.00±0.25. Results of pH determination after one hour incubation of macerated paper in distilled water are summarized in Table 3.1.1.

Surface roughness was measured using a Parker Print Surf testing machine, which clamps the paper's surface and measures the resistance of airflow past the clamp to determine an average

surface roughness measurement in µm. Surface roughness measurements are summarized in Table 3.1.1.

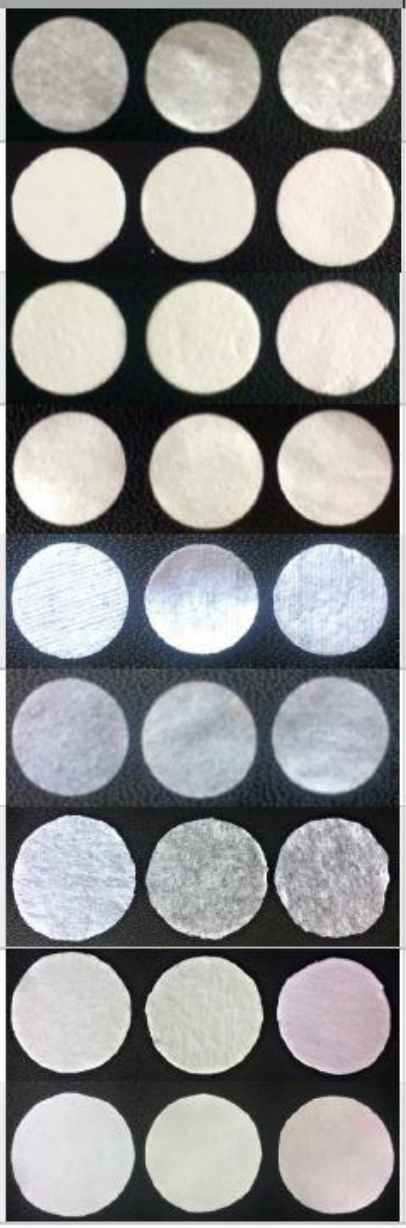
Paper thickness was measured with a Mitutoyo Absolute digital micrometer. Results are summarized in Table 3.1.1. The thickest papers included the Strathmore Bristol multimedia art paper, poster, Whatman 3MM chromatography paper, and Forest Biomaterials blotter paper. The thinnest papers were the Whatman weigh paper and Fisherbrand lens paper.

Paper materials of construction were also requested from the distributors/manufacturers. Some companies, like those that manufacture office supply and art paper, were unable to provide that information. Legion YUPO® translucent is a synthetic art paper made entirely of polypropylene. Oxford stone paper is also a synthetic paper but is made from limestone and polyethylene. Materials of construction are listed in Table 3.1.1.

Table 3.1.1. Paper characterization results.

Paper	Materials of Construction	Mass (mg/cm^2) (N=3)	Before Autoclave / After Autoclave / After Incubation (5d EMEM)	Cold Water Extraction pH (N=2)	Surface Roughness (µm, N=5)	Thickness (µm, N=3)
Staples Multi-purpose Paper	No Data	7.11 ± 0.001		8.81 ± 0.12	7.54 ± 0.16	107.7 ± 0.5
Staples Loose Leaf Paper	No Data	5.27 ± 0.001		9.52 ± 0.06	5.93 ± 0.10	74.6 ± 0.5
Oxford Stone Paper	80-90% Limestone, Polyethylene (PE)	6.15 ± 0.006		Insufficient sample	3.94 ± 0.09	101.0 ± 5.0
Legion YUPO® Translucent	Poly-propylene	15.53 ± 0.001		9.21 ± 0.04	3.40 ± 0.10	151.7 ± 0.5
Strathmore 300 Series Bristol Smooth	No Data	28.17 ± 0.001		8.73 ± 0.24	6.37 ± 0.15	361.3 ± 14.7

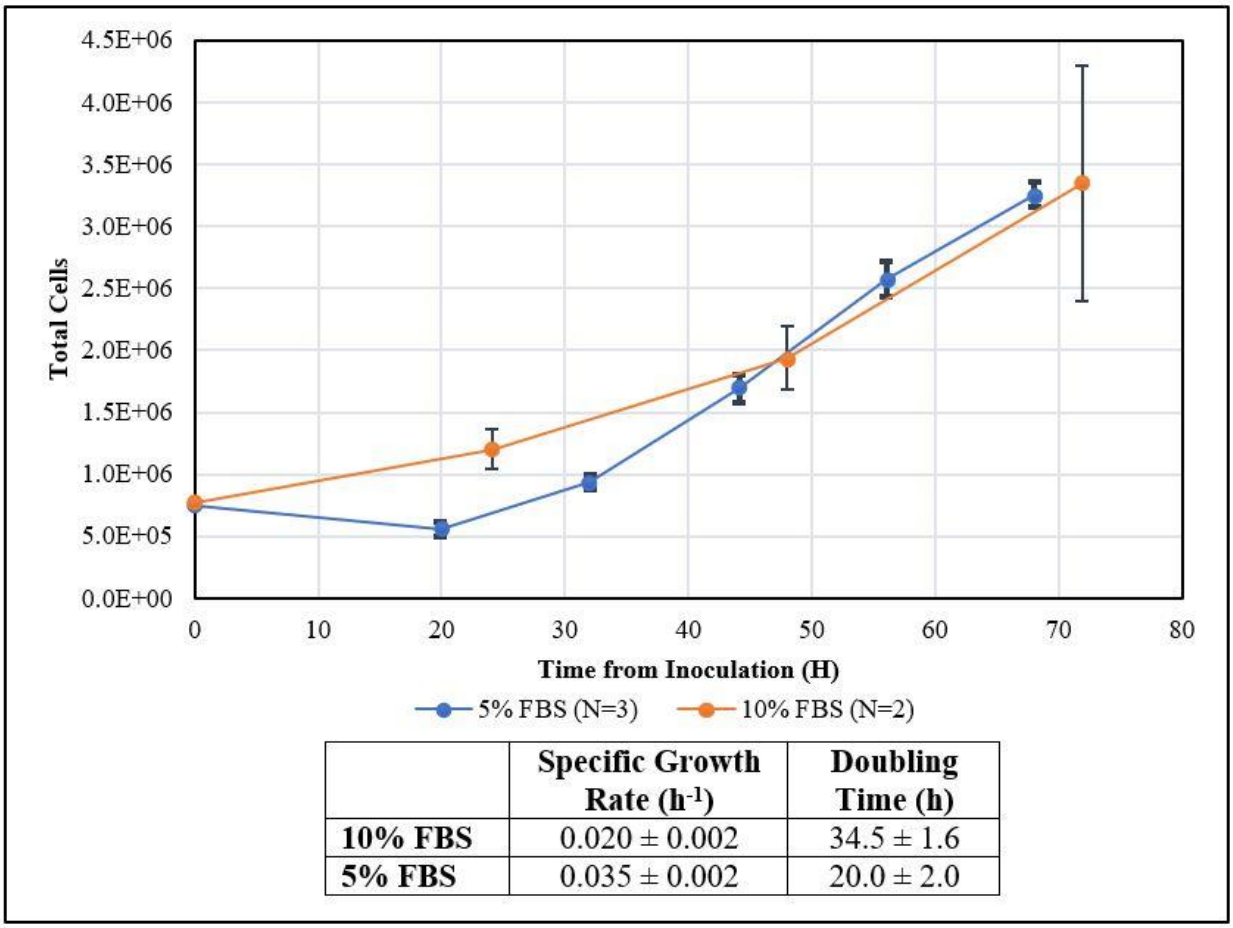
Table 3.1.1 (continued).

Paper	Materials of Construction	Mass (mg/cm^2) (N=3)	Before Autoclave / After Autoclave / After Incubation (5d EMEM)	Cold Water Extraction pH (N=2)	Surface Roughness (µm, N=5)	Thickness (µm, N=3)
Kirkland's Parchment Paper	No Data	1.84 ± 0.001		7.40 ± 0.08	394.36 ± 34.66	61.7 ± 3.1
Poster	No Data	29.75 ± 0.001		7.34 ± 0.01	Top: 2.51 ± 0.10 Bottom: 7.48 ± 0.11	393.3 ± 10.4
Whatman 3MM Chromato-graphy	100% Cellulose	18.69 ± 0.001		6.56 ± 0.22	646.43 ± 14.28	343.7 ± 5.9
Fisherbrand Autoclave Pouch	No Data	4.48 ± 0.002		6.73 ± 0.06	7.80 ± 0.06	88.3 ± 2.5
Texwipe VersaWipe	55% Cellulose, 45% Polyester, Nonwoven	6.32 ± 0.001		6.52 ± 0.15	534.76 ± 18.54	275.0 ± 9.4
Whatman Weigh Paper	Cellulose-based Parchment Paper	1.42 ± 0.002		6.84 ± 0.12	1.70 ± 0.02	38.3 ± 3.3
Fisherbrand Lens Paper	Natural Pulp	1.37 ± 0.017		5.56 ± 0.03	5.00 ± 0.21	39.3 ± 1.7
Forest Bioaterials Blotter Paper	No Data	27.58 ± 0.671		6.64 ± 0.05	11.59 ± 0.37	563.3 ± 13.1
Fisherbrand Bibulous Paper	Natural Pulp	7.73 ± 0.075		9.16 ± 0.10	8.39 ± 0.74	128.3 ± 2.1

3.2. Weaning of MDCK from 10% to 5% FBS

MDCK, originally adapted to media supplemented with 10% FBS, were weaned to 5% FBS by gradually decreasing FBS levels to 5% over several passages. A batch growth study was performed on 10% serum-adapted MDCK and 5% serum-adapted MDCK to compare growth rates before and after serum reduction (Figure 3.2.1).

The growth curve comparison showed that 5% serum-adapted MDCK had a lag phase after inoculation but had a greater growth rate once the exponential growth phase was reached. The 10% serum-adapted MDCK went through exponential growth from T=0 hours from inoculation through T=72 hours from inoculation, while the 5% serum-adapted cells went through exponential growth from T=20 hours from inoculation through T=56 hours from inoculation. The 10% serum-adapted MDCK had an exponential growth rate of 0.020 ± 0.002 h^{-1} and a corresponding doubling time of 34.5 ± 1.6 hours. The 5% serum-adapted MDCK had an exponential growth rate of 0.035 ± 0.002 h^{-1} and a doubling time of 20.0 ± 2.00 hours. Both cell lines reached a similar final total cell count around 70 hours post-inoculation before growth plateaued and then declined.



	Specific Growth Rate (h^{-1})	Doubling Time (h)
10% FBS	0.020 ± 0.002	34.5 ± 1.6
5% FBS	0.035 ± 0.002	20.0 ± 2.0

Figure 3.2.1. Growth curve comparison of 10% serum-adapted MDCK and 5% serum-adapted MDCK.

3.3. Growth of 5% Serum-Adapted MDCK on Paper

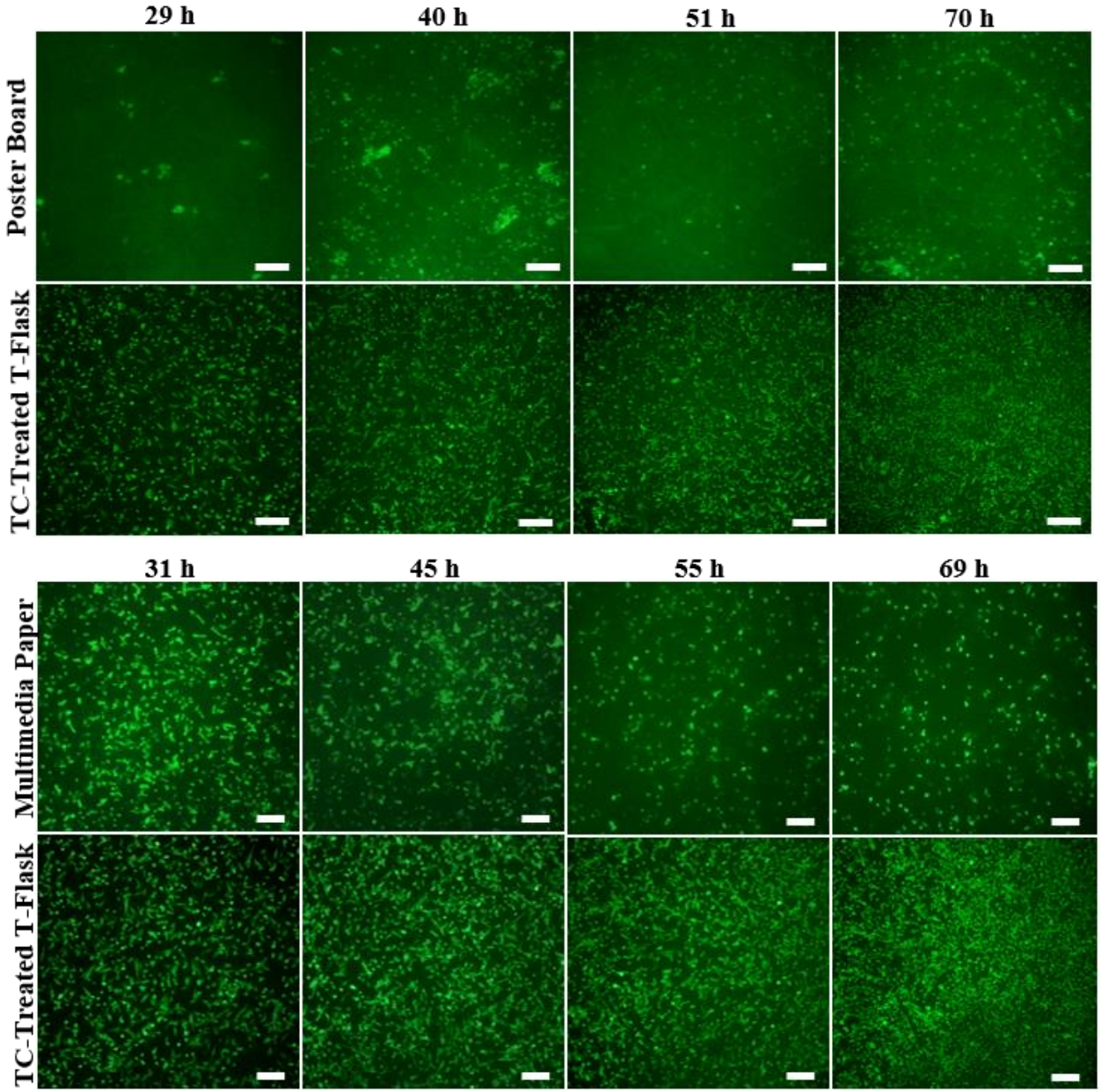
5% serum-adapted MDCK were grown on poster, multimedia art paper, weigh paper, blotter paper, autoclave pouch paper, lens paper, and bibulous paper to determine the best paper to promote cell adhesion, spreading, and proliferation. Adhesion and confluence on paper was compared to adhesion and confluence in T-25 tissue culture treated flasks via fluorescent staining, imaging, and image confluence analysis. Each growth study on paper was accompanied by a representative growth study in T-25 flasks, which were imaged alongside the paper for qualitative

