ABSTRACT

THONGTHAI, WOR. Effects of Photobleaching on Membrane Fusion and Infectivity of Dye Labeled Sindbis Virus. (Under the direction of Keith Weninger.)

Membrane fusion has long been accepted as a common cell entry mechanism for enveloped viruses. We have observed membrane fusion between Sindbis virus (SIN) and liposomes formed from purified lipids using a fluorescence dequenching assay. We verified that labeling Sindbis virus with R18, DiI, and DiD fluorescent dyes did not decrease its infectivity as determined by plaque assays. We found that infectivity was eliminated after extensive photobleaching of R18-labeled Sindbis virus, and in contrast, DiI and DiD labeled Sindbis both maintain infectivity following bleaching. Even though photobleached R18-labeled Sindbis was not infectious, we verified that it is still fully competent for low pH triggered membrane fusion using a liposome assay where fusion was indicated by the dequenching of a second membrane dye incorporated into the liposome. We attribute the inactivation of Sindbis virus to the propensity of R18 to translocate to the inner leaflet of the viral membrane, thus exposing the viral genome to the byproducts of photobleaching. Possible mechanisms of photoinactivation are discussed.

Effects of Photobleaching on Membrane Fusion and Infectivity of Dye Labeled Sindbis Virus

by
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DEDICATION

I would like to thank my mom and dad for all their love and support (and a whole lot of patience). I thank them for supporting me in my decisions, where my pursuit a degree in physics is one of them.

BIOGRAPHY

Wor Thongthai was born in Philadelphia, Pennsylvania. He moved with his parents to Bangkok, Thailand at the age of three and completed his primary education there. He moved back to United States in the 11^{th} grade and finished his high school education in Shelly, Idaho. He received his Bachelor's degree in Physics and Mathematics from the University of Idaho.

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I would like to thank my advisor, Keith Weninger, for his guidance, advice, assistance, and for his patience of putting up with my often time excruciatingly slow progress. I also would like to thank everyone in the Weninger Lab: Brandon Choi, Trevor Anderson, John Sakon, and Laura Wessels, for all their help and support.

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I want to thank my friends of the Raleigh University Ward for all their support and relentless questions on the status of my thesis. Also special thanks to Brittany Walker, the only person outside my lab who was willing enough to go through my entire thesis and edit it.

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INTRODUCTION

A more complete knowledge of the initiation of the virus reproduction cycle may lead to better understating of cell functions and more effective means of combating viral infection. Because membrane fusion is an integral part in cell entry during initiation of the virus reproduction cycle in many viruses, a great deal of experimental effort has been expected to explore the relationship between membrane fusion and the viral infection process [1-4]. This thesis reports our experiments characterizing the effects, on infection, of photobleaching fluorescent dyes commonly added to viruses to study cell entry mechanisms.

1.I Membrane fusion

Membrane fusion is a process by which two distinct lipid membrane bilayers merge to form one continuous membrane bilayer. Membrane fusion allows the encased content to be transferred across the membranes. Figure 1 shows a simplified model of membrane fusion. The figures on the right show two distinct vesicles fusing to form one vesicle and figures on the left show fusion from the molecular perspective of the lipid bilayers. One of the key features of membrane fusion that is essential for the experiments described in this thesis is the continuity established between the two initially distinct membranes by fusion. Since biological lipid membranes are fluid in the plan of the membrane at normal temperature, this continuity allows diffusive lipid mixing of the two membranes following fusion.

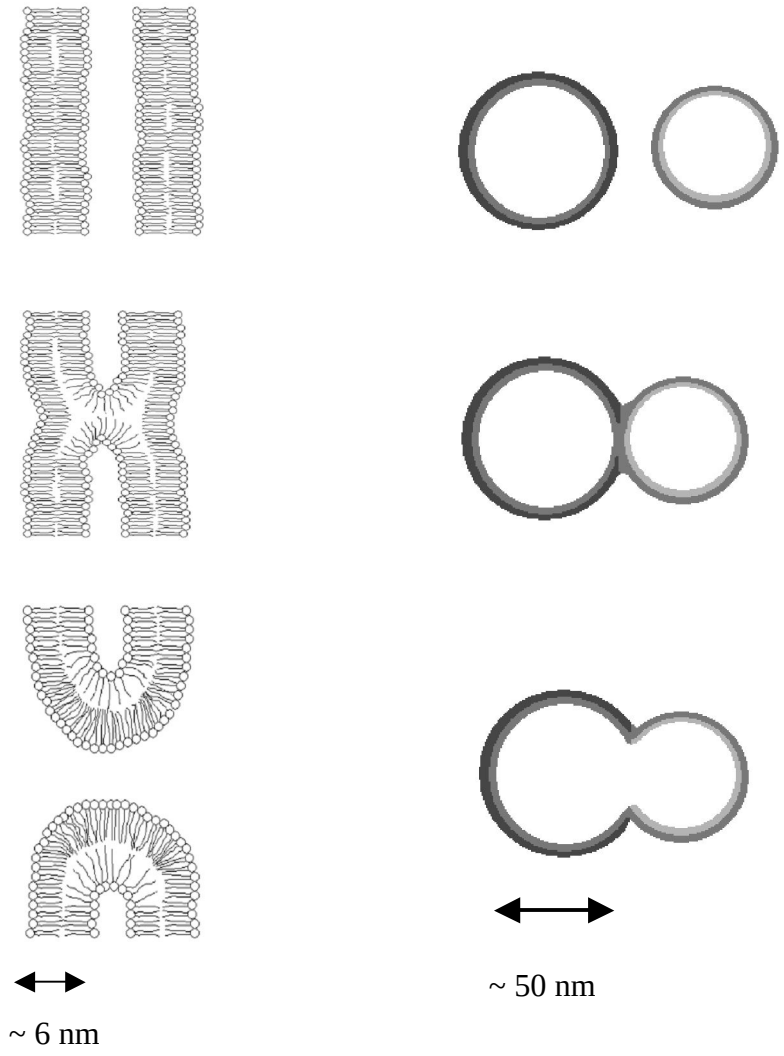


Figure 1. Membrane fusion. The left column shows a simplified diagram of two fusing lipid bilayers, with phospholipid schematically represented as a hydrophilic headgroup (circle) and two hydrophobic acyl chains (2 lines). The right column shows two distinct vesicles fusing into one vesicle, with the inner and outer leaflets shown in distinct colors.

Membrane fusion is one of the most important and fundamental biological processes in cell biology. For example, it is responsible for basic cell functions such as endocytosis and exocytosis, and it plays a role in specialized operations such as neurotransmitter release at neuronal synapses [5]. Studies of membrane fusion range from early work exploring chemically induced cell fusion [6, 7], to examinations of the governing mechanism of SNARE proteins [8], and to the application of liposome-based carriers as target delivery vehicles for specific compounds [9].

1.II Fluorescent dyes

One of the most useful tools for studying membrane fusion in many settings is fluorescent dye labeling of the membrane. One important property of fluorescent dyes is that they each have specific absorption spectra with corresponding red-shifted emission spectra due to internal molecular vibration, which makes filtering unwanted signals possible. For example, Brandenburg et al. took advantage of advances in the sensitivity of fluorescence microscopy to perform single particle tracking experiment using two distinct fluorescent dyes and monitored RNA release from viral capsid *in vivo* at the single-virion level [1]. Fluorescent dyes can also be used to study membrane fusion in at least two different ways: resonance energy transfer and fluorescence dequenching.

1.II.a Resonance energy transfer

Resonance energy transfer is a phenomenon that occurs when fluorescent dyes of different colors transfer energy, which was absorbed from photoexcitation, to each other. The efficiency of transfer is strongly dependent on the distance: inversely proportional to

the sixth power of the distance between the dyes [10]. Early works by Struck et al. used a fluorescent energy transfer assay to study fusion by labeling each of the two constituents with dyes of different colors, and only exciting one of the dyes [11]. Prior to fusion only the emission spectra of the excited dye can be detected. When membrane fusion occurs the two dyes are brought into close proximity with each other due to lipid membrane diffusive mixing, at which point energy transfer from the excited dye to the non-excited dye occurs. This energy transfer results in decrease in emission intensity of the directly excited dye and increase in the emission intensity of the non-directly excited dye.

1.II.b Fluorescence dequenching

Another common use of fluorescent dyes in the study of surface membrane fusion utilizes fluorescent dye quenching. When the concentration of fluorescent dye exceeds a certain threshold, dye quenching occurs, which reduces the dye emission intensity due to either dye dimerization into a non-fluorescent form or through resonant energy transfer mechanisms. In fluorescence dequenching assays, a lipid-like fluorescent dye is incorporated into the membrane of one of the fusing structures at a sufficiently high concentration that the dye self-quenches. When a labeled membrane fuses with a fluorophore-free target membrane, the concentration of dye is decreased, the quenching is released, and the intensity of fluorescence emission increases (Figure 2). Early work by Hoekstra et al. explored membrane fusion between influenza virus and phospholipid vesicles by taking advantage of the dye quenching mechanism [12]. This assay has been particularly effective in characterizing the dependence of membrane fusion capacity on many parameters, including buffer and lipid compositions in a variety of systems.

More recently, single particle tracking approaches combined with fluorescence dequenching techniques, have been used to determine the spatial trajectory of enveloped virus within live cells and have directly revealed fusion events along viral infection pathways. This method is being applied to studies of enveloped viruses that are labeled with quenching concentrations of lipid-like dyes in their membrane and observed as they enter living cells [2, 4, 13, 14]. While it is possible to study fusion in a variety of viruses, the focus of the studies in this thesis was on Sindbis virus due to its relatively simple composition and structure.

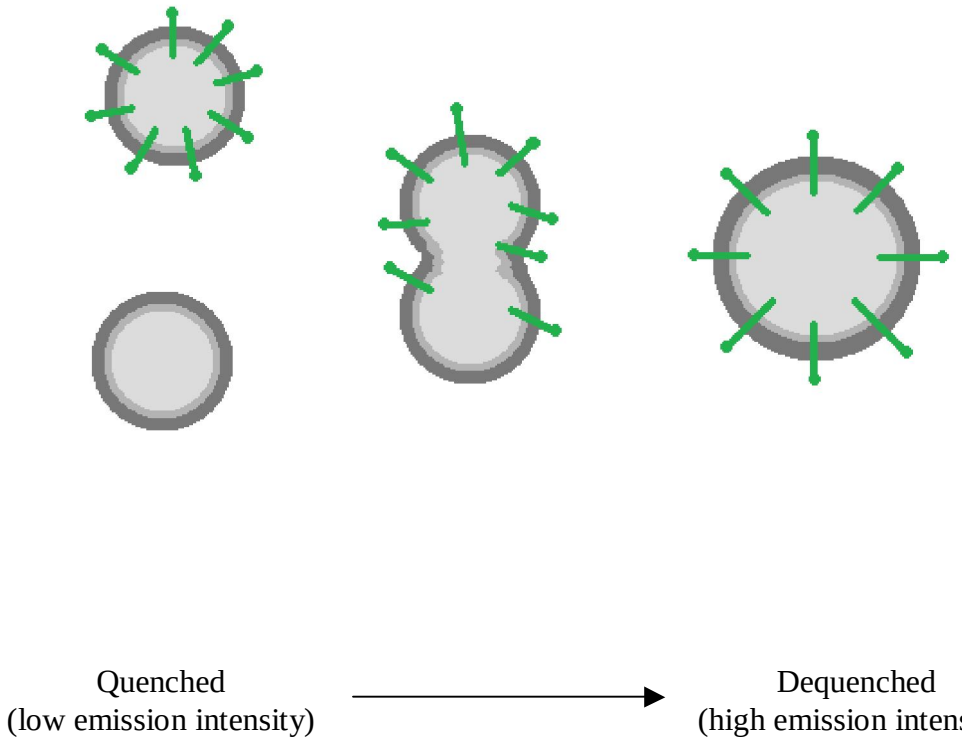


Figure 2. Fluorescence dequenching. The dye is restricted to one vesicle before fusion, but may spread by diffusion into the other, dye-free, membrane after vesicle fuses. The emission intensity of the fluorescent dye rises as the distance between dyes increase with the increased surface area after membrane fusion.

1.III Sindbis Virus

Sindbis virus (SIN) is a virus in the alphavirus genus in the Togaviridae family. It was first isolated in the Sindbis health district, Egypt in 1952, and has been well-studied. Sindbis virus is now considered a prototype of the alphavirus genus [15, 16]. A Sindbis virus particle is 70 nm in diameter and is composed from three distinct structural proteins: Envelope glycoproteins 1 and 2 (E1, E2) and capsid in a 1:1:1 stoichiometric ratio with the outer shell constructed from E1:E2 dimers rearranged into trimers [17]. The E1, E2 proteins are imbedded in the viral lipid bilayer composed of lipid membrane derived from the previous host. Both the E1, E2 protein envelope and the capsid core have T=4 icosahedral symmetry, with the E1 and E2 transmembrane domains traversing the lipid bilayer envelope and contacting the capsid [18-20]. Sindbis virus can infect a variety of cells without being pathogenic to humans while related viruses are the cause of many human diseases such as yellow fever and West Nile fever [21].

1.III.a Sindbis virus and membrane fusion

The topic of cell entry and RNA release mechanisms of Sindbis virus has been a subject of interest because its relatively simple structure and composition may aid research in virus-host interaction due to limited variables. Figure 3 shows the basic steps of the most commonly accepted model of RNA release thought to be employed by Sindbis virus: The virus particle enters the cell interior by endocytosis and the RNA is released within the cell through fusion between the viral membrane and endosome formed from cell membrane. The ability of Sindbis virus to deliver RNA into cells has led to it being used in gene therapy and cancer therapy. Tseng et al., for example, found

that Sindbis virus could efficiently target tumors *in vivo* under certain conditions [22]. Other studies have also demonstrated that Sindbis virus can be altered to infect human cells [23] and engineered to carry recombinant engineered genomes [24, 25].

While classical virology has been able to establish virus-receptor binding, membrane fusion/capsid membrane penetration, and viral genome replication as the important steps in the virus life cycle, many details such as the process or location of membrane fusion and the virus-host interactions are unanswered [3]. For example, it has been shown that low-pH is a necessary and sufficient condition for Sindbis-liposome fusion [26], but it has also been shown that low-pH exposure is not required for Sindbis virus to infect mosquito cells [27]. This apparent contradiction exposed the weakness in the classical model of virus since it suggested a much more complex relationship between surface membrane fusion and virus infectivity. Ongoing research combining fluorescence dequenching and single particle tracking in live cells is attempting to better understand the entry mechanisms of Sindbis virus by exploiting the benefits of single particle observation to reveal details that may be lost when averaging over an ensemble [28].