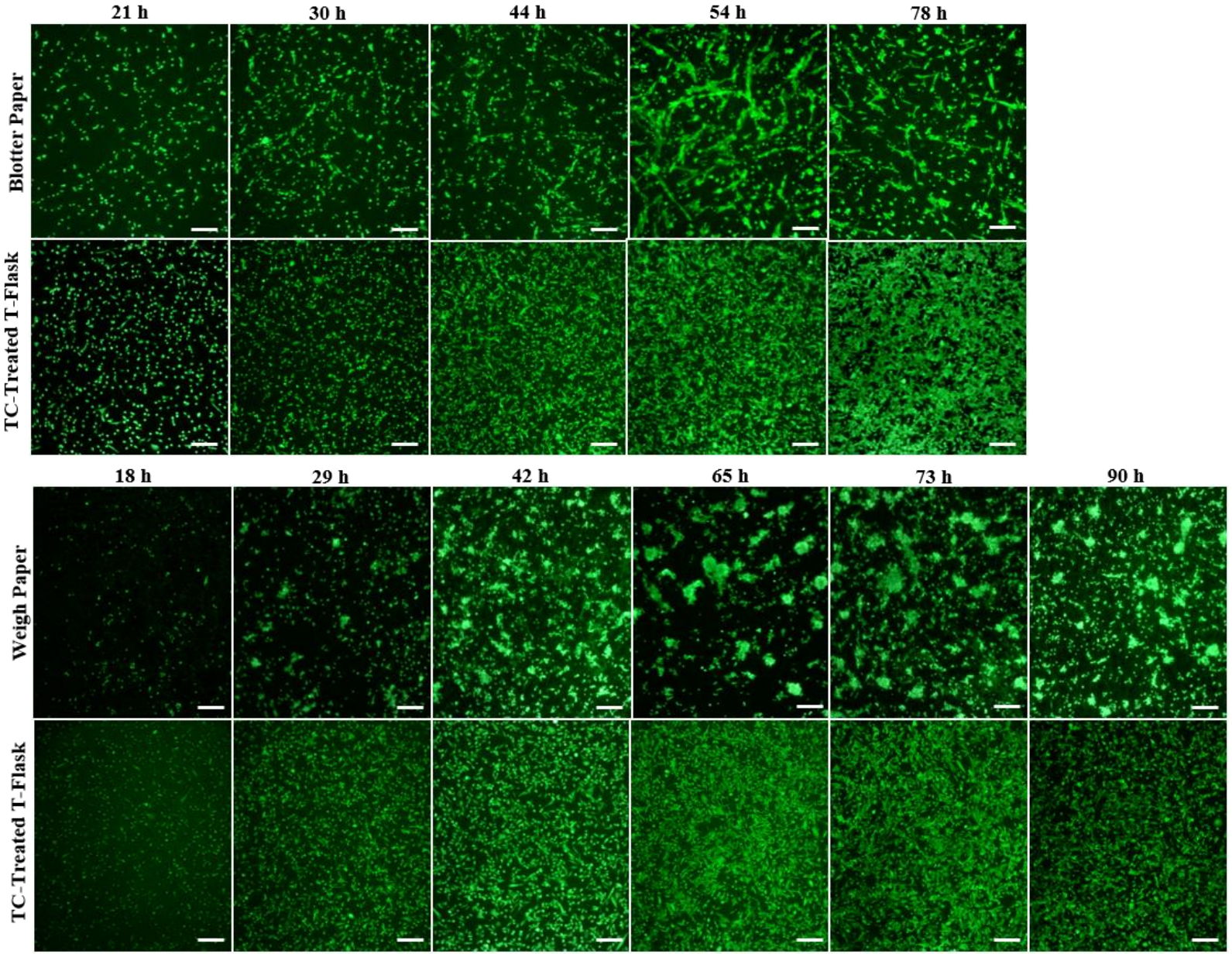
comparison purposes (Figure 3.3.1). A representative T-25 flask positive control was also generated, and the confluence values of this flask were quantitatively compared with confluence values for each paper type (Figure 3.3.2).

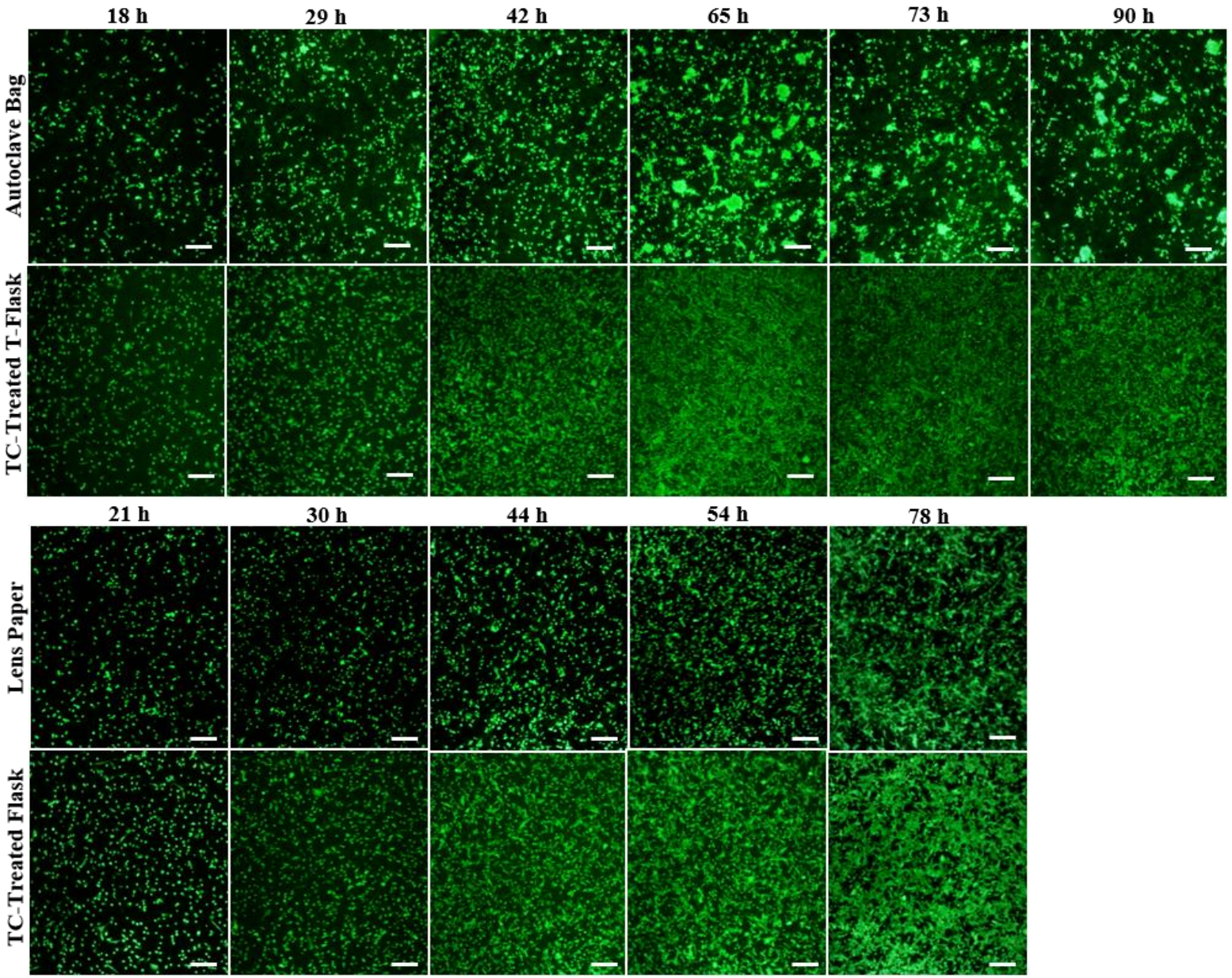
The least successful paper was poster. Cells struggled to attach and were never able to proliferate over a span of 70 hours. Bristol multimedia art paper was also unsuccessful. Cells were initially able to attach to the paper, and at 31 hours post-inoculation reached a confluence of 23%. However, between 31 hours and 69 hours, the cells declined down to 8% confluence. Weigh paper, blotter paper, and autoclave pouch paper all performed similarly, all reaching maximum confluence of around 30% before declining. Weigh paper and autoclave pouch paper caused cells to group and grow in large clumps, rather than colonizing the entire paper surface. On blotter paper, the cells clumped and grew along the cellulose fibers, but weren't able to fully colonize the surface. Lens paper proved to be the second best paper, gradually reaching a confluence of 40% after 68 hours of incubation. However, the large gaps in the paper structure and the thinness of the paper gave the cells less surface to colonize. Cells grew best on the bibulous paper, reaching 48% confluence after an incubation of 49 hours. Images of MDCK growing on bibulous paper depict cells that are the most morphologically similar to cells grown on tissue culture-treated polystyrene. The cells do not clump, and have similar elongated shapes as those grown on polystyrene.

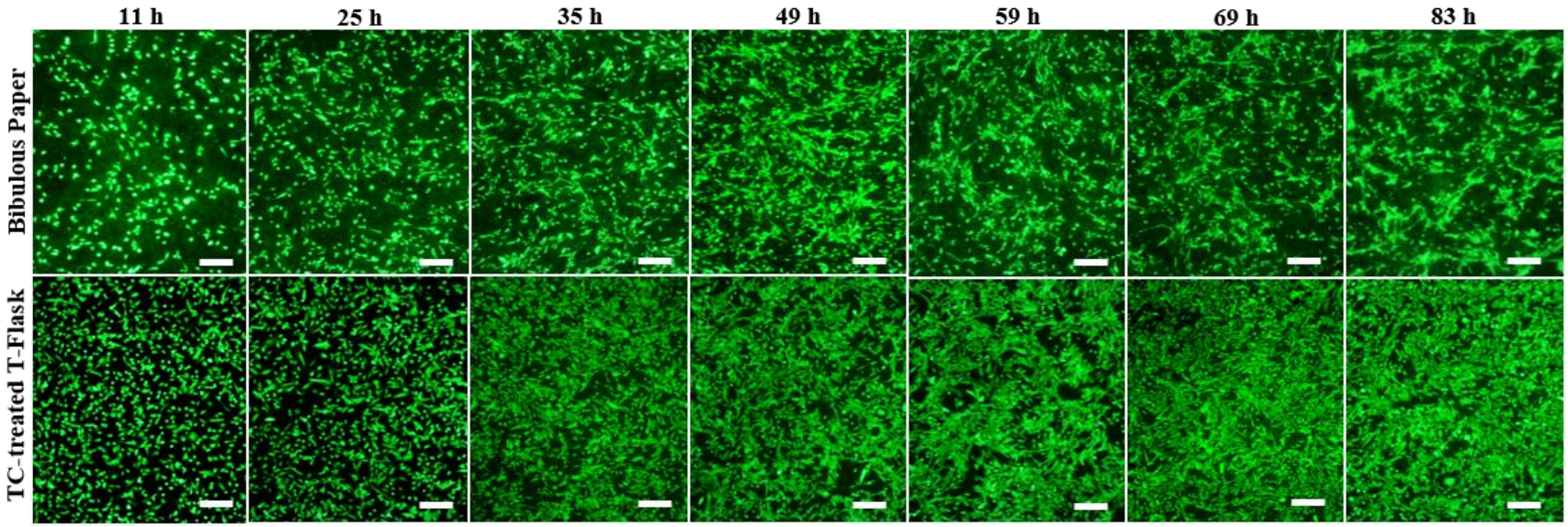
Additional images of MDCK grown on paper can be found in Appendix G.

Figure 3.3.1. Fluorescently stained and imaged MDCK growth on paper. (1) poster, (2) Strathmore Bristol 300 Series Smooth Surface Multimedia Art Paper, (3) Forest Biomaterials Blotter Paper, (4) Whatman Weigh Paper, (5) Fisherbrand Autoclave Pouch, (6) Fisherbrand Lens Paper, and (7) Fisherbrand Bibulous Paper. Each time course of images is aligned with a positive control of fluorescently imaged MDCK on tissue culture-treated T-flasks grown alongside the MDCK on paper. Scale bars represent 200 µm.


29 h
40 h
51 h
70 h
Poster Board
TC-Treated T-Flask
31 h
45 h
55 h
69 h
Multimedia Paper
TC-Treated T-Flask


21 h
30 h
44 h
54 h
78 h
Blotter Paper
TC-Treated T-Flask
18 h
29 h
42 h
65 h
73 h
90 h
Weigh Paper
TC-Treated T-Flask


18 h
29 h
42 h
65 h
73 h
90 h
Autoclave Bag
TC-Treated T-Flask
21 h
30 h
44 h
54 h
78 h
Lens Paper
TC-Treated Flask


11 h
25 h
35 h
49 h
59 h
69 h
83 h
Bibulous Paper
TC-treated T-Flask

A growth curve of the confluence of MDCK on paper over time was generated for each paper and compared with MDCK growth in T-25 flasks (Figure 3.3.2). Bibulous paper was identified as having growth characteristics closest to those of MDCK grown on tissue culture-treated polystyrene before the number of viable cells deteriorated after 49 hours of culture.