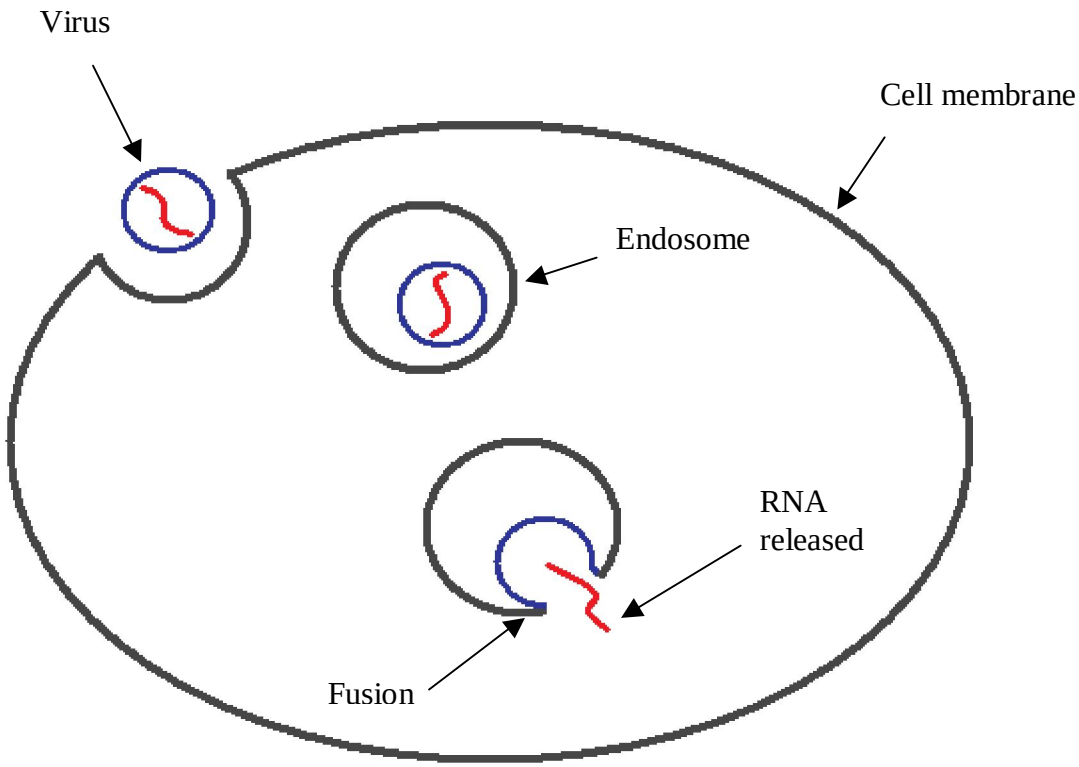


Figure 3. Sindbis RNA release mechanism. The basic steps in the most common model of RNA release mechanism employed by Sindbis virus: The virus particle enters the cell interior by endocytosis and the RNA (red) is released within the cell through fusion between the viral membrane (blue), and endosome formed from cell membrane (gray). Fusion is believed to be triggered by the acidification of the endosomal interior.

1.III.b Effect of fluorescent dyes

Labeling Sindbis virus with fluorescent dye is required to perform single particle tracking and fluorescence dequenching assays. However, addition of fluorescent dyes and observation by photon excitation may alter the virus or cell-virus interaction. Our own experiments have shown that photobleaching R18-labeled Sindbis virus can lead to photoinactivation, which is a phenomenon where a pathogen becomes noninfectious after exposure to light. In our case photoinactivation was achieved through labeling Sindbis virus with octadecyl rhodamine B chloride (R18) fluorescent dye and overexposing it to green laser light (532 nm). This and similar studies, which aim to determine modes of infection in live cells as opposed to biophysical aspects of membrane fusion, can be complicated by toxic byproducts of photobleaching. Experiments have shown that dye labeling of the nucleic acids within virus particles commonly leads to inactivation upon photobleaching, an effect that is under development for clinical application (known as photodynamic therapy). Single particle tracking studies using fluorescent dyes to label enveloped viruses with the goal of examining the role of membrane fusion in cell entry could be complicated by possible photoinactivation. We sought to examine the relationship between photoinactivation of a dye-labeled envelope virus, Sindbis virus, and membrane fusion capacity.

1.IV Goals

In the following work we employed a dual dye version of the fluorescence dequenching assay to investigate the fusion capability between liposomes formed from

purified lipids (total liver extract) and Sindbis virus under various labeling conditions. We first incorporated a dye into the virus membrane. This dye can be photobleached before membrane fusion is tested with liposome formed from purified lipids. To observe fusion, a fluorescent dye of distinct color from the dye in the virus is incorporated into the liposome and a dequenching signal is obtained upon virus fusion.

With this assay, we tested three common dyes used for labeling enveloped viruses: octadecyl rhodamine B chloride (R18), 1,1'-dioctadecyl-3,3,3',3' tetramethylindo-carbocyanine (DiI), and 1,1'-dioctadecyl-3,3,3',3' tetramethylindodicarbocyanine (DiD). Using plaque assays, we found that Sindbis viruses labeled with these dyes, but not photobleached, maintained the infectivity of unlabeled viruses. When the dyes were photobleached, the infectivity of DiI and DiD-labeled Sindbis virus did not change, but in sharp contrast, the infectivity of bleached R18-labeled Sindbis virus decreased at least five orders of magnitude. This decrease in infectivity is not a result of different membrane fusion capacity, because we observed that for all three dyes used, the bleached and unbleached samples fused with the same kinetics and efficiency. We suggest that the difference in the inactivation of R18 compared to DiI and DiD is related to the ability of R18 to “flip-flop” between the inner and outer leaflets of the virus membrane to allow reactive by-products of R18 bleaching to access the viral genome.

MATERIALS AND METHODS

2.I Sindbis virus and dye labeling

Sindbis virus, which was provided by professor Dennis Brown, NCSU, was grown in baby hamster kidney cells (BHK-21) cultured by standard methods in minimal essential media with Earl's salts containing 10% fetal bovine serum, 5% tryptose phosphate broth, and 2 mM glutamine. Cells were inoculated with Sindbis virus and incubated for 12 h at 37˚C. Supernatant was then collected and clarified by low-speed centrifugation. Sindbis virus was purified from the clarified supernatant by ultracentrifugation on a step density gradient followed by a continuous density gradient in phosphate-buffered saline (PBS, 10 mM phosphate, 140 mM sodium chloride, pH 7.4), containing variable amounts (15–35%) of potassium tartrate to adjust the density. Purified Sindbis virus solutions were adjusted to a concentration of 2×10^{12} particles/ml as calibrated by BCA Assay (Pierce Biotechnology, Rockford, IL). Sindbis virus was used immediately or stored at –80˚C without noticeable loss of fusion capacity [29].

Sindbis virus was dye-labeled with hydrophobic fluorescent dye; either R18 (Figure 4a) or DiI (Figure 4b) from Molecular Probes (Invitrogen, Carlsbad, CA). Aliquots of Sindbis virus (100 µl at 2×10^{12} particles/ml) were rapidly mixed at room temperature with 3 µl of dye dissolved in ethanol at 1.4 mM in case of R18 and 10 mM in case of DiI. The dye-virus mix was incubated on ice for 2 h. The dye-labeled virus was purified from unincorporated dye by gel filtration (NAP 5, G.E. Biosciences, Piscataway, NJ) at room temperature in Hepes-buffered saline (HBS, 5 mM Hepes, 150 mM NaCl,

pH 7.3). We estimate that gel filtration in NAP 5 columns diluted the samples to ~1/3 the starting concentration (0.7×10^{12} particles/ml). Unlabeled Sindbis virus used in control experiments was also passed through the NAP 5 column to maintain consistent concentration. Labeled/unlabeled virus was then stored in ice prior to use.

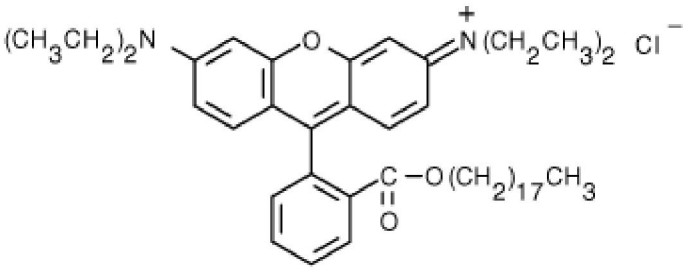
2.II Liposomes

Total liver lipid extract (25 mg/ml) was purchased from Avanti Polar Lipids (Alabaster, AL). Chloroform was removed from solutions of lipids under flowing argon leaving a film on the surface of a glass tube. The lipid films were placed in vacuum for at least 2 h and then hydrated with Hepes-buffered saline (HBS, 5 mM Hepes, 150 mM NaCl, pH 7.3) [30].

Liposomes were dye-labeled with DiD (1,1'-dioctadecyl-3,3,3',3' tetramethy-lindodicarbocyanine, Figure 4c). Total liver extract in chloroform was rapidly mixed at room temperature with DiD dissolved in ethanol to achieve final dye/lipid mass ratio of 5%. Chloroform and ethanol were removed from the mixed solution under flowing argon leaving a film on the surface of a glass tube. The film was then rehydrated with additional chloroform before again being dried under flowing argon to ensure both chloroform and ethanol was thoroughly evaporated. The film was placed in vacuum for at least 2 h and hydrated with HBS.

Large unilamellar vesicles (LUV) were prepared by extrusion (31 times) through 100 nm-pore filters (miniextruder, Avanti Polar Lipids) at 5 mg/ml (total lipid).

(a)



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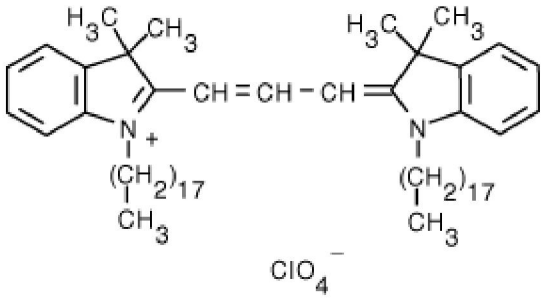

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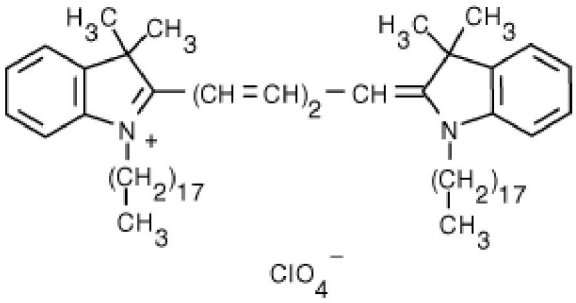

Figure 4. Chemical structures of fluorescent dyes. a) R18, b) DiI, c) DiD (Invitrogen™, www.invitrogen.com)

2.III Fluorescence dequenching assays

The fluorescent-liposome solution was diluted to 1/100 its original concentration to allow the beam from the spectrophotometer to penetrate the entire volume of the cuvette. Equal volumes of liposome solution (0.05 mg/ml) and virus solution (~0.7×10^{12} particles/ml) were mixed in a fluorescence cuvette. A fluorescence spectrophotometer (Hitachi F4500, Kyoto, Japan) detected fluorescence emission from the cuvette using excitation at 550 nm and emission at 600 nm for R18/DiI, and excitation, emission wavelength of 650 nm and 700 nm, respectively, for DiD. The slit width of the excitation and emission wavelength was set to 5 nm and 10 nm, and the spectrophotometer parameter was set to have PMT voltage of 950 V and a response time of 0.5 seconds. After the initial emission intensity (I_0) was recorded, the mixture was acidified by addition of an equal volume of an acidic buffer pretitrated to yield the desired final pH and corresponding intensity (I*). The emission intensity (I) was recorded for 5 min. Toward the end of the experiment, ~1% vol/vol of 100 mM dodecyl-maltoside was added to dissolve the samples and yield the unquenched intensity (I_f) (Figure 5).

Acidification buffer used to reach desired pH of 5.3 was 12 mM MES, 150 mM NaCl, pH 4.1 (MES). Control experiments at neutral pH used HBS (pH 7.3) in place of MES. The bulk fluorescence experiments were all performed at room temperature. Control experiments with unlabeled virus were performed at both low and neutral pH using the same method described above.

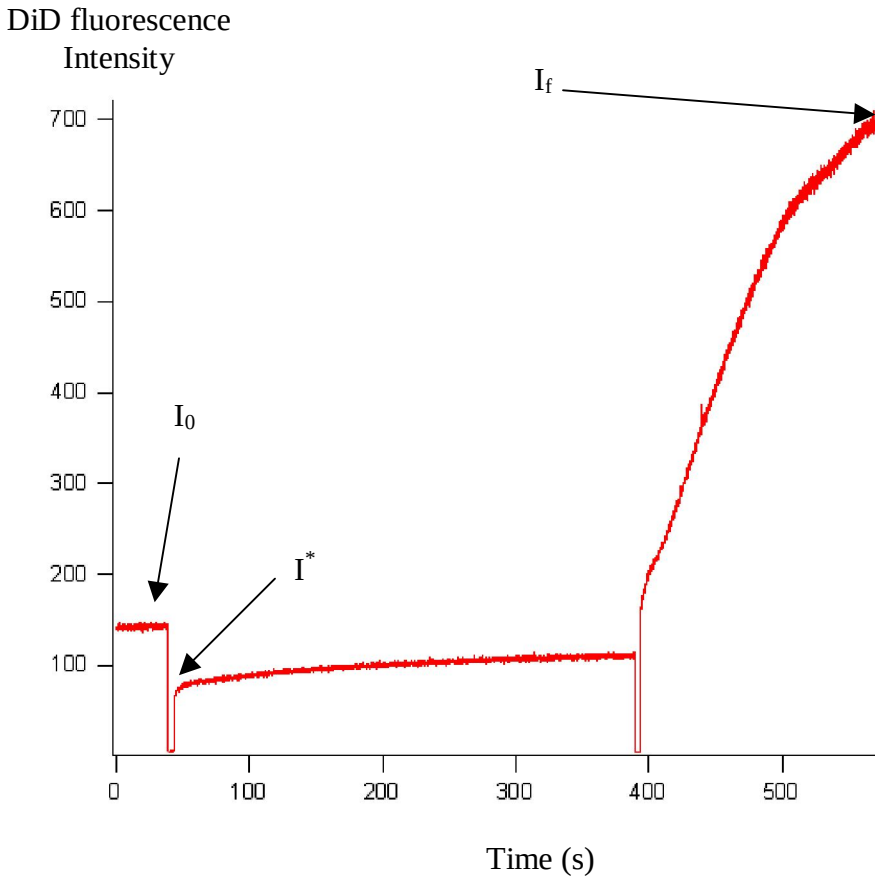

Figure 5. Raw data from fluorescence dequenching assay. I_0 is the initial emission intensity of the fluorescent dye, I^* is the intensity after the volume of the sample in the cuvette is doubled by adding equal volume of buffer to change the pH, and I_f is the intensity at infinite dilution. The two dips in intensity between I_0 and I_f were when the detector was shut off when the additional buffer and detergent were added, respectively. The unit for intensity is arbitrary as are the raw A-to-D output of the spectrophotometer.