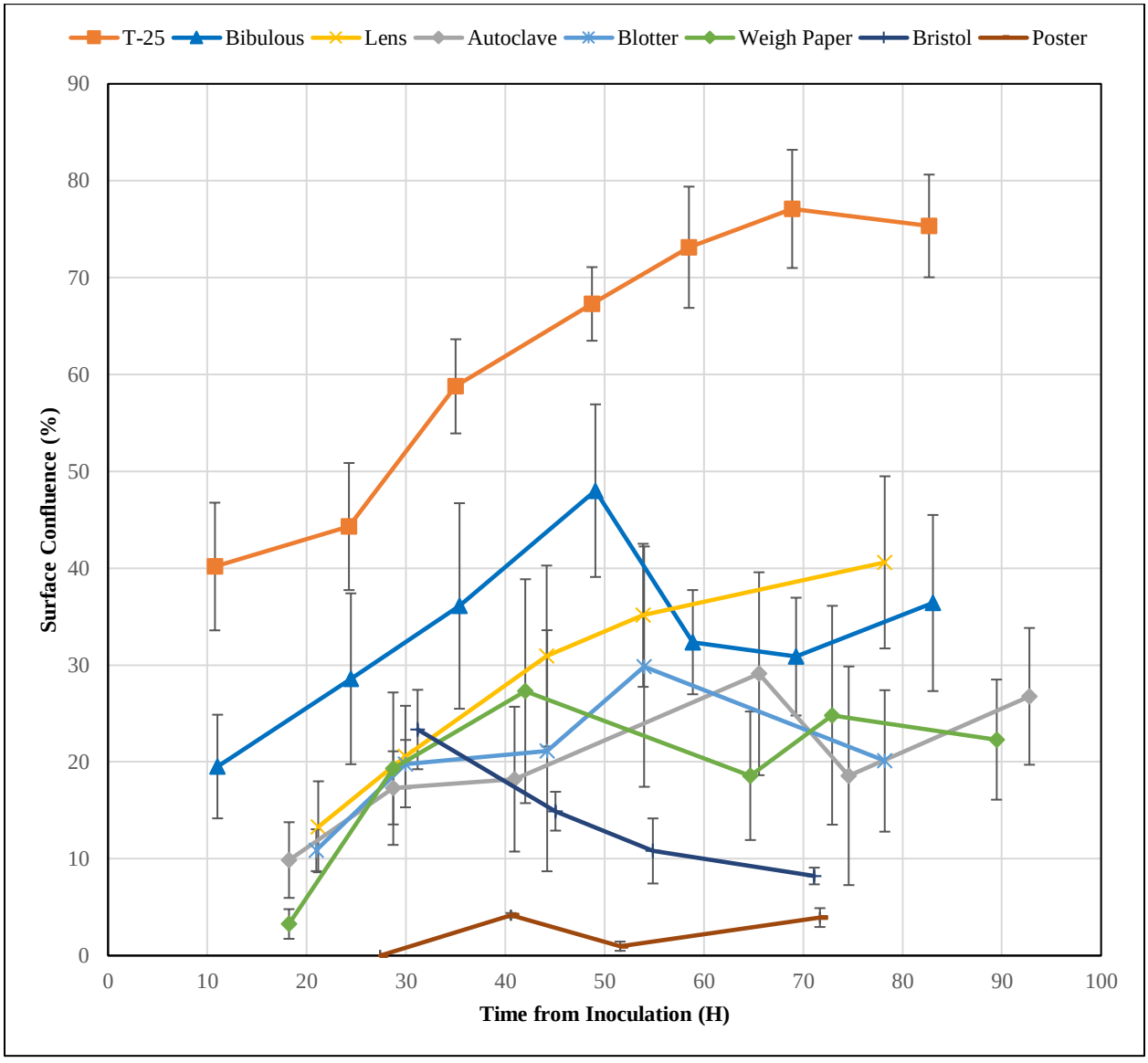


Figure 3.3.2. Surface confluence of MDCK batch-grown on papers versus MDCK batch-grown in T-flasks. N=6.

3.4. Simulated Plug Flow Reactor (SPFR) Growth of MDCK on Bibulous Paper

Combining continuous manufacturing with a paper-based manufacturing platform is of great interest, so a continuous plug flow reactor was simulated with MDCK grown in T-flasks and on bibulous paper. This was done by removing spent media every 24 hours starting 48 hours from inoculation. Images of fluorescently stained MDCK were taken and they were analyzed for confluence (Figure 3.4.1). MDCK under SPFR conditions in T-flasks reached 80% confluence around 67 hours after inoculation, and MDCK on bibulous paper reached 80% confluence about 131 hours after inoculation. Images of MDCK grown on bibulous paper with the SPFR method are shown in Figure 3.4.2.

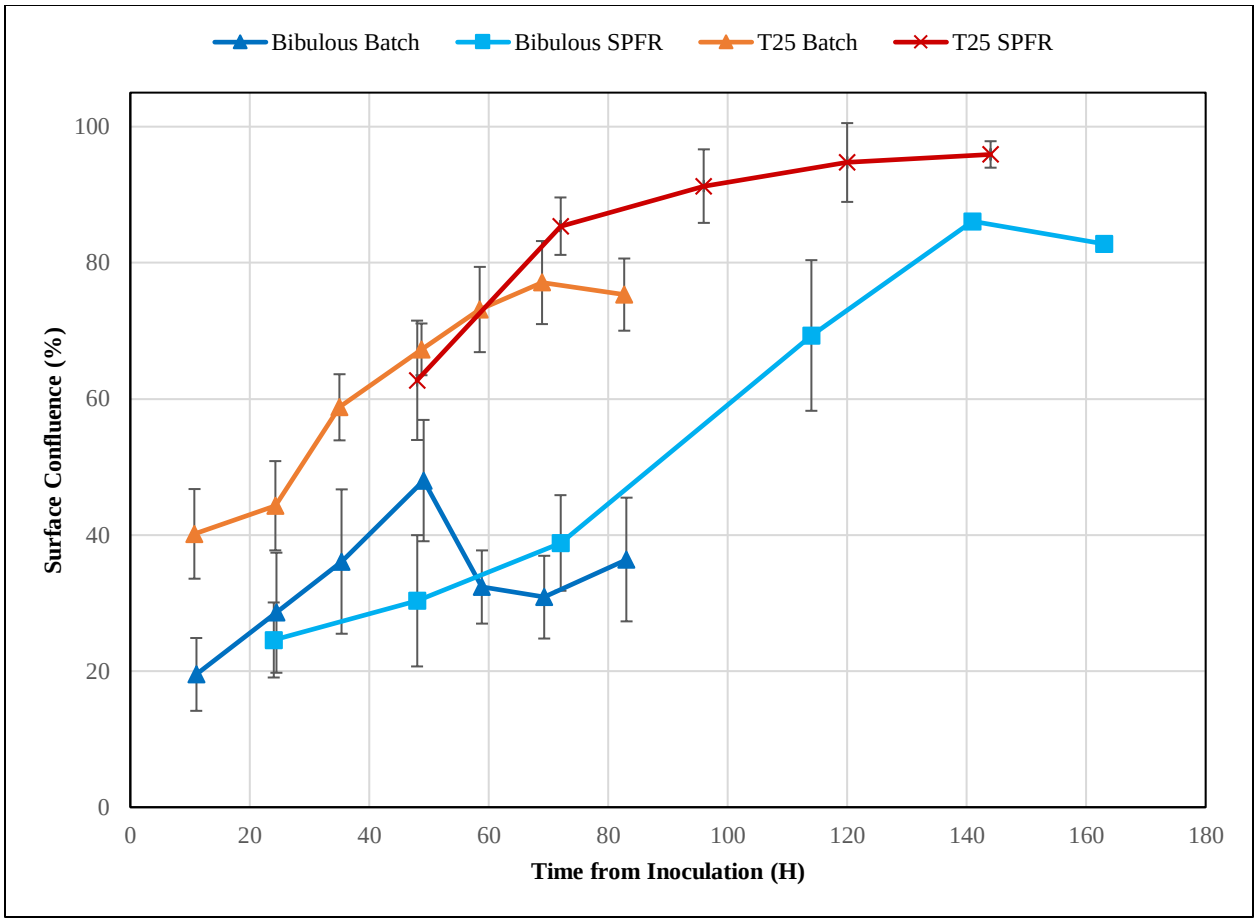


Figure 3.4.1. Simulated plug flow growth of MDCK in T-25 flasks and on bibulous paper compared to batch growth of MDCK in T-25 flasks and on bibulous paper. N=6.

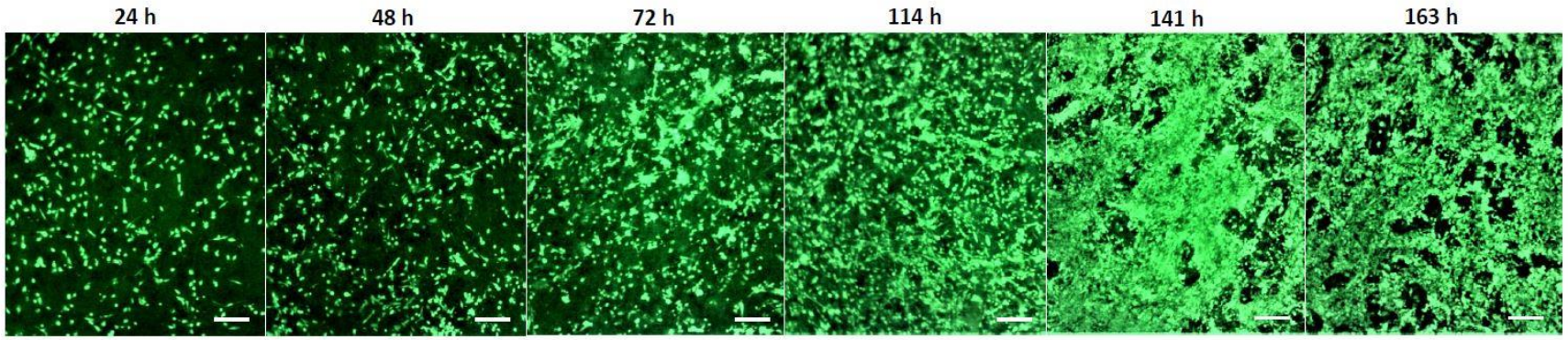


Figure 3.4.2. Simulated plug flow reactor growth of MDCK on bibulous paper. Scale bars represent 200 μm

3.5. Confocal Microscopy of MDCK on Bibulous Paper

Confocal microscopy was used to image fluorescently stained MDCK to characterize growth on bibulous paper (Figure 3.5.1). Bibulous paper had a natural blue fluorescence, and so was able to be fluorescently imaged using a DAPI channel. Confocal images were taken at 24-hour intervals during an SPFR growth study, with images beginning at 72 hours post-inoculation and continuing through 192 hours post-inoculation. For each timepoint, two samples were imaged two to three times each. Images were collected from both the top and bottom of the paper to determine if cells had growth through the paper or attached to the bottom surface. No significant cell growth was seen on the bottom of the paper. On average, the confocal microscope was able to image 63μm into the paper, and bibulous paper is 128.3 ± 2.1 μm thick. Orthogonal view of the z-stacked images (Figure 3.5.1.C.) was used to view the depth at which cells are able to colonize the paper. In all confocal images, cells were observed on the top surface of the paper, but no cells were observed to penetrate through the paper. Additional confocal images can be found in Appendix H.

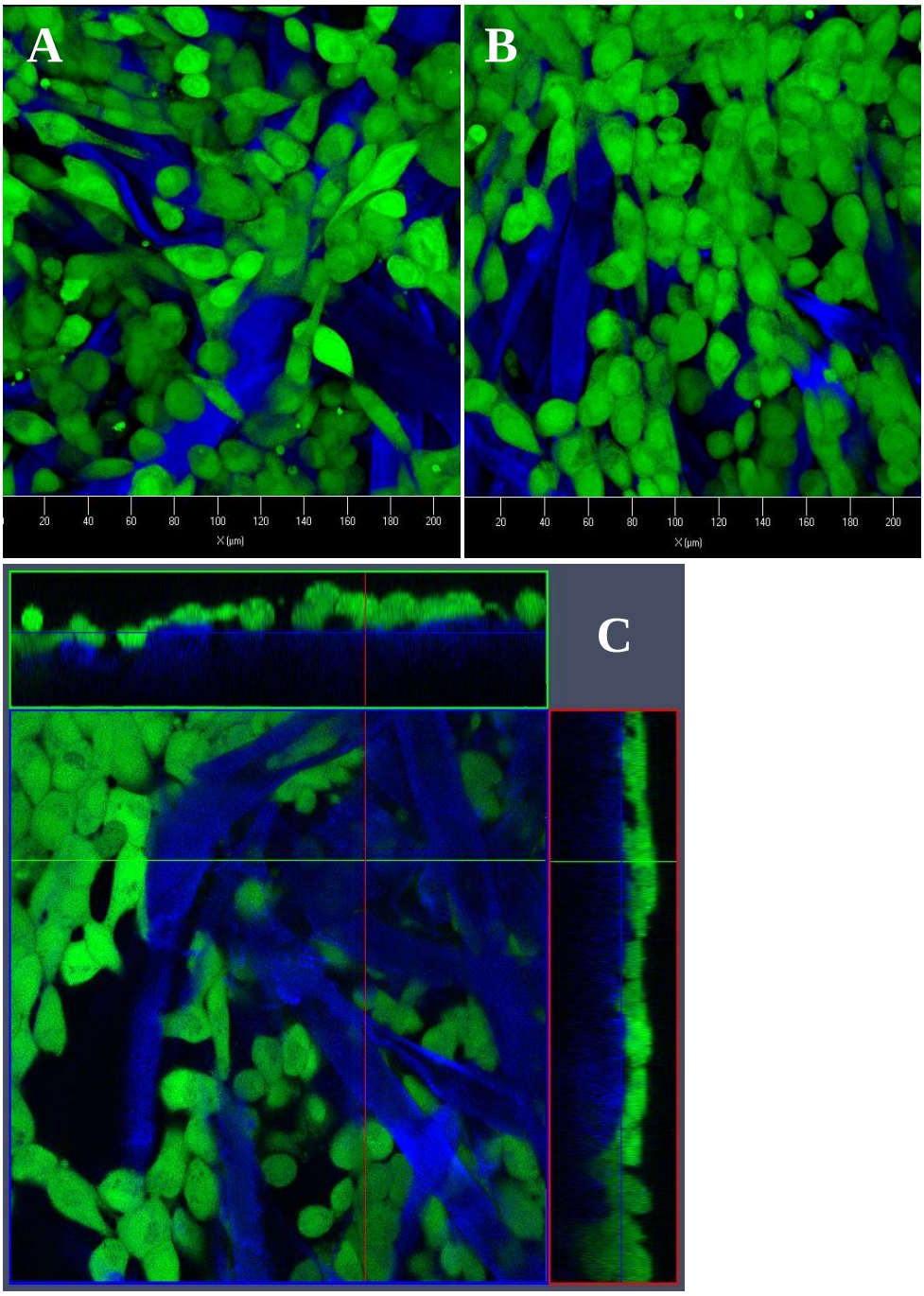


Figure 3.5.1. Top-down z-stacked view of MDCK growth on the top surface of bibulous paper. A.) SPFR MDCK 168 hours (7 days) post-inoculation. 40X magnification, 2µm slices, 66µm total thickness. B.) SPFR MDCK 192 hours (8 days) post-inoculation. 40X magnification, 2µm slices, 50µm total thickness. C.) Orthogonal view of SPFR MDCK 192 hours post-inoculation. 40X magnification, 2µm slices, 50µm total thickness. Ortho view depicts 22 µm depth from surface of z-stack.