2.IV Effect of pH on DiD emission intensity

Fusion of viruses with liposomes in bulk solution has been studied extensively with fluorescence dequenching in the past [26], and it is important to establish that our Sindbis viruses samples exhibit dequenching due to fusion that is consistent with this past work. In order to compare the result between various experiments, we normalized the DiD emission intensity with respect to I_f and obtained a DiD fluorescence dequenching percentage. We chose to normalize our data with respect to I_f because I_f is simply a function of the amount of dye added to liposome, which was held constant. However, we noticed that the emission intensity of DiD was affected by pH. In our experimental protocol, we doubled the volume in the cuvette by adding either acidic or neutral buffer, which should decrease the intensity by a factor of two simply due to the dilution. However, Figure 6a shows that the intensity we observed after adding acidification buffer was reduced to less than $I_0/2$ while the intensity after adding HBS (pH 7.3) was nearly $I_0/2$ (Figure 6b). This shows that the emission intensity of the dye is sensitive to the pH of the solution. We corrected our data for the effect of low pH on the fluorescent dyes in order to verify fusion as described below.

We measured the pH effect on the dyes in control experiments using dye-labeled liposome without virus. Data from many experiments where dye-liposome solutions were mixed with MES to lower the pH or HBS for neutral pH were collected and the average intensity drops were calculated. The average intensity of the dye-liposome after adding acidification buffer was $0.43I_0 \pm 0.01I_0$ while the average intensity after adding HBS was $0.54I_0 \pm 0.03I_0$. Using these values we can correct the DiD dequenching in

virus experiment using the formula $(I-(0.54*I_o))/I_f$ for neutral pH and $(I-(0.43*I_o))/I_f$ for low pH (Figure 6c), where I is the DiD emission intensity as a function of time during the experiment.

Because fusion can be triggered by introduction of low pH, quantitative analysis of fluorescence dequenching can be carried out by setting the dequenching value to zero immediately after lowering the pH by adding MES and scaling the intensity to 100% at infinite dilution. Infinite dilution is achieved when detergent is added to the mixture, causing the liposomes to dissolve and the distance between dyes to become large enough that the quenching effect is essentially eliminated. Complete dequenching is reached when the DiD emission intensity no longer increases. Control experiments were performed identical to fusion experiments with the exception that neutral pH HBS was added in place of low pH MES. An example of a scaled dequenching curve between labeled liver liposomes and Sindbis virus is shown in Figure 7c. All results throughout the rest of this thesis is scaled to % dequenching following this procedure.

2.V Photobleaching R18/DiI-labeled Sindbis vius

Sindbis virus labeled with R18 or DiI was contained inside a 200 μl pipet tip, which was placed in the path of a 532 nm solid state laser operating at 6.75 to 6.85 mW and focused with a lens to avoid beam contact with the plastic for at least 1 hour at room temperature to simulate laser exposure when under real-time cell-tracking experiments. The extent of photobleaching can be determined through the level of fluorescence dequenching (Figure 11). The extent of photobleaching was assessed by comparing the unquenched intensity

of photobleached samples to the unquenched intensity of non-bleached counterpart by addition of detergent.

2.VI Infectivity measurements

Infectivity of Sindbis virus was assessed with standard viral plaque assays performed by the members of Professor Dennis Brown's group at NCSU. Briefly, plaque assays were performed by applying serial dilutions of virus to identical monolayers of BHK-21 cells, which were then overlaid with agarose/media and grown for one day at 37°C. Plaques were stained with neutral red and counted by hand after two days. Virus concentration was measured by the BCA assay to allow calculation of plaque forming unit/ml (pfu/ml) for each sample. More in depth details of this assay are described elsewhere [31].