As the SPFR samples of MDCK on bibulous paper were prepared for confocal microscopy, a sample preparation mistake was made. The bibulous paper for the 8-day SPFR sample was not soaked for 24 hours prior to cell inoculation. These samples grew noticeably faster than all other SPFR samples, which had been soaked for 24 hours prior to inoculation. These fast-growing cells can be seen in the "192 h" image of Figure 3.5.2.

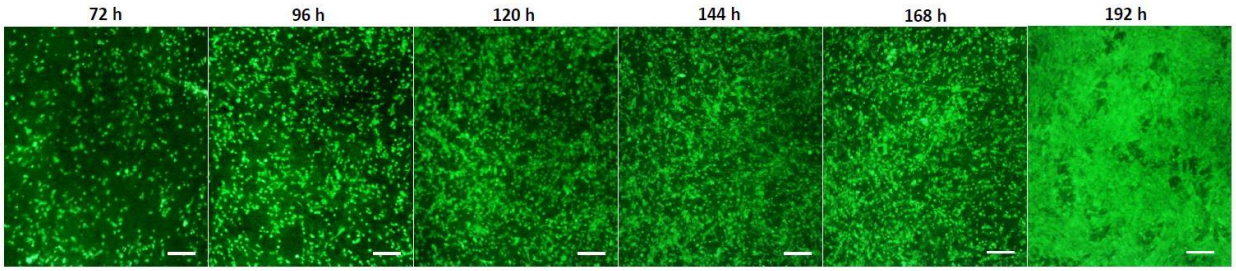


Figure 3.5.2. MDCK on bibulous paper generated with the SPFR method prior to confocal microscopy. Scale bars represent 200 µm.

The growth curve in Figure 3.5.3. shows the measured surface confluence of the SPFR samples before confocal microscopy. The Red arrow indicates the 8-day sample, which reached a 93.45% confluence. This event triggered an investigation into the effect of paper soak time on cell growth and surface confluence in order to optimize the process of growing MDCK on bibulous paper.

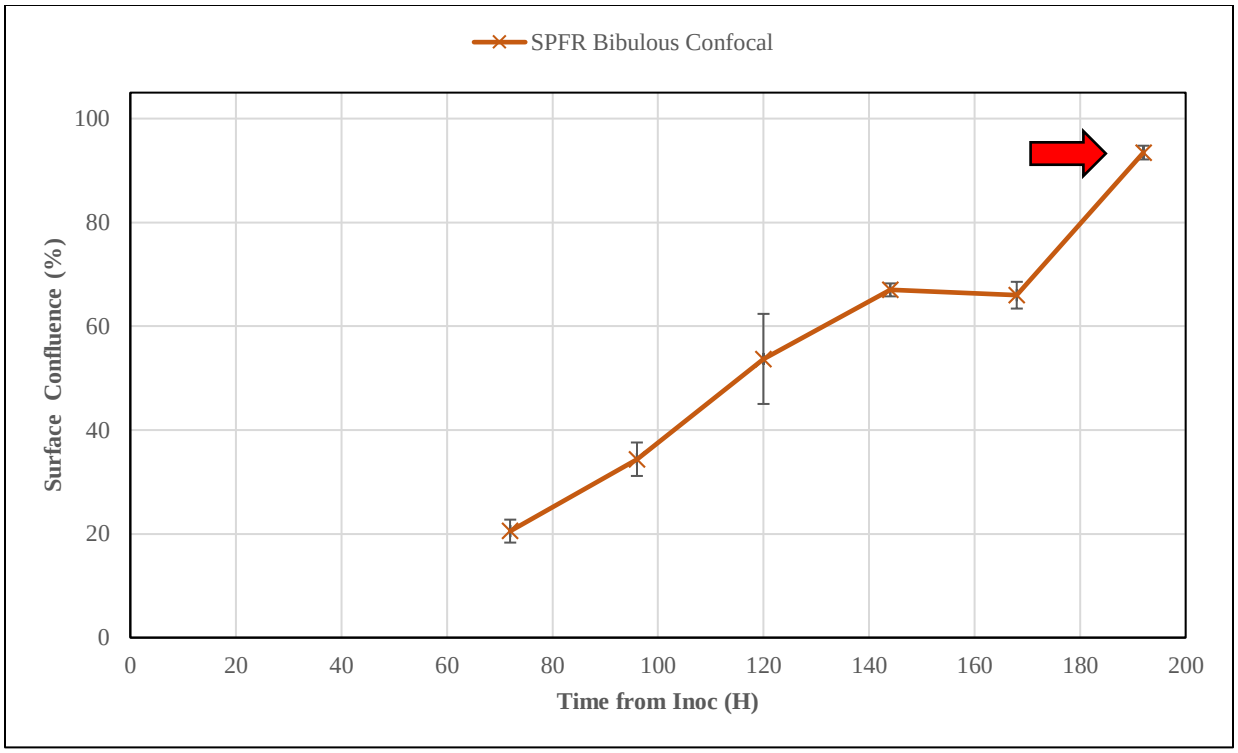


Figure 3.5.3. Surface confluence of confocal microscopy samples of SPFR MDCK on bibulous paper.

3.6. Effect of Paper Soak Time on SPFR Growth of MDCK on Bibulous Paper

Due to variability in growth curve data for SPFR MDCK on bibulous paper, the effect of the soak time of autoclaved paper in media before inoculation was investigated. Soak times of 24 hours and 0 hours were studied. A growth curve of surface confluence over time of the two groups was compared (Figure 3.6.1). The growth curves closely mirrored each other, but at 24 hours post-inoculation, the 24-hour soak samples had an average surface confluence of 27.7% while the 0-hour soak samples had an average surface confluence of 43.2%. The 24-hour soak samples reached a maximum surface confluence of 66.4% and the 0-hour soak samples reached a maximum surface confluence of 85.4%.

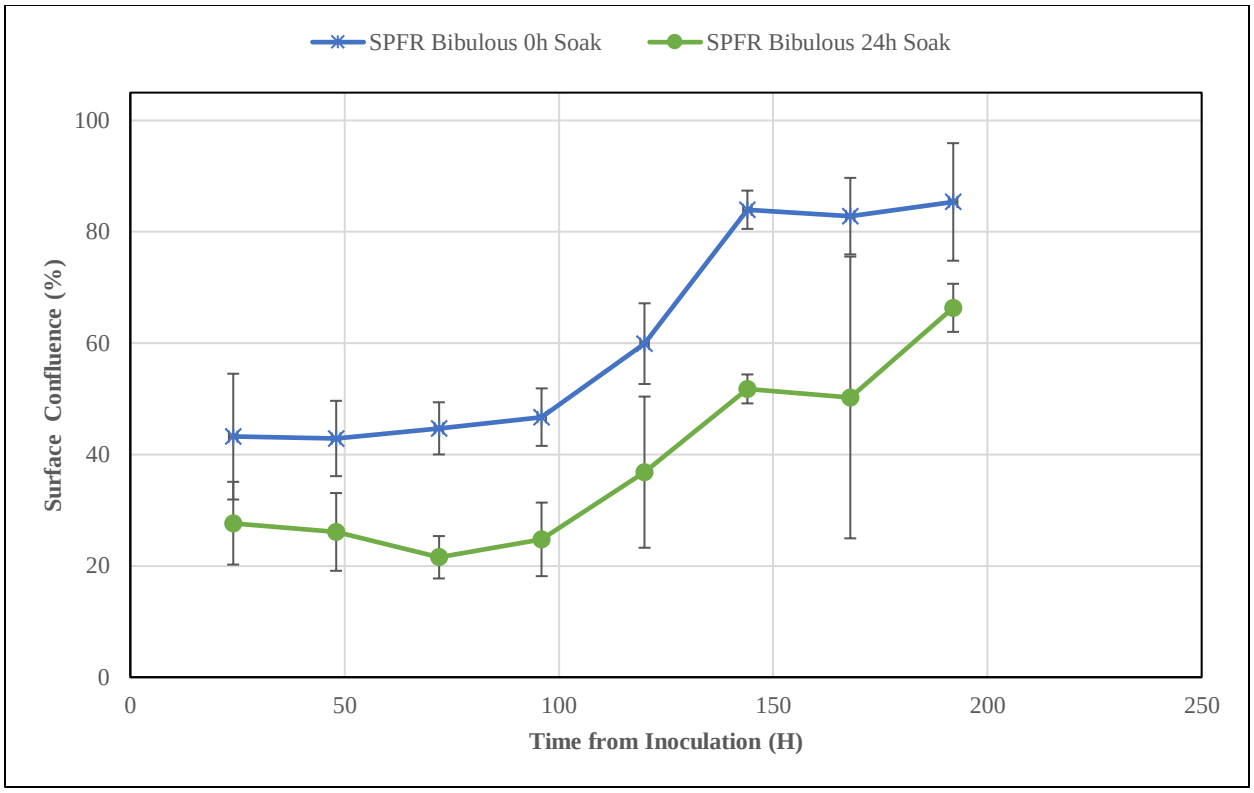


Figure 3.6.1. The effect of pre-inoculation soaking time on surface confluence of SPFR MDCK on bibulous paper. N=6.

To determine a possible cause for the discrepancies in surface confluence between the 24-hour and 0-hour groups, the pH and glutamine levels of fresh media versus those of media soaked for 24 hours with bibulous paper. This data is summarized in Table 3.6.1. There was negligible difference between the pH of the two media, but the media that had been incubated for 24 hours had a slightly lower glutamine concentration than the fresh media.

Table 3.6.1. pH and Glutamine concentration of complete EMEM and complete EMEM soaked for 24 hours with bibulous paper.

	pH	**Glutamine (mmol/L)**
Complete EMEM soaked 24 hours with bibulous paper	7.31 ± 0.03	5.19 ± 0.06
Complete EMEM	7.28 ± 0.02	5.47 ± 0.08

CHAPTER 4: Discussion

4.1. Paper Characterization

Of the fourteen papers tested, all were capable of being sterilized by autoclaving for one hour at 122˚C, as evidenced by no turbidity or sudden drop in media pH during the five-day incubation after autoclaving. It was further shown that sterile paper can be aseptically transferred from one plate to another using autoclaved forceps. These methods form the foundation of subsequent cell culture studies on paper.

The two synthetic papers, Legion YUPO® and Oxford Stone paper, were excluded first from consideration as a cell growth substrate on the basis of their composition. As the purpose of using paper in pharmaceutical manufacturing is that it is eco-friendly, biodegradable, cheap, and has a large surface area for cell growth, the use of synthetic plastic papers would be counter to that purpose.

The next consideration taken before selecting papers for cell culture was the durability of the paper after a 5-day incubation in cell culture media and under cell culture conditions (Table 3.1.1). Both Staples multipurpose paper and loose-leaf paper were extremely delicate after incubation, and tore as they were being removed from the wells. Therefore, those papers were excluded from consideration.

The pH of each paper was tested using the cold water extraction method, an industry standard for measuring the pH of paper.[18] Results are summarized in Table 3.1.1. Papers resulting in a final pH above 8.0, like Staples multipurpose paper, Staples loose-leaf paper, YUPO® synthetic multimedia paper, and Strathmore Bristol multimedia art paper, were considered unlikely to promote good cell growth, as the physiological pH preferred by mammalian cells is 7.2 – 7.4.

Previous studies that investigated the growth of mammalian cells directly on different paper surfaces found that cells prefer the smoothest surface possible, as it is a good substitute for polystyrene. Park *et al.* found that human adipose-derived stem cells (hADSCs) preferentially grew on weigh paper as opposed to chromatography paper or wiping tissue.[13] They hypothesized that the cells preferred weigh paper as it was the smoothest option.[13] Juvonen *et al.* compared four proprietary blends of paper and latex, and found that human retinal pigment epithelial cells (ARPE-19) grew best on the smoothest of the four papers.[12] This agrees with previous research by Baharloo *et al.*, who showed that epithelial cells preferentially grow on smooth surfaces and do not adhere or grow well on rough surfaces.[19]

In light of this previous research and the fact that MDCK cells are epithelial cells, the surface roughness of each type of paper was measured using a Parker Print Surf testing machine (Table 3.1.1). The roughest papers were the Whatman 3MM chromatography paper, Texwipe VersaWipe, and Kirkland's parchment paper, and those were accordingly excluded from cell growth studies. The smoothest papers, including Strathmore Bristol multimedia art paper, poster paper, Fisherbrand autoclave pouch paper, Whatman weigh paper, Fisher lens paper, and Fisher bibulous paper, were considered for cell growth studies.

After all characterization and consideration, 7 of the original 14 papers were chosen as substrates for MDCK in batch growth studies. The 7 papers were Fisherbrand autoclave pouch paper, Fisher lens paper, Fisher bibulous paper, blotter paper made by the Department of Forest Biomaterials, Whatman weigh paper, poster, and Strathmore Bristol multimedia art paper.

4.2. Weaning of MDCK from 10% to 5% FBS

The MDCK cell line obtained for this study was adapted to 10% FBS (Figure 3.2.1). In order to conserve serum, the cell line was first weaned to 5% serum before banking. Both the 10% and 5% serum-adapted MDCK obtained similar final viable cell counts after about 70 hours of growth, both entered the stationary growth phase around the same time post-inoculation, and both had very similar overall growth rates from inoculation to stationary phase. Therefore, the 5% serum-adapted cell line was deemed a successful substitute for the 10% serum-adapted cell line, and a working cell bank was established. However, the 5% serum-adapted cell line experienced a lag phase for the first 20 hours after inoculation. This signifies that the cells had difficulty adhering to the tissue culture-treated polystyrene in the presence of less serum. This supposition is supported by previously documented evidence showing that mammalian cells require the presence of two major serum components – albumin and α-globulin – for cell attachment, cell growth in a monolayer, and cell proliferation.[20] However, pharmaceutical regulatory agencies restrict the use of animal-derived materials in commercial processes. As such, production cell lines are adapted to serum-free media, which is supplemented with recombinant growth factors. As this technology continues, the MDCK cell line can be adapted to serum free media.

4.3. Growth of 5% Serum-Adapted MDCK on Paper

MDCK were grown in a batch growth study on each of the 7 selected papers, and MDCK grown on tissue culture-treated T-25 flasks were used as a positive control. Results were assessed both qualitatively, by comparing side-by-side images of positive control cells with cells on paper (Figure 3.3.1), and quantitatively, by using confluence analysis software to track surface confluence over time of positive control cells versus cells on paper (Figure 3.3.2). From both a visual and numerical assessment, cells reached the highest surface confluence when grown on

bibulous paper. Cells grown on bibulous paper also looked morphologically similar to cells grown in T-flasks.

Unexpectedly, cells grew the worst on the top surface of the poster, which had the second smoothest surface after weigh paper. The poster also had a pH very close to physiological pH. The cells may have not been able to attach to the top surface of the poster as it has a glossy, hydrophobic finish that may deter adherence. Strathmore Bristol paper was also a poor cell growth substrate. This is less surprising, as its pH was 8.73. The Fisherbrand autoclave pouch paper and the Whatman weigh paper, while smooth, also did not promote cell growth and spreading as well as expected. This may be due to the fact that these lightweight papers floated on the media surface and were very difficult to submerge. Therefore, when cells were inoculated they may not have had a chance to attach to the paper. The Forest Biomaterials blotter paper was the roughest paper tested, and it promoted cell growth on par with the autoclave and weigh paper. The cellulose structure in the blotter paper was pronounced enough that cells grew along fibers rather than in a flat plain as on other papers. Fisher lens paper was the second most successful cell growth substrate and allowed cells to reach a final surface confluence of 40.6%. However, the most successful substrate was the Fisher bibulous paper. MDCK grown on the bibulous paper reached a maximum surface confluence of 48.0%, but then declined after 50 hours post-inoculation.

As Fisher bibulous paper was the most promising cell growth substrate, it was selected as the paper for all subsequent growth studies. Because the surface confluence decreased after 50 hours, it was decided that media needed to be replenished after 48 hours post-inoculation to try to prevent cell death. This led to the development of the simulated plug flow reactor growth studies, a way to simulate a continuous cell culture process.

4.4. Simulated Plug Flow Reactor (SPFR) Growth of MDCK on Bibulous Paper

As surface confluence of batch-grown MDCK on bibulous paper steeply declines after 50 hours of culture, the possibility of combining a paper-based manufacturing platform with continuous cell culture technologies was considered. Continuous cell culture is achieved in the industry by continuously supplying fresh media while simultaneously removing spent media, thus maintaining a steady state environment rich in metabolites but low in waste products. Continuous cell culture is often leveraged to reach high viable cell densities before the production phase of the manufacturing process occurs, or during production to continuously remove product from the cells.

In the case of MDCK used to produce influenza virus, continuous technologies would be best used to increase the cell density to reach a cell density appropriate for infection and production. This is due to the fact that the MDCK cells are infected at a low multiplicity of infection (MOI), often with a value of $10^{-3} – 10^{-6}$.[21] Therefore, one in every one thousand to one in every one million cells is initially infected with an influenza virus. The virus then reproduces within the cell, and the cell eventually lyses and releases more virus, which go on to infect the rest of the cells. Utilizing a continuous process during virus production would remove virus particles and prevent them from infecting the entire culture, thus drastically decreasing virus yield. However, continuous processing could potentially be used to promote cell growth prior to infection. An example of how this process could be made to be continuous is depicted in Figure 4.4.1. In this example, cells are grown on sterile bibulous paper that is continuously fed, as a moving bed, into a plug flow reactor. Within this reactor, fresh media is constantly supplied and spent media is constantly removed. An initial inoculum of MDCK is added as paper is first fed into the reactor, and the velocity of the moving bed is controlled to reach the desired surface confluence upon emerging from the reactor.

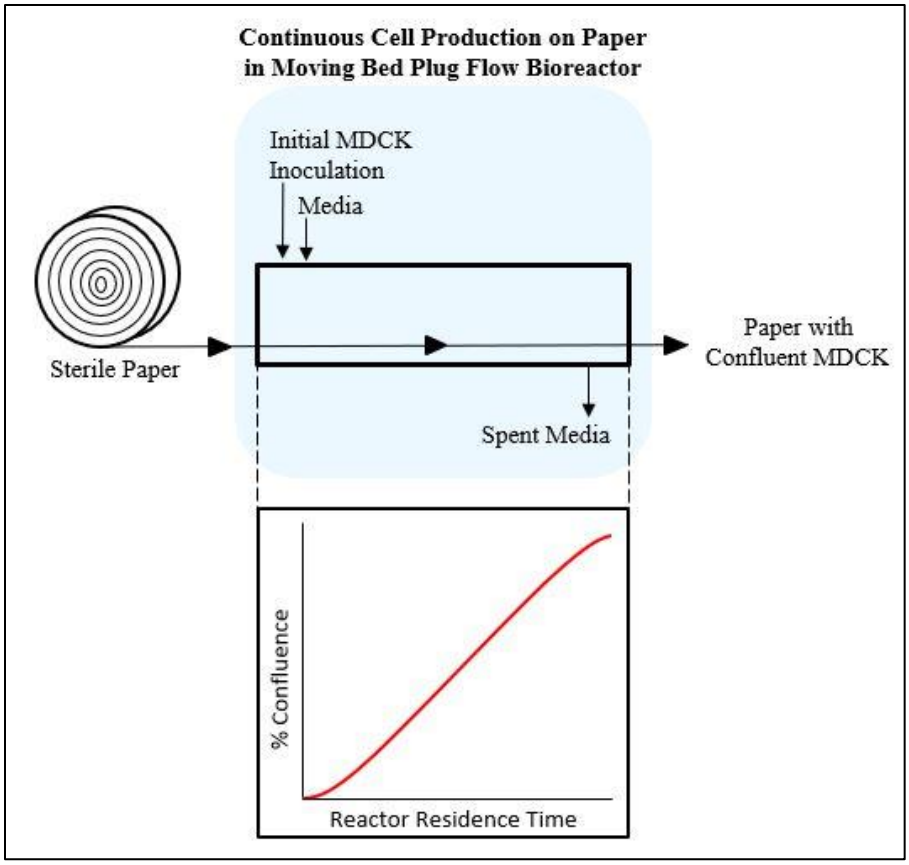


Figure 4.4.1. Moving bed plug flow bioreactor for continuous cell production.

To test this theory, a continuous plug flow reactor was simulated with MDCK grown on bibulous paper to see if the final surface confluence could be increased (Figure 3.4.1). At 48 hours post-inoculation, spent media was replaced with fresh media every 24 hours to simulate continuous media replacement. This study design was based on a study done at Amgen that used spin tubes to simulate continuous mammalian suspension cultures at small scale.[22] In the Amgen study, suspension cells were cultured in 50 mL centrifuge tubes with agitation. Every day, the tubes were centrifuged, and the spent media was removed from the cell pellet. The cells were then resuspended with fresh media.[22] In this way, Amgen was able to quickly and cheaply test the interaction between media formulation and continuous cell culture in process development.[22]

This method was easily adapted for an adherent cell line to simulate a continuous process by simply removing all spent media and replacing it with fresh media every 24 hours. The effect of this simulated plug flow reactor growth of MDCK on bibulous paper was investigated in comparison with SPFR growth of MDCK in T-25 flasks (Figure 3.4.1). With the simulated continuous growth, MDCK grown in T-25 flasks reached 80% confluence after 67 hours and MDCK grown on bibulous paper reached 80% confluence after 131 hours.

Using the moving bed plug flow reactor example in Figure 4.4.1, the residence time of the paper in the bioreactor could be controlled to be 131 hours to achieve paper with 80% confluent MDCK. Based on future virus production studies identifying the optimal confluence at which to infect with virus, the residence time or length of the cell growth bioreactor could be altered.

4.5. Confocal Microscopy of MDCK on Bibulous Paper

Confocal microscopy was used to further visualize the growth of MDCK on bibulous paper. Of particular interest was whether the cells formed the characteristic cell size and shape and if they were able to penetrate the 128.3 μm depth of the paper, thus reaching a very high cell density undetectable from just imaging the paper surface. Confocal microscopy z-stack images of SPFR-grown MDCK on bibulous paper at 3, 4, 5, 6, 7, and 8 days post-inoculation were analyzed. It was shown that the cells were not colonizing the paper or growing on the bottom surface of the paper, but instead grew in a roughly flat monolayer on the top surface of the paper (Figure 3.5.1). However, one of the major benefits of paper is its ease of physical modification. As such, the weave of the paper could be optimized to allow cells to penetrate the entire depth of the paper and further maximize the viable cell density beyond that possible with 2D surfaces like polystyrene cell scaffolds.

4.6. The Effect of Paper Soak Time on SPFR Growth of MDCK on Bibulous Paper

As the time-course of SPFR samples of MDCK on bibulous paper were prepared for confocal microscopy, a mistake was made. The bibulous paper for the 8-day SPFR sample was not soaked for 24 hours prior to cell inoculation. These samples grew noticeably faster than all other SPFR samples, which had been soaked for 24 hours prior to inoculation (Figure 3.5.2). This triggered an investigation into the effect of paper soak time on cell growth and surface confluence in order to optimize the process of growing MDCK on bibulous paper. Soak times of 0 hours and 24 hours were compared (Figure 3.6.1). The paper that was not pre-soaked prior to inoculation had a significantly higher starting confluence, but the rest of the growth curves closely mirrored each other. This suggests that pre-soaking the paper has an effect on cell attachment to the paper surface.

To determine a possible cause for the discrepancy in surface confluence, the difference between pH and glutamine concentration of media incubated with bibulous paper for 24 hours was compared to that of fresh media (Table 3.6.1). The results showed no significant difference in pH and only a very slightly lower glutamine concentration in the media that had been incubated for 24 hours. However, the glutamine concentration would not have an effect on cell attachment, as it is used in cell metabolism. Another possible cause of the lessened cell attachment onto pre-soaked bibulous paper is the degradation of the components of FBS that promote cell attachment, like albumin and α-globulin.[20]

CHAPTER 5: Future Work

The optimization of growth of an adherent MDCK cell line on a paper substrate is the first step towards creating a continuous plug flow reactor with a moving bed of MDCK stabilized on a paper surface. Figure 5.1.1 depicts a 3-stage manufacturing process based on continuous plug flow moving bed bioreactors. In the first stage of the process (Figure 5.1.1.A), sterile paper is slowly fed through the plug flow bioreactor. Media is supplied at the start of the linear bioreactor, and spent media is expelled at the end. An initial MDCK inoculation occurs at the beginning of the cell growth bioreactor. The speed of the moving bed is adjusted such that the MDCK have time to spread and reach an optimal confluence for viral infection as the paper leaves the cell growth bioreactor. Future studies can determine the optimal MDCK confluence on paper to maximize virus production in the virus production stage to determine the speed of the moving bed and the length of the cell production bioreactor.

One of the advantages of using paper as a cell growth substrate is the ease with which it is physically and chemically modified. To increase the stability of MDCK grown on bibulous paper, the paper surface can be chemically altered to promote adherence. Park, H. *et al.* demonstrated that by coating weigh paper with glycidyl methacrylate (GMA), which contains an epoxide group, they were able to promote cell attachment to the paper surface.[13] The epoxide group is highly reactive with amine groups found in serum proteins.[13] The serum proteins bind to GMA and then help mammalian cells to attach to the surface.[13] In future, bibulous paper can be treated with GMA or a similar chemical to promote cell attachment.

Because the FDA does not allow materials of animal origin to be used in pharmaceutical manufacturing, further studies need to adapt the MDCK cell line to a fully serum-free growth

media. To substitute for the absence of serum, the media will be supplemented with a hydrolysate such as wheat, and then further supplemented with insulin and transferrin.

As an alternative to immediately infecting the cells, in the second stage of the proposed 3-stage manufacturing scheme the paper with attached MDCK can be stored long-term (Figure 5.1.1.B). The paper can be continually frozen or lyopreserved and then stored either roll-to-roll or cut and inserted into wave bags. The stored paper can be shipped to multiple manufacturing sites, and then thawed or rehydrated when needed to manufacture influenza virus.

A potentially alternative storage option to freezing is lyopreservation by controlled drying. Studies done by Chakraborty, N *et al.* in a collaborator lab have demonstrated progress towards the ability to dry mammalian cells for long-term storage.[29,30] The MDCK cells could be engineered to express the *TRET1* gene, which produces a trehalose transporter membrane protein native to African insect larvae.[29,30] Trehalose, a sugar normally unable to enter mammalian cells, can then be actively transported into the cells. Trehalose has been found to make up a large percentage of the body weight of desiccated larvae, and it protects the cellular structure against the stress of drying.[29,30] By transfecting MDCK cells with the *TRET1* gene, they could withstand controlled drying and then be rehydrated.[29,30] In combination with the stabilization of manufacturing mammalian cell lines on paper, the controlled drying process takes much of the energy out of long-term storage and takes the water and cold chain out of shipping live cells. These advantages, although far in the future, could revolutionize the way that manufacturing cell lines are stored and transported.

In the third phase of the proposed influenza vaccine manufacturing process, the MDCK on paper is fed into another moving bed continuous plug flow reactor for viral infection (Figure 5.1.1. C). Media is again fed into the beginning of the bioreactor, but a bolus of live influenza virus is

also added. The virus infects the MDCK cells, which then lyse and release more virus particles that are removed at the outlet of the bioreactor with the spent media. The length of the bioreactor and the paper residence time are adjusted to maximize the recovery of virus. A small amount of the produced virus must be cycled back to the beginning of the bioreactor to infect cells as they continue to feed into the bioreactor. The rest of the virus can be sent to downstream processing for final vaccine formulation.

The final suggested stage of the manufacturing process is the possibility of an integrated continuous virus purification process (Figure 5.1.1. D). This third stage would provide a clarification or capture step at the end of virus production. The first option is to allow the potentially non-virus-binding bibulous paper to release all virus particles into the media for harvest, thus clarifying the virus from insoluble cellular debris. If the paper is physically and chemically modified to strongly bind cellular membranes, this clarification is possible. At the end of the virus production stage, the spent paper is discarded and the spent media with virus particles are processed for virus purification and inactivation.

The second option for a partially integrated continuous process is to incorporate a capture step into the virus production bioreactor. In this strategy, the paper is modified to selectively bind influenza virus. Holstein *et al.* have demonstrated the immobilization of affinity-proteins for the influenza HA protein to paper for the purpose of influenza diagnostics.[31] The HA affinity protein selectively binds the stem portion of the HA antigen, thus capturing it and adhering it to the paper.[31] In this process design, the paper is washed or shredded after virus production to remove cells, debris, and impurities. The influenza virus could then be isolated by the affinity protein and released by a low pH rinse. In this way, an affinity capture step could be incorporated at the very beginning of the process and be coupled with removal of large solid MDCK cell debris.

In sum, future studies of the growth of MDCK on bibulous paper need to explore (1) the possibility of optimizing MDCK attachment to the paper surface, (2) the adaptation of the MDCK cells to serum-free media, (3) the ability to freeze MDCK on paper and then thaw, (4) the ability of MDCK attached to paper to produce virus, and (5) the ability of paper to be used as a clarification or capture step during virus production.