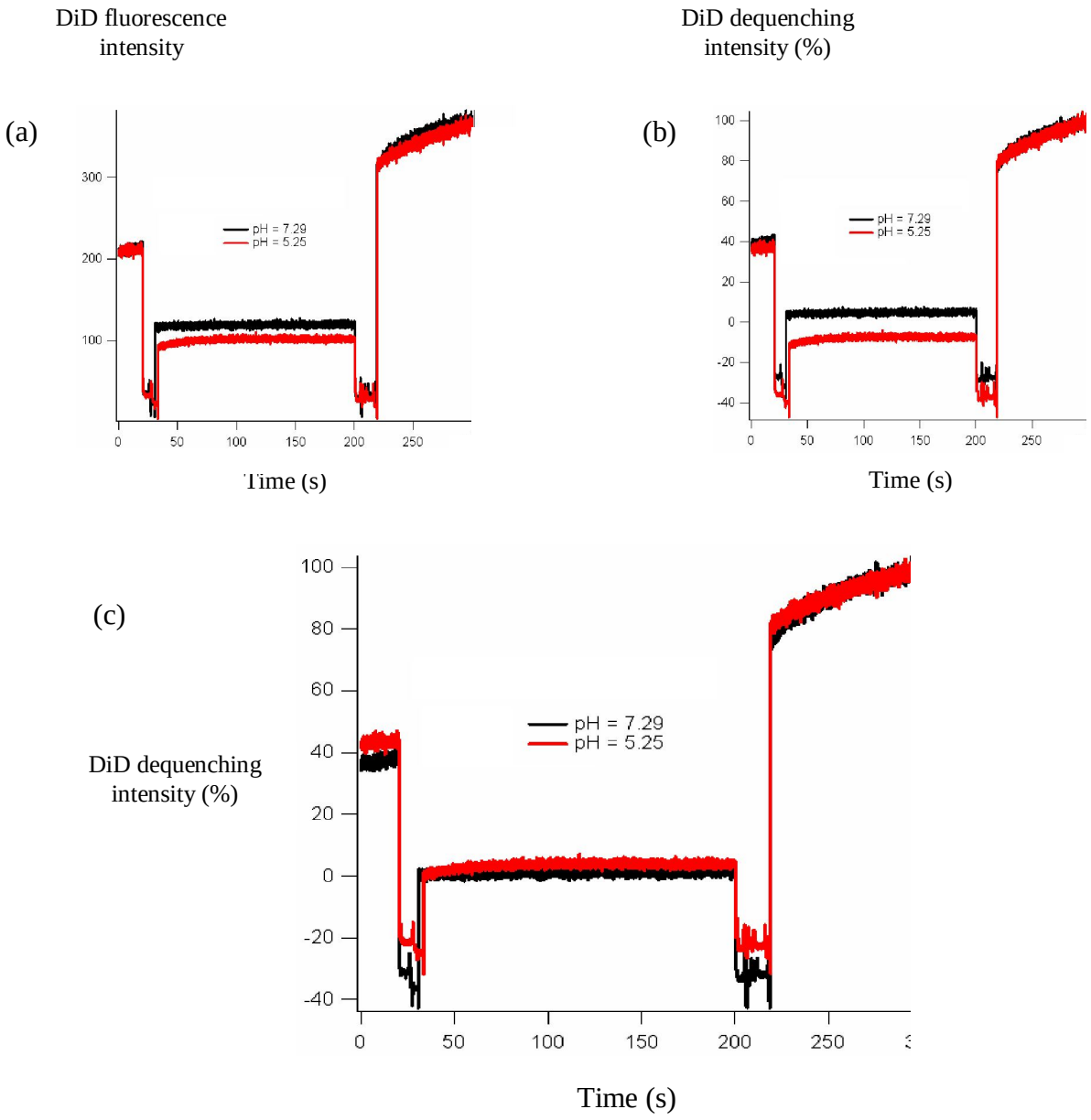

Figure 6. Correcting pH-dependent fluorescence. (a) Unscaled fluorescence of DiD-labeled liposomes with addition of neutral (black) and low (red) pH buffer at time ~30 sec, (b) fluorescence of DiD-labeled liposomes after a simple scaling, ignoring the pH effects on the dye, (c) fluorescence of DiD-labeled liver liposomes using new scaling values corrected for the pH effects on dyes.

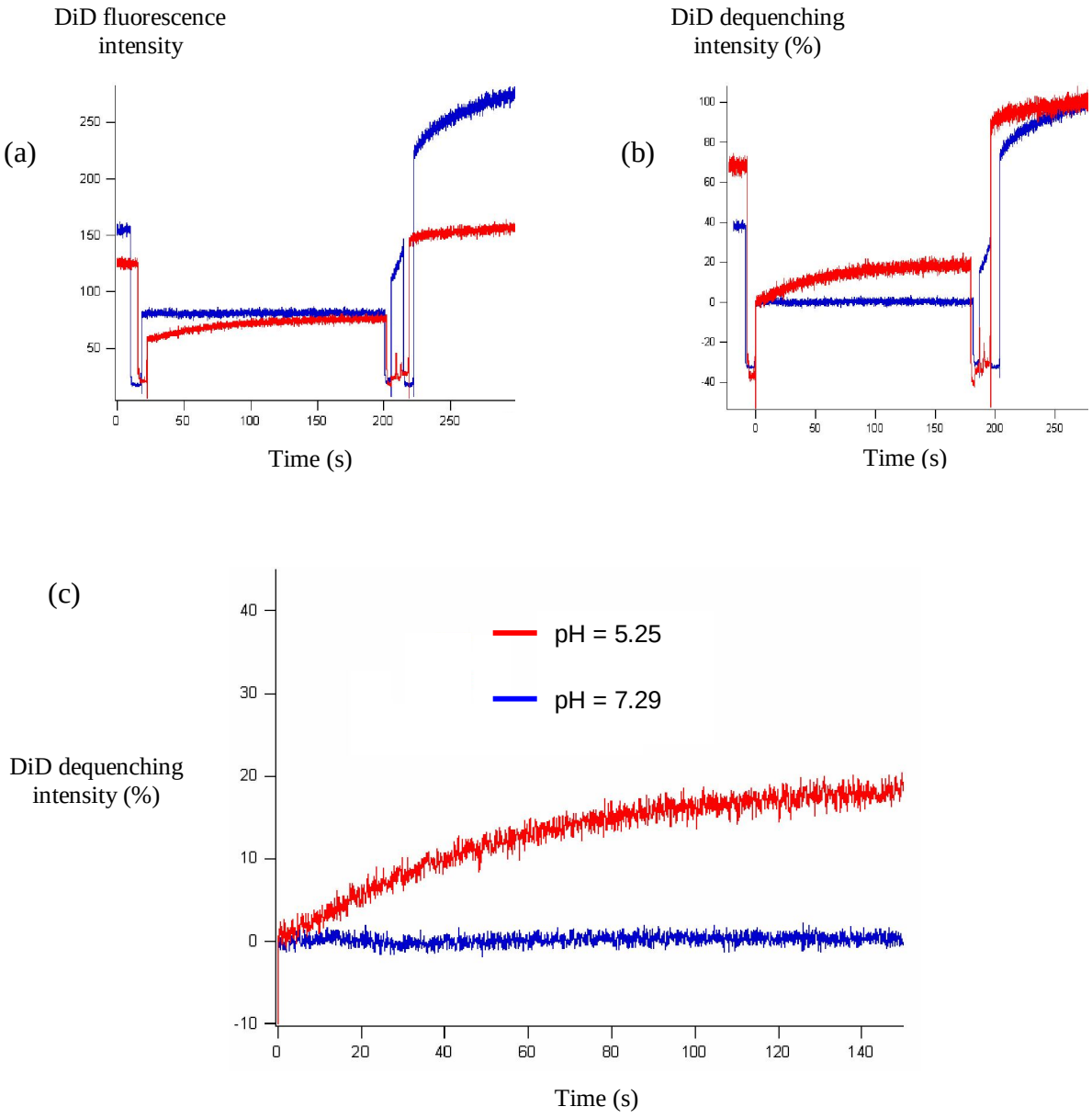

Figure 7. Scaling process. Red is low pH curve and blue is neutral. (a) Raw input data, (b) scaled data, (c) scaled data in (b) displaying only the time interval relevant to viral membrane fusion. The curve shows fusion between DiD- labeled liposome and unlabeled Sindbis virus.

2.VII Gel studies

SDS-PAGE Phastgels (GE Biosciences, 10-15% gradient gel) were used to separate viral proteins by electrophoresis according to manufacturer instructions. Gels were silver stained following manufacturer instruction. Low Molecular Weight (LMW) Standards (GE Biosciences) were used as molecular weight calibration standard.

RNA was extracted from bleached and unbleached samples of Sindbis virus by cesium chloride precipitation. The recovered RNA was radiolabeled with [5′-^{32}P]pCp using 3′-terminal labeling with T4 RNA ligase [32]. The labeled RNA samples were separated on a denaturing agarose gel (1% agarose, 2.2M formaldehyde) in standard buffer and imaged with a phosophoimager.

RESULTS

3.I Fusion capabilities of dye-labeled virus

Our initial experiments focused on exploring the effect of dye labeling on membrane fusion of Sindbis virus. Membrane fusion experiments were performed on mixtures of virus and liposomes in a bulk fluorimeter as described in section 2.III of the materials and methods section. First, experiments were performed between liposomes, which were formed from liver extract and labeled with quenched concentration of DiD as described in the previous section, and unlabeled Sindbis virus. Membrane fusion of viruses with liposomes was detected by fluorescence dequenching of DiD in the liposome bilayer. When fusion events occur, the increase in surface area will lead to decrease in DiD concentration and an increase in emission intensity. The fusion process was triggered by decreasing the pH of the system to 5.3 with the addition of MES buffer.