If the creation of this bioreactor were achieved, it could offer significant process intensification to virus manufacturing. In a 2015 study, researchers adapted the same MDCK cell line used in this study (ATCC CCL-34) to a serum-free media and then to a suspension culture.[32] From these cells in a 1L culture, they were able to produce virus yields of $22.02 \pm 3.57 * 10^9$ virions/mL and cell-specific virus yields of 51,700 ± 9,900 virions/cell.[32] In a t-flask of 70% confluence, this study found that there were 138,000 cells/cm^2. Because the MDCK cells are 20μm in diameter, and bibulous paper is 128μm thick, bibulous paper can theoretically support 6 times the number of cells that the 2D polystyrene can. Using these assumptions, and the assumption that the same virus yield can be achieved from adherent, serum-free adapted MDCK, it would take a piece of bibulous paper with an area of 514.4 cm^2 to achieve the same virus production as the 1L culture of suspension cells. This is slightly smaller than the size of a 8.5” by 11” piece of paper. Because media requirements for cells on paper are less than those of cells grown in suspension, this represents a significant reduction in footprint and resource consumption.

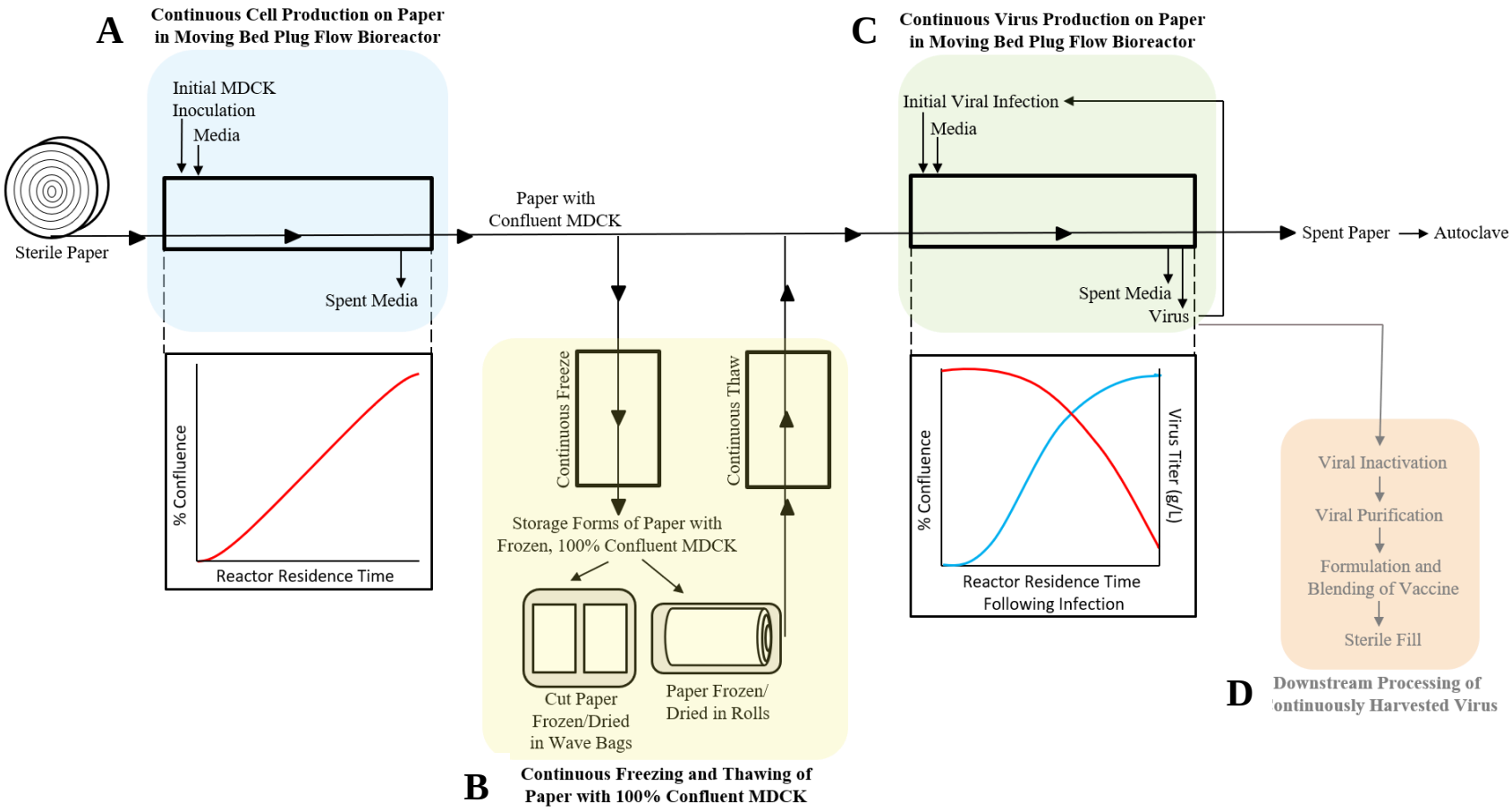


Figure 5.1.1. Integrated continuous influenza virus manufacturing using anchorage-dependent MDCK cells on a paper substrate. A.) Stage 1, continuous MDCK production from a moving bed plug flow bioreactor. B.) Stage 2, longterm storage of MDCK stabilized on paper. C.) Stage 3, continuous virus production from a moving bed plug flow bioreactor. D.) Stage 4, downstream purification and inactivation of virus for vaccines.

CHAPTER 6: Conclusion

This proof-of-concept study successfully demonstrated growth of a manufacturing cell line on a paper substrate for the purpose of developing a continuous paper-based flu virus manufacturing platform. Adherent MDCK were grown in 5% FBS on seven types of paper. Fisherbrand bibulous paper was determined to be the best cell growth substrate. Scale-down continuous cell culture techniques were successfully used to increase the surface confluence of MDCK grown on bibulous paper. Confocal microscopy was used to image cell size, shape, and growth during the growth of MDCK on the surface of the paper. A future adaptation could explore physical modification of the paper structure (porosity, pore size) to allow cells to colonize the entire depth of the paper, thus significantly increasing the final viable cell density. Finally, the growth of MDCK on bibulous paper was optimized by investigating the effect of paper soak time prior to inoculation.

A paper-based cell culture platform could offer expedient, cost effective, and flexible continuous manufacturing of biopharmaceuticals using anchorage-dependent mammalian cells, with a particular emphasis on the influenza vaccine. With further development, this technology could be leveraged to create an integrated, continuous plug flow manufacturing platform based on a high density of MDCK cells stabilized on rolls of paper that can be shipped to multiple global manufacturing sites to respond to pandemics and offer more reproducible and accessible vaccine production technology to under-developed areas currently using eggs to manufacture the flu vaccine.

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APPENDICES

APPENDIX A: Vial Thaw of Adherent MDCK Cells

Protocol adapted from protocols shared by Laurie Overton and Melody Lao.

A.1. Materials

- Frozen vial of adherent MDCK cells
- Corning tissue culture-treated T-flask (T-25, T-75, T-225)
- Eagle's Minimum Essential Medium (ATCC, Cat# 30-2003, 500 mL)
- L-Glutamine (Gibco, Cat# A2916801, 200 mM, 100 mL)
- Glucose (400 g/L)
- Fetal Bovine Serum (Gibco, Cat# 10099-141)
- 15 mL Falcon conical tube
- 37˚C bead bath
- Centrifuge
- VI-Cell XR Cell Viability Analyzer
- Humidified incubator (37˚C, 5% CO_2, static)

A.2. Complete Media Preparation

Table A.2.1. Complete EMEM Formula (+5% FBS, +4 mM Glutamine, +3.5 g/L Glucose).

Component	**Quantity Required**
Eagle's Minimum Essential Medium	500 mL
Fetal Bovine Serum	25 mL
L-Glutamine	10 mL
Glucose	4.375 mL

1. In a biosafety cabinet (BSC), add the quantities of Fetal Bovine Serum (FBS), L-Glutamine, and Glucose listed in Table A.1. to a bottle of Eagle's Minimum Essential Medium (EMEM).

2. Clearly label the medium bottle with your initials, the date of preparation, and the additions made (+5% FBS, +4 mM Glutamine, +3.5 g/L Glucose).
3. Store the complete medium at 4˚C and use for up to a month after the preparation date.

A.3. Procedure

1. Before thawing cells, place a bottle of complete EMEM in the humidified incubator to heat the medium to 37˚C.
2. In a BSC, aliquot 9 mL of pre-warmed complete EMEM into a sterile 15 mL conical tube.
3. Retrieve a frozen cryovial of adherent MDCK from the liquid Nitrogen freezer.
4. Thaw the cells by swirling the cryovial in a 37˚C bead bath until only a small sliver of ice remains.
5. Transfer the cryovial into the BSC and immediately transfer the entire contents of the cryovial via pipet into the 9 mL of aliquoted media by slowly dropping them into the media.
6. Very gently mix the cell suspension by slowly pipetting up and down.
7. Centrifuge the cells for 5 minutes at 1500 rpm.
8. Back in the BSC, aspirate the supernatant from the 15 mL conical tube.
9. Add 5 mL of complete EMEM to the cell pellet and gently resuspend by slowly pipetting up and down.
10. Add another 5 mL of complete EMEM to the cell suspension, and gently mix.
11. Take a 0.5 mL sample of the cell suspension and use the Vi-Cell to determine the VCD. Record the total number of cells recovered from the cryovial and the post-thaw viability.

12. Determine the total T-flask surface area that can be seeded with the thawed cells at 30,000 cells/cm^2 by solving Equation A.1.

Equation A.3.1.: $$Total\ surface\ area\ = \frac{VCD\left(\frac{cells}{mL}\right) * V(mL)}{30{,}000\ (\frac{cells}{cm^2})}$$

13. Choose the appropriate T-flask(s) to seed based on the total surface area that can be seeded.
14. Determine the appropriate volume of cell suspension needed to seed a new T-flask at 30,000 cells/cm^2 by solving Equation A.2. for V_1, where C_1 is the concentration of cells in the cell suspension, the desired cells/cm^2 is 30,000, and the surface area of the flask is either 25 cm^2, 75 cm^2, or 225 cm^2 depending on the T-flask used.

Equation A.3.2.: $$C_1V_1 = (Desired\ cells/cm^2)(Surface\ area\ of\ flask)$$

15. Before seeding the T-flask, add the appropriate volume of complete EMEM to the flask so that the final volume after seeding falls within the working volume range listed in Table A.2.

Table A.3.1. T-flask working volumes.

T-flask Size	**Working Volume**
T-25	5 - 7.5 mL
T-75	15 - 25 mL
T-225	45 - 67.5 mL

16. Seed the T-flask by adding the determined volume of cell suspension.
17. Use a North, South, East, West motion to evenly distribute cells across the bottom of the T-flask.
18. Place flask inside the incubator to grow.

APPENDIX B: Cell Passaging and Maintenance of Adherent MDCK Cells

Protocol adapted from the thesis of Stephen White.[19]

B.1. Materials

- T-flask of MDCK, 80-90% confluent
- Corning tissue culture-treated T-flask (T-25, T-75, T-225)
- Complete EMEM (Preparation detailed in Appendix A.2.)
- PBS (Gibco, Cat# 10010-023, 1X, pH 7.4)
- TrypLE Select (Gibco, Cat# 12563-011, 1X)
- 15 mL Falcon conical tube
- Centrifuge
- VI-Cell XR Cell Viability Analyzer
- Inverted light microscope
- Humidified incubator (37˚C, 5% CO_2, static)

B.2. Procedure

1. Before passaging cells, place a bottle of complete EMEM in the humidified incubator to heat the medium to 37˚C.
2. Obtain a T-flask of adherent MDCK and ensure that the confluence is 80-90% by visually inspecting the bottom of the flask using an inverted microscope.
3. If the T-flask is ≥80% confluent, place the flask in a BSC. If not, return the flask to the humidified incubator to continue growing to ≥80% confluence.
4. Inside the BSC, aspirate the old media from the T-flask.
5. Add 3 mL of PBS to a T-25 flask, 5 mL of PBS to a T-75 flask, or 15 mL of PBS to a T-225 flask. Seal the cap and rock to gently wash the bottom of the flask.

6. Aspirate the PBS from the T-flask.
7. Add 2 mL of TrypLE to a T-25 flask, 3 mL of TrypLE to a T-75 flask, or 10 mL of TrypLE to a T-225 flask. Seal the cap.
8. Place the T-flask in the incubator for 5 minutes.
9. After 5 minutes, remove the T-flask from the incubator and check for cell detachment using the inverted microscope. Gently tap the bottom of the flask and wash with TrypLE until all cells have detached, and then place the flask back inside the BSC.
10. Add an equal volume of complete EMEM to the flask to inactivate the TrypLE.
11. Aspirate all of liquid from the T-flask and add to a sterile 15 mL conical tube.
12. Centrifuge the cells for 5 minutes at 1500 rpm.
13. Back in the BSC, aspirate the supernatant from the 15 mL conical tube.
14. Add 5 mL of complete EMEM to the cell pellet and gently resuspend by slowly pipetting up and down.
15. Add another 5 mL of complete EMEM to the cell suspension, and gently mix.
16. Take a 0.5 mL sample of the cell suspension and use the Vi-Cell to determine the VCD. Record the total number of cells harvested, the number of cells harvested per cm^2 of the T-flask, and the viability.
17. Determine the appropriate volume of cell suspension needed to seed a new T-flask at 30,000 cells/cm^2 by solving Equation B.1. for V_1, where C_1 is the concentration of cells in the cell suspension, the desired cells/cm^2 is 30,000, and the surface area of the flask is either 25 cm^2, 75 cm^2, or 225 cm^2 depending on the T-flask used.

Equation B.2.1.: $C_1V_1 = (Desired\ cells/cm^2)(Surface\ area\ of\ flask)$

18. Before seeding the T-flask, add the appropriate volume of complete EMEM to the flask so that the final volume after seeding falls within the working volume range listed in Table B.2.1.

Table B.2.1. T-flask working volumes.

T-flask Size	**Working Volume**
T-25	5 - 7.5 mL
T-75	15 - 25 mL
T-225	45 - 67.5 mL

19. Seed the T-flask by adding the determined volume of cell suspension.
20. Use a North, South, East, West motion to evenly distribute cells across the bottom of the T-flask.
21. Place flask inside the incubator to grow.
22. 48 hours after seeding, fully replace the spent media with fresh complete media. Passage the T-flask within 48 hours of re-feeding for optimal results.
23. If MDCK are needed to seed a growth study, the seeding density of MDCK in the T-flask used to generate cells for the study can be adjusted based on how many days from seeding to harvest. Table B.3. indicates the ideal seeding density based on the number of days between seeding and harvest of the T-flask. For each of these cases, replace spent media with fresh media 24 hours prior to harvesting the T-flask.

Table B.2.2. Ideal T-flask seeding density based on the number of days until harvest.

Days Prior to T-flask Harvest	**Seeding Density**
2	40,000 cells/cm^2
3	30,000 cells/cm^2
4	20,000 cells/cm^2

APPENDIX C: Banking of Adherent MDCK Cells

Protocol adapted from a protocol provided by Laurie Overton.

C.1. Materials

- T-flask(s) of MDCK, ~80% confluent
- Complete EMEM (Preparation detailed in Appendix A.2.)
- DMSO (Sigma, Cat# D2650-5x5ML, sterile filtered)
- PBS (Gibco, Cat# 10010-023, 1X, pH 7.4)
- TrypLE Select (Gibco, Cat# 12563-011, 1X)
- 15 mL Falcon conical tube 37˚C bead bath
- Centrifuge
- VI-Cell XR Cell Viability Analyzer
- Cryovials
- Mr. Frosty
- Humidified incubator (37˚C, 5% CO_2, static)
- -80˚C freezer
- Liquid Nitrogen freezer

C.2. Freeze Media Preparation

Table C.2.1. Freeze Media Formula.

Component	**Quantity Required**
Complete Eagle's Minimum Essential Medium	80%
DMSO	20%

1. Directly before harvesting the intended T-flasks for banking, make freeze medium by combining 80% complete EMEM and 20% DMSO by volume.

2. Keep the freeze medium chilled until use.

C.3. Procedure

1. Harvest all T-flasks to be banked following the procedure detailed in Appendix B.2., steps 1-10.
2. After detaching cells, take a 0.5 mL sample of the cell suspension and use the Vi-Cell to determine the total number of cells harvested from all T-flasks combined.
3. Transfer the detached cells to 15 mL conical tubes and centrifuge for 5 minutes at 1500 rpm.
4. Based on the total number of cells harvested from all flasks combined, determine the amount of complete EMEM to resuspend the cell pellet in to reach a VCD of $10*10^6$ cells/mL.
5. After centrifuging, remove the supernatant from the cell pellet, and resuspend the cell pellet in the pre-determined volume of complete EMEM to create a cell suspension with a VCD of $10*10^6$ cells/mL.
6. Slowly add the appropriate volume of freeze medium to the cell suspension to create a 1:2 dilution with a final VCD of $5*10^6$ cells/mL and a final DMSO content of 10%.
7. Aliquot 1 mL of this cell suspension into pre-labeled 2 mL cryovials with name, date, cell line, and VCD.
8. Put all cryovials into a Mr. Frosty and place the Mr. Frosty on the bottom shelf of a -80˚C freezer overnight.
9. The next morning, transfer the cryovials to the gas-phase of a liquid Nitrogen freezer for long-term storage. One cryovial can be thawed and analyzed using the Vi-Cell to determine the post-thaw viability of the bank.

APPENDIX D: Batch Growth Study of Adherent MDCK on Paper vs. T-25 Flasks

D.1. Materials

- T-flask(s) of MDCK, 80-90% confluent
- Complete EMEM (Preparation detailed in Appendix A.2.)
- Paper
- Autoclave Pouches (Fisherbrand, Cat# 01-812-54)
- Metal Forceps
- 3 Corning ultra-low attachment 6-well plates
- 2 Corning tissue culture-treated 6-well plates
- 18-24 Corning tissue culture-treated T-25 flasks
- PBS (Gibco, Cat# 10010-023, 1X, pH 7.4)
- TrypLE Select (Gibco, Cat# 12563-011, 1X)
- 15 mL Falcon conical tube
- Centrifuge
- VI-Cell XR Cell Viability Analyzer
- Inverted light microscope
- Humidified incubator (37˚C, 5% CO_2, static)
- Calcein AM (Invitrogen, Cat# C3099, 1 mg/mL in anhydrous DMSO)
- Dino-Eye Digital Eye-Piece
- Fluorescence Illuminator (Nikon Intensilight C-HGFI)
- Fluorescence Filter Cube (Nikon FITC-HYQ, Excitation 460-500 nm)
- Olympus CKX-CCSW Cell Confluence Checker software

D.2. Procedure

D.2.1. Setting up the growth study by seeding T-25 flasks and paper with MDCK

1. Make 32 mm diameter disks of paper using a punch.
2. Put paper disks into autoclave pouches, 7 disks to a pouch.
3. Put metal forceps individually into autoclave pouches.
4. Autoclave paper disks and forceps for 60 minutes at 121˚C to sterilize the paper and forceps.
5. After autoclaving, transfer autoclave pouches to a BSC. Using the sterilized forceps, transfer one paper disk into each well of 3 Corning ultra-low attachment 6-well plates and 1 Corning tissue culture-treated 6-well plate (negative control).
6. Add 2 mL of complete EMEM to each well of the 4 plates and forcibly submerge the paper with the tip of a pipet if necessary.
7. Place the plates in the incubator for at least 2 hours for the paper to be fully wetted and the media to be warmed.
8. After 2 hours, obtain a T-flask(s) of adherent MDCK, and ensure that the confluence is 80-90% by visually inspecting the bottom of the flask using an inverted microscope.
9. Harvest the cells from the T-flask(s) by following the procedure outlined in Appendix B.2., steps 4-14.
10. After resuspending the cell pellet in 5 mL of complete EMEM, take a 0.5 mL sample of the cell suspension and use the Vi-Cell to determine the VCD. Record the total number of cells harvested, the number of cells harvested per cm^2 of the T-flask, and the viability.
11. Seed 18-24 T-25 flasks at a seeding density of 30,000 cells/cm^2 following step 17 in Appendix B.2. This is the positive control of this growth study for comparison with

MDCK growth on paper. Bring the final volume of the flasks to 7 mL. Record the time of seeding.

12. Determine the appropriate volume of cell suspension needed to seed the paper disks (ultra-low attachment plates) at 30,000 cells/cm^2 by solving Equation D.1. for V_1, where C_1 is the concentration of cells in the cell suspension, the desired cells/cm^2 is 30,000, and the surface area of the 32 mm paper disk is 8.04 cm^2.

Equation D.2.1.1.: $C_1V_1 = (Desired\ cells/cm^2)(Surface\ area\ of\ paper\ disk)$

13. Before seeding the paper, add the appropriate volume of complete EMEM to each well so that the final volume after seeding equals 3.3 mL.
14. Using a micropipette, seed each paper disk with the determined volume of cell suspension necessary for a seeding density of 30,000 cm^2.
15. Gently rock the plate to evenly disperse the cells in the wells.
16. Place the plates in the incubator and record the time of seeding.
17. Do not seed the paper disks in the 1 tissue culture-treated 6-well plate. This will be used as a negative control.
18. If this growth study on paper is not done alongside the T-25 flasks positive control study, seed the wells of another tissue culture-treated 6-well plate, without paper, at 30,000 cm^2. This will serve as a positive control.

D.2.2. Imaging Stained Cells in T-25 flasks and on Paper

1. Ideally at 12 hour intervals after seeding the growth study, image cells in both the T-25 flasks and on paper disks.
2. Create a 1 µM calcein AM solution by adding 1 µL of calcein AM per mL of complete EMEM.

3. Remove spent media from all flasks and wells to be stained.
4. Add 2 mL of the 1 μM calcein AM solution to each T-25 flask and 1 mL of 1 uM calcein AM solution to each well of the 6-well plate. Stain 3 T-flasks, 3 wells with cells on paper, and 1 negative control well at the time of each imaging.
5. Place the stained cells in the incubator for 20 minutes.
6. After 20 minutes, image fluorescently stained cells using an inverted microscope at a 4X objective and fluorescence. Capture images using the Dino-Eye microscope camera.
7. For the T-25 flasks, take images in 2 places on the bottom of the flask. In each location, take an image with and without fluorescence.
8. Before imaging paper, gently remove paper from the wells using forceps. Gently invert the paper and place, top-side down, in a new 6-well plate.
9. Take fluorescent images in 2 locations on each paper disk, including the negative control paper without cells.

D.2.3. Image Analysis to Determine Surface Confluence

1. Olympus CKX-CCSW Cell Confluence Checker software is used to analyze each image of fluorescent cells to determine the surface confluence.
2. Open the images in the software. Images may need to be adjusted before analysis to increase contrast for ease of analysis.
3. Calibrate the confluence checker before analysis of each image to ensure that the software is correctly distinguishing background and cells.
4. Record the percentage of surface confluence that the software determines for each image.

APPENDIX E: Simulated Plug Flow Reactor Growth Study of Adherent MDCK on Bibulous Paper vs. T-25 Flasks

E.1. Materials

- T-flask(s) of MDCK, 80-90% confluent
- Complete EMEM (Preparation detailed in Appendix A.2.)
- Bibulous paper (Fisher, Cat#)
- Autoclave Pouches (Fisherbrand, Cat# 01-812-54)
- Metal forceps
- 5 Corning ultra-low attachment 6-well plates
- 2 Corning tissue culture-treated 6-well plates
- 21 Corning tissue culture-treated T-25 flasks
- PBS (Gibco, Cat# 10010-023, 1X, pH 7.4)
- TrypLE Select (Gibco, Cat# 12563-011, 1X)
- 15 mL Falcon conical tube
- Centrifuge
- VI-Cell XR Cell Viability Analyzer
- Nova Bioprofile 400 Metabolite Analyzer
- Roche Cedex Bio-HT
- Inverted light microscope
- Humidified incubator (37˚C, 5% CO_2, static)
- Calcein AM (Invitrogen, Cat# C3099, 1 mg/mL in anhydrous DMSO)
- Dino-Eye Digital Eye-Piece
- Fluorescence Illuminator (Nikon Intensilight C-HGFI)

- Fluorescence Filter Cube (Nikon FITC-HYQ, Excitation 460-500 nm)
- Olympus CKX-CCSW Cell Confluence Checker software

E.2. Procedure

E.2.1. Setting up the growth study by seeding T-25 flasks and paper with MDCK

1. Follow the procedure in Appendix D.2.1. to make bibulous paper disks, autoclave paper and forceps, transfer paper to 5 Corning ultra-low attachment 6-well plates and 2 Corning tissue culture-treated 6-well plates, and seed 21 T-25 flasks and the 5 ultra-low attachment 6-well plates at 30,000 cells/cm^2. The 2 tissue culture-treated 6-well plates with paper will serve as negative controls.

E.2.2. Imaging Stained Cells in T-25 flasks and on Paper

1. At 24 hour intervals after seeding the growth study, image cells in both the T-25 flasks and on paper disks.
2. Create a 1 μM calcein AM solution by adding 1 μL of calcein AM per mL of complete EMEM.
3. Remove spent media from all flasks and wells to be stained. Stain 3 T-flasks, 3 wells with cells on paper, and 1 negative control well at the time of each imaging.
4. Combine the spent media into a representative sample for both the T-25 and paper disks. Analyze the samples on the Vi-Cell, Nova, and Bio-HT for VCD, viability, pH, glucose, glutamine, ammonium, and lactate.
5. To stain cells, add 2 mL of the 1 μM calcein AM solution to each T-25 flask and 1.5 mL of 1 μM calcein AM solution to each well of the 6-well plate.
6. Place the stained cells in the incubator for 30 minutes.

7. After 30 minutes, image fluorescently stained cells using an inverted microscope at a 4X objective and fluorescence. Capture images using the Dino-Eye microscope camera.
8. For the T-25 flasks, take images in 2 places on the bottom of the flask. In each location, take an image with and without fluorescence.
9. Before imaging paper, gently remove paper from the wells using forceps. Gently invert the paper discs and place, top-side down, in a new 6-well plate.
10. Take fluorescent images in 2 locations on each paper disk, including the negative control paper without cells.

E.2.3. Simulating a Plug Flow Reactor

1. 48 hours after seeding the T-flasks and paper disks, begin simulating a plug flow reactor.
2. After staining 3 flasks/wells, remove all spent media from the remaining flasks and plates.
3. Replace spent media with an equal volume of fresh, pre-warmed complete EMEM.
4. Repeat this process every 24 hours after the first time at hour 48 post-inoculation.

E.2.4. Image Analysis to Determine Surface Confluence

1. Analyze images of fluorescent cells using Olympus CKX-CCSW Cell Confluence Checker software, as described in Appendix D.2.3.

APPENDIX F: Operation of the LSM 880 for Confocal Microscopy of Adherent MDCK on Bibulous Paper

Protocol written by Colleen Duffy based on training provided by Mariusz Zareba at the NC State Cellular and Molecular Imaging Center.

F.1. Procedure

F.1.1. Turning on the LSM 880 microscope

1. Follow the numbered sequence of labels on the microscope to turn on in the correct order
2. The mercury lamp is #1, hit the on switch
3. Turn on #2 and then wait until you hear a click and everything is powered on before hitting #3
4. #3 powers on many microscope components and the touch pad. The touch pad must load completely before proceeding.
5. Once touch pad is on home screen, switch #4 on and then immediately hit #5 which is located on modem under the computer desk
6. Once computer turns on choose "LSM User"
7. Wait for the desktop to load completely before proceeding, as all drivers need to load. Wait ~1 minute.
8. Choose Zen Black icon then click "start system"
9. In Zen program, go to "Acquisition" tab and set the Argon laser to Power → ON because it takes several minutes for that laser to warm up. Once it isn't red it is ready to use. Prepare samples while laser is turning on.

F.1.2. Loading sample into microscope

1. Push microscope head back

2. On touch screen, hit “Load Position”
3. Move microscope slides to cradle sample dish without touching lens
4. Put microscope head back then press the triangle button on the touch pad to put the microscope back into working position
5. Move microscope head back again and remove the sample
6. On the touch screen go to “Control” and choose the first 40X W objective (this one is better for long distances)
7. Use the water dropper bottle to put one drop on the lens. Add water to the lens every 2-3 samples. Don’t go from immersion lens to 20X without cleaning water from the bottom of the dish because 20X objective is not water immersion.
8. Press “back” on the touch screen to keep the microscope at 10X
9. Choose “load position” again and reload the sample, lowering the microscope head afterwards
10. Turn lights off in the room

F.1.3. Getting the microscope ready for imaging

1. In the “reflector” tab on the touchpad, “position 6” is for normal light for bright field microscopy. “TL” (transmitted light) must be turned on on the touchpad for the light to be turned on on the microscope.
2. In “XYZ” tab on the touchpad you can see the z position of the lens. 0 is where the microscope was positioned when it was turned off. For z focusing of the lens, you can use the knob on the touchpad or on the microscope. Go down first to make sure that the lens doesn’t touch the dish, then go up to find the focus plane. To focus down turn the knob

towards you, to focus up turn knob away from you. The 10X can go up 2mm and the 40X can go up 0.6 mm.