Figure 8 shows the DiD dequenching percentage from fusion between DiD-labeled liposomes and unlabeled Sindbis virus as well as Sindbis virus labeled with R18 and DiI. We observed no significant variation between the dequenching values of the unlabeled (green), R18-labeled (red), and DiI-labeled Sindbis virus (blue). Figure 9 compares the results of each low-pH triggered experiment with its neutral pH counterpart. The lack of fusion at neutral pH verifies that the fusion process is only triggered by lowering the pH. Figure 10 shows results of averaging together the final dequenching values of several repeated experiments. The fluorescence dequenching of

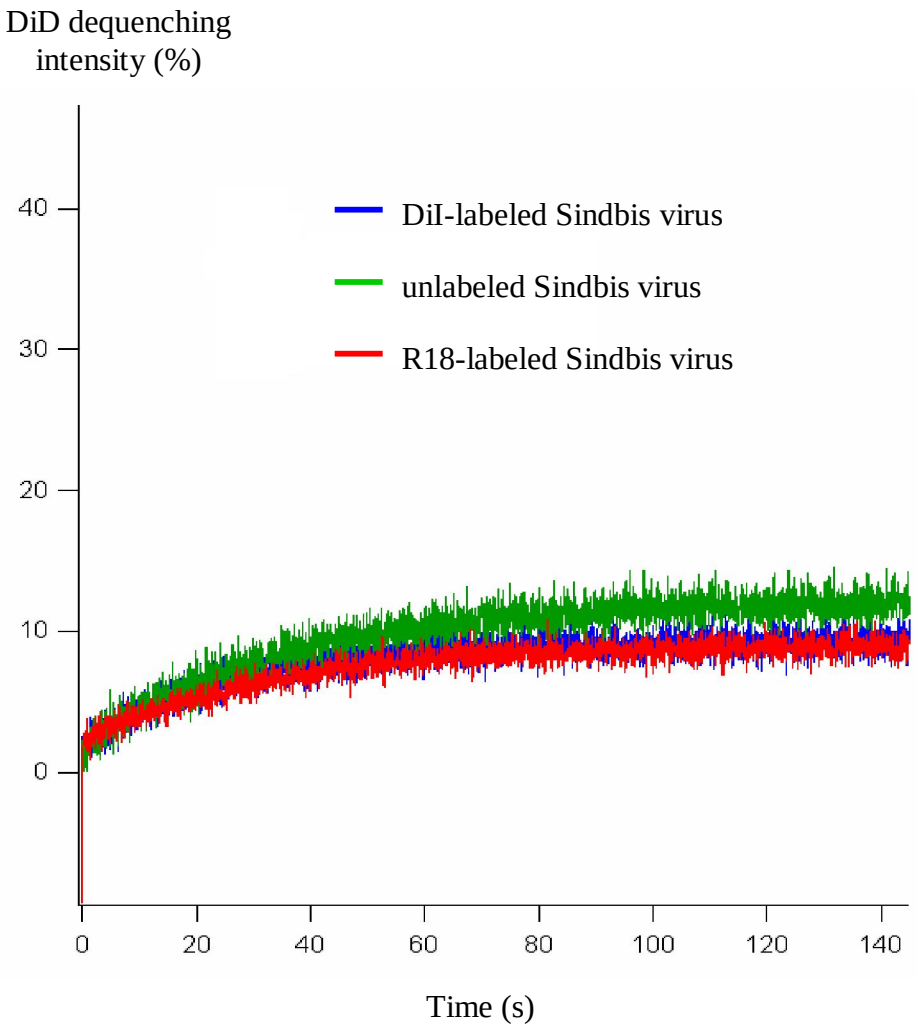

Figure 8. Effect of fluorescent dyes on fusion. Fluorescence dequenching assay intensity curve comparison shows similar DiD emission intensity from fusion between DiD-labeled liver liposome and Sindbis virus without fluorescent dye (green) and Sindbis virus labeled with R18 (red) and DiI (blue). We conclude that labeling Sindbis virus with these fluorescence dyes have no effect on membrane fusion.

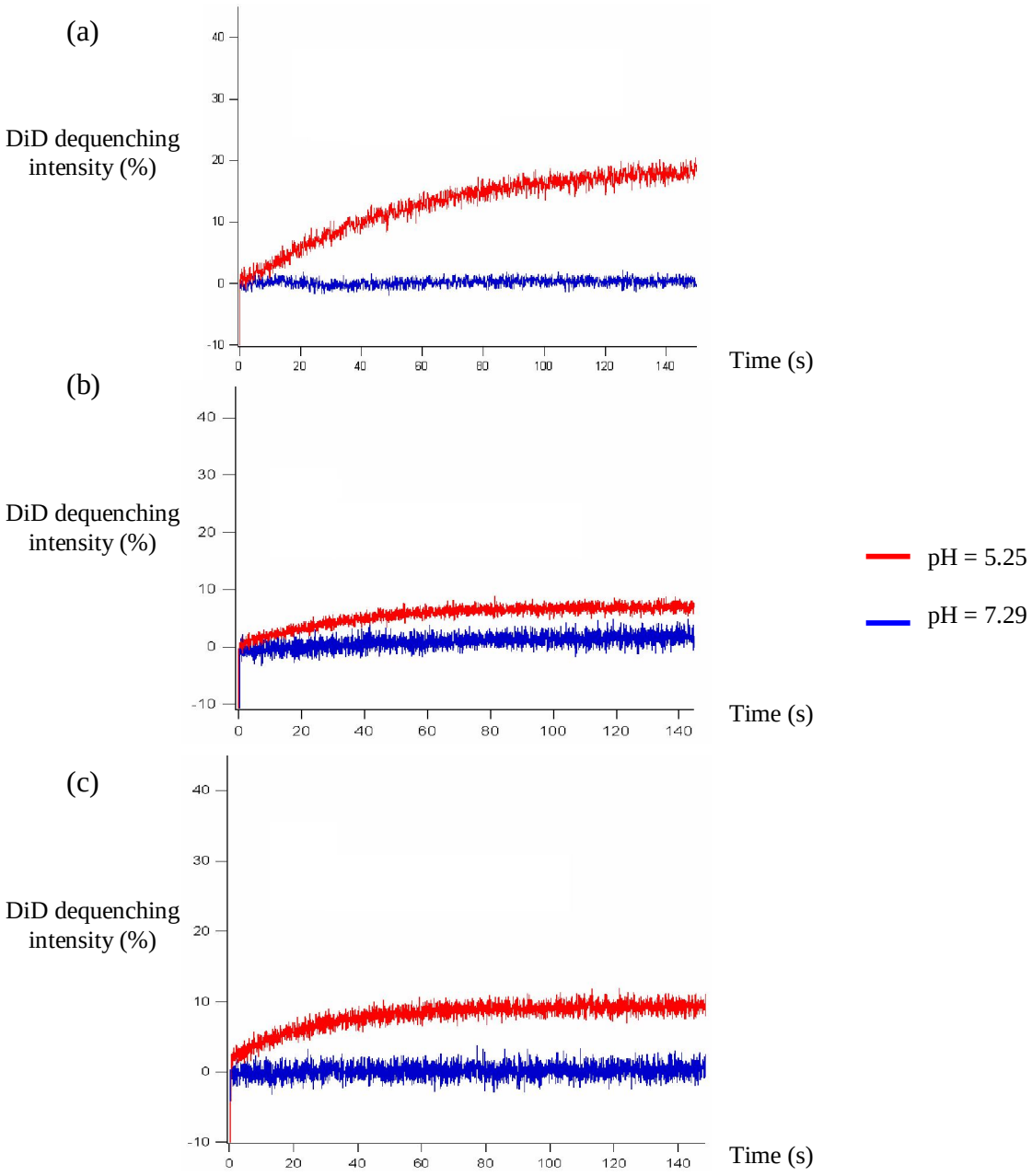

Figure 9. Verifying low-pH triggered fusion. Diagrams show fusion between liposome and a) unlabeled, b) R18 labeled, and c) DiI labeled Sindbis virus. All diagrams show increase in emission intensity only after adding low-pH buffer. We conclude that lowering pH triggers the membrane fusion.