3. Go to the "Control" tab on the touchpad and turn on different light filters to focus on your sample. The AF488 is green and Dapi is blue.
4. Switch to 40X objective and reexamine imaging area. May have to adjust the Argon lamp intensity down, can do that with the knob on #1

F.1.4. Imaging (on the desktop)

1. You can open up previous imaging files and click "Reuse" under the image to load those imaging settings. Check that the pinhole is still set to 1. Will have to reset the first and last images. If not, follow steps 2 through 6.
2. In the "Acquisition" tab, start by selecting imaging parameters in "Imaging Setup." The folder in the top right can load pre-set configurations. 405/488 is for blue and green excitation. In the green you can actually select calcein. In 405 track you can select Dapi. Check below the color spectrum that your wavelengths by the dish icons match those in "Channels"
3. In "Channels" you set image parameters. You have to set a pinhole, which excludes unwanted light for sectioning. Only set the pinhole for the 488 wavelength since it gives the most crucial information – the cells. Check the 488 laser box. Click on the "1 AU" button and it automatically sets the pinhole size to 1 AU.
4. Adjusting laser power: Turn lasers on and off by checking the box in the "Channels" tab. We turn one laser off to adjust power individually to avoid bleaching. Go into live mode. Move the focus up and down to make sure that laser power is constant throughout each plane. Check the "Range Indicator" box under the live image. All red is where the image

is over-exposed. Laser power is by the yellow triangle with the red star icon. Move that bar until you see some red but not much. Use “Digital Offset” to control the amount of black pixels in the live image. Increase the value until you only have a few blue pixels. Adjust the other laser. To avoid bleaching the sample, you can adjust laser power in one area and then move to another for imaging. Keep laser power low, because you can adjust the histogram of the image afterwards. If there is one very bright cell or fiber, may move view frames.

5. Switch to the “locate” tab to view through the microscope. The pinhole also varies when you change between objectives, so you have to switch the pinhole back to 1 AU.
6. In the “Acquisition Mode” tab, adjust the image quality. Click “optimal” first. Last time we used 1024x1024 and that was good enough. Recommended to have the averaging number between 2 and 5. We put averaging number on 2.
7. Check the “z-stack” box. Go to live mode and then “set first” for the bottom plane and “set last” for the top plane. The interval controls the interval at which slices are taken. Set the interval to 2 µm. Click “start experiment” to take images. Multiply the number of slices by the scan time to estimate how long the imaging will take.
8. Immediately save images before viewing them or making any adjustments

F.1.4. Cleanup

1. After imaging, check the microscope calendar before turning the Argon laser off. If someone is on the calendar after you, put the laser in standby. Always turn the laser off after the appointment or in standby.
2. Using z position in touchpad, put the lens back in the 0 position
3. Put microscope back in 10X position before removing sample. Remove sample.

4. Put microscope back in 40X to wick water off of the 40X W lens with a kim-wipe, not coming into contact with the lens with the wipe
5. Leave microscope in 10X objective
6. Save all images before exiting software.
7. Turn off all components in reverse order. Before turning off #2 and #3 make sure that the fan stops because the laser has to cool down.

APPENDIX G: Batch Growth of MDCK on Paper Substrates

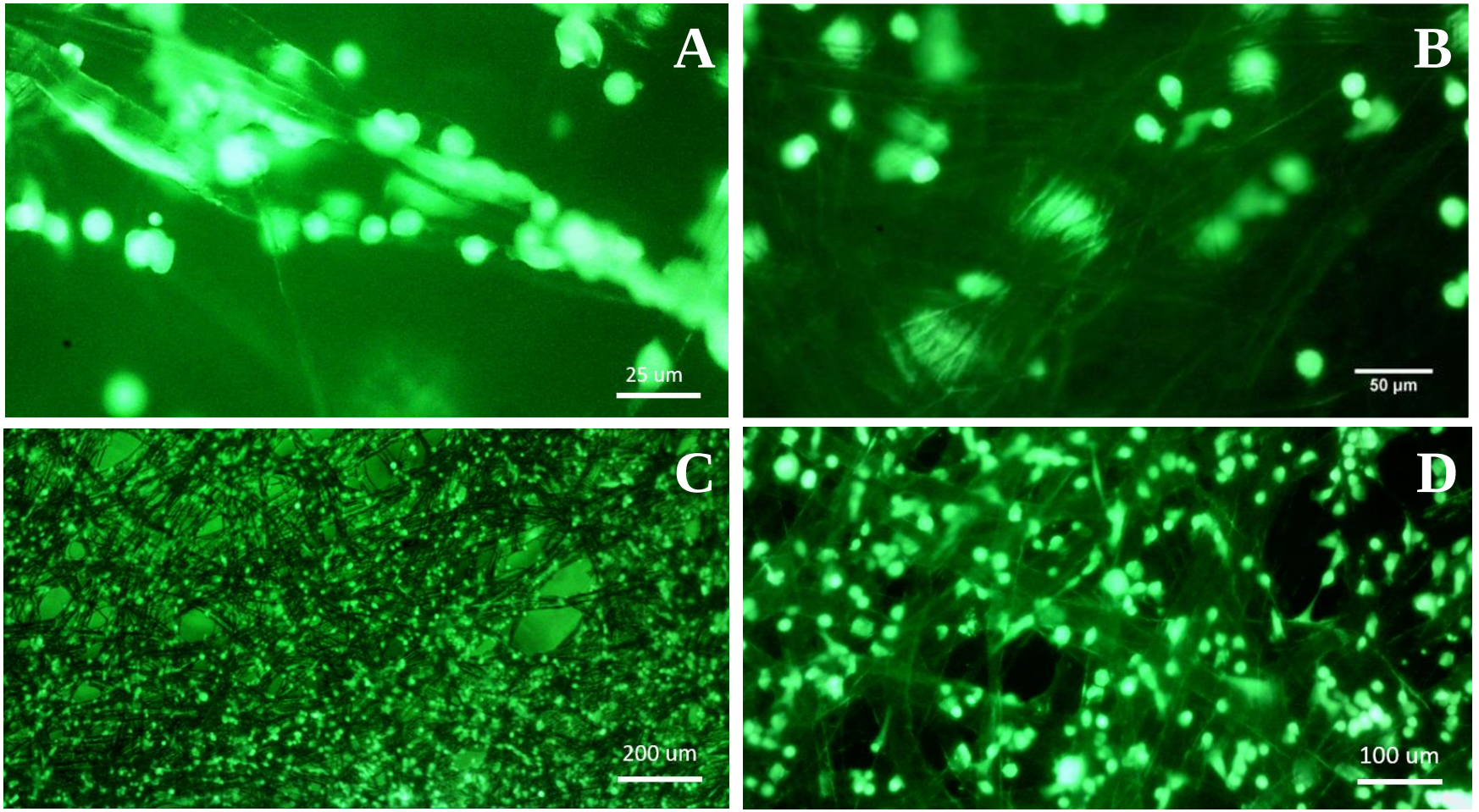

Figure G.1. Batch growth of MDCK on Paper substrates. (A) MDCK growing along the fibers of Forest Biomaterials blotter paper, 40X magnification. (B) MDCK growing on and underneath the fibers of Fisherbrand autoclave paper, 20X. (C-D) MDCK growing on Fisherbrand lens paper, 4X and 10X respectively.

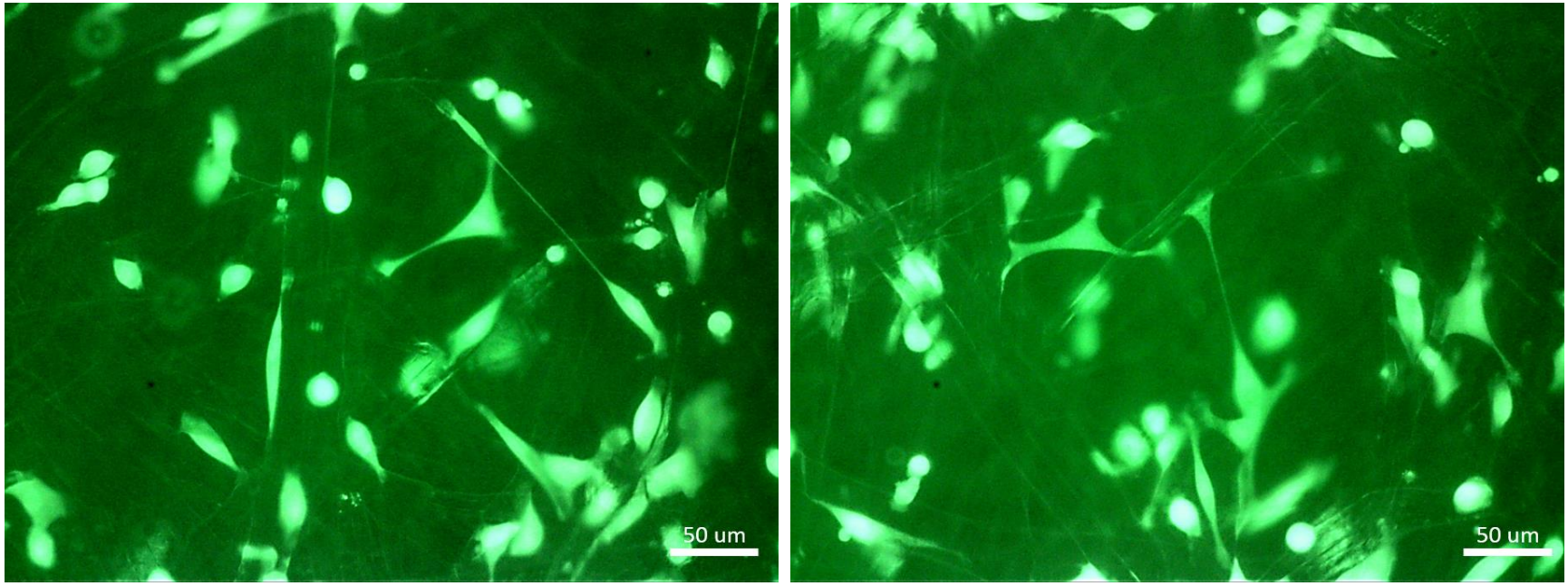

Figure G.2. Batch growth of MDCK on bibulous paper. 20X objective.

APPENDIX H: Confocal Microscopy of SPFR-grown MDCK on Bibulous Paper

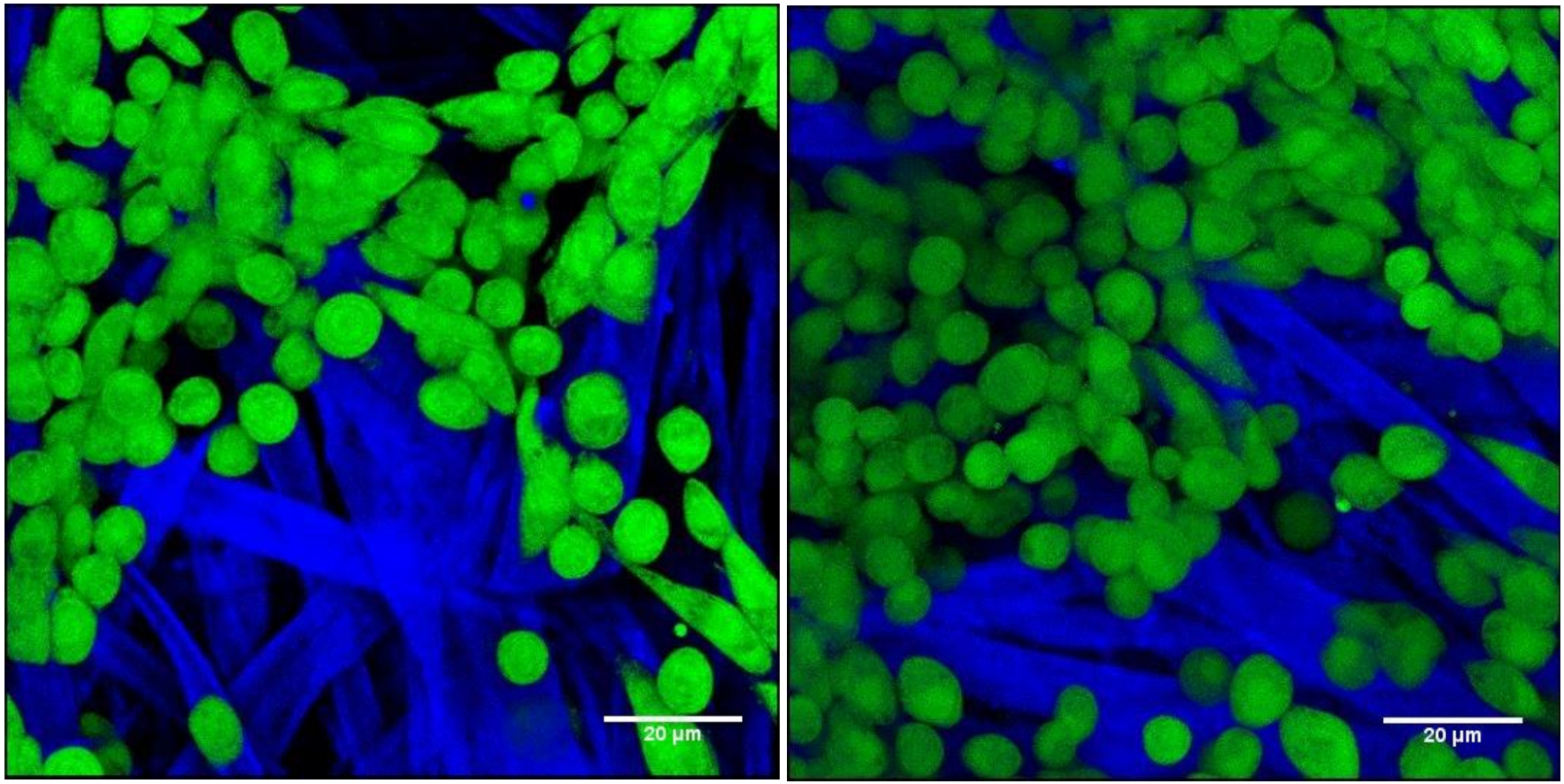

Figure H.1. Z-stacked views of MDCK growth on the top surface of bibulous paper. SPFR MDCK are 168 hours (7 days) post-inoculation, 40X magnification, 2µm slices