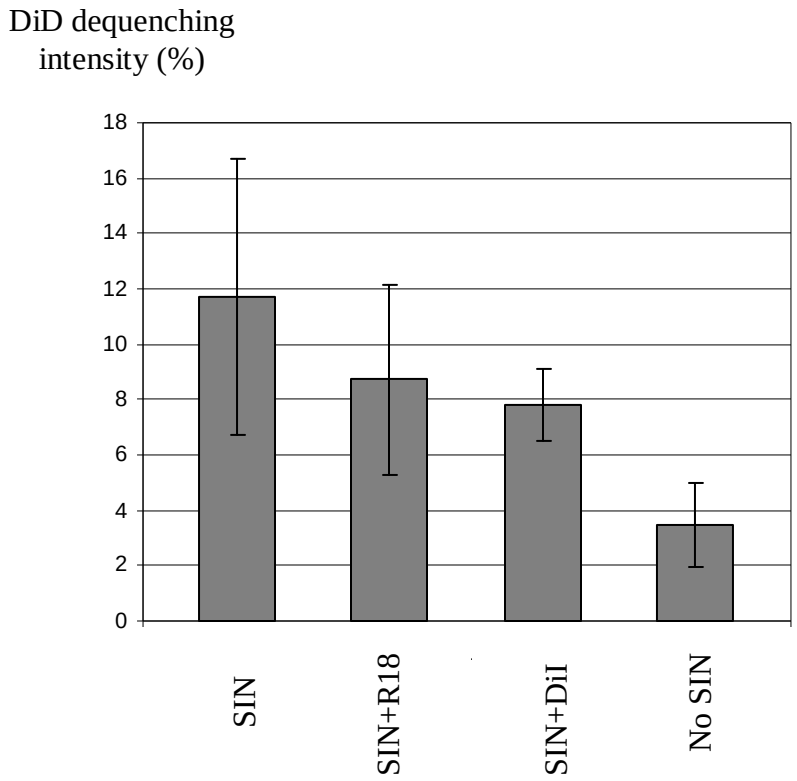

Figure 10. Average DiD dequenching. Fluorescence dequenching after fusion between DiD-labeled liposome with unlabeled SIN is 12%±5% compared to 8.7%±3% and 7.8%±1% of R18 and DiI-labeled SIN respectively. Intensity rise in the absence of SIN is only 3.5%±1.6% and is attributed to the use of averaged correction values and slight dequenching due to bleaching during observation. The values are represented as (average)±(1 std dev), and the error bars correspond to ±1 standard deviation of the averaged final dequenching values within each set of experiments.

DiD-labeled liposomes with unlabeled Sindbis viruses was 12%±5%, compared to 8.7%±3% and 7.8%±1% of R18 and DiI-labeled Sindbis virus respectively. We note that the final dequenching value taken from each experiment was the averaged intensity over the last five seconds when the traces became steady. The value of the dequenching reported above is the mean of the average of each set of repeated experiments. The error of the average presented as ±1 standard deviation, where the standard deviation was obtained by calculating the standard deviation of the averaged final intensities within each set of repeated experiments.

We verified that the rise in DiD intensity was due to membrane fusion rather than the effects of low pH on DiD-labeled liposomes. For these control experiments we performed the fluorescence dequenching assay with DiD-labeled liposome and omitted Sindbis virus. The low pH decreases the emission intensity of (Figure 5), but after the initial adjustment, no time dependent increase as seen in fusion experiment (Figure 8) was observed in the absent of virus (Figure 6). We corrected our data accordingly, as discussed in detail in the materials and methods section.

It is important to note, however, that while the dequenching mechanism allowed the detection of membrane fusion, the often non-linear dequenching characteristic of fluorescent dyes prohibits relating DiD dequenching percentage directly to the extent of fusion.

3.II Effect of photobleaching R18/DiI on fusion capability

We wanted to test fusion capability of labeled Sindbis virus that has been thoroughly photobleached prior to fusion assay. We exposed the virus solutions to a 532nm laser light (~10 mW) for approximately 1 hour before testing with fusion assay. This exposure was selected to simulate the exposure during cell tracking experiments. We first measured the extent of bleaching R18 or DiI in viral membranes by performing the fluorescence dequenching assay on laser-exposed, labeled Sindbis virus without liposomes present. Figure 11 verifies photobleaching by comparing the unquenched fluorescence emission intensity of bleached and nonbleached dye. We found that the emission intensity of R18 and DiI was reduced by 83%±6% and 80%±3% by our bleaching procedure before use in the fluorescence dequenching assay.

The results of subsequent use of labeled and pre-photobleached Sindbis virus in the fluorescence dequenching assay with liposomes are shown in Figure 12. Very little effect on fusion was observed from prebleaching R18 or DiI viruses. Figure 13 shows that the average final extent of fluorescence dequenching of the bleached dyes from several repeated experiments compared to the non-bleached dye experiments was 10%±3% and 8.7%±3% for R18, and 9.5%±1% and 7.8%±1% for DiI, respectively. Errors are ±1 standard deviation of the repeated experiments. We conclude that extensive photobleaching of R18/DiI-labeled Sindbis virus before use in low-pH triggered fusion experiments did not inhibit fusion between Sindbis virus and liposomes as diagnosed by DiD fluorescence dequenching in liposomes.

3.III Effect of photobleaching R18/DiI on infectivity

Infectivity of each sample used in these fusion experiments was determined using standard plaque assay (see materials and methods). Figure 14 shows plaque assay results grouped by the dye that was used for labeling. The first two bars in each group portray unlabeled virus and labeled virus. None of the dyes that were tested decreased the infectivity of the virus to a degree larger than the error of the plaque assay. The third bar in each dye group shows the infectivity results for dye-labeled samples following extensive photobleaching. Photobleaching of DiI and DiD labeled Sindbis did not significantly decrease the titer of the virus samples. In contrast, the bleaching of R18-labeled virus led to a dramatic loss of infectivity of Sindbis virus particles by at least five orders of magnitude. These results were repeated at least 3 times with the same trends. Only one example of each experiment is displayed where a common aliquot was split for all the samples to control the titer among the different treatments. Independent repetitions of the experiments showed similar trends although the overall titers differed due to variations in initial virus concentration. Additional control experiments shown on the far right in Figure 14b were carried out to investigate other factors that might be responsible for inactivation. In one experiment, unlabeled Sindbis virus was left in the plastic pipet tip for 1 hour at room temperature, while the other experiment exposed the Sindbis virus in the pipet to laser identical to the photobleaching procedure described earlier. Our results show that contribution from the pipet tip and the bleaching process applied to un-dyed virus also had negligible effects on the infectivity of Sindbis virus.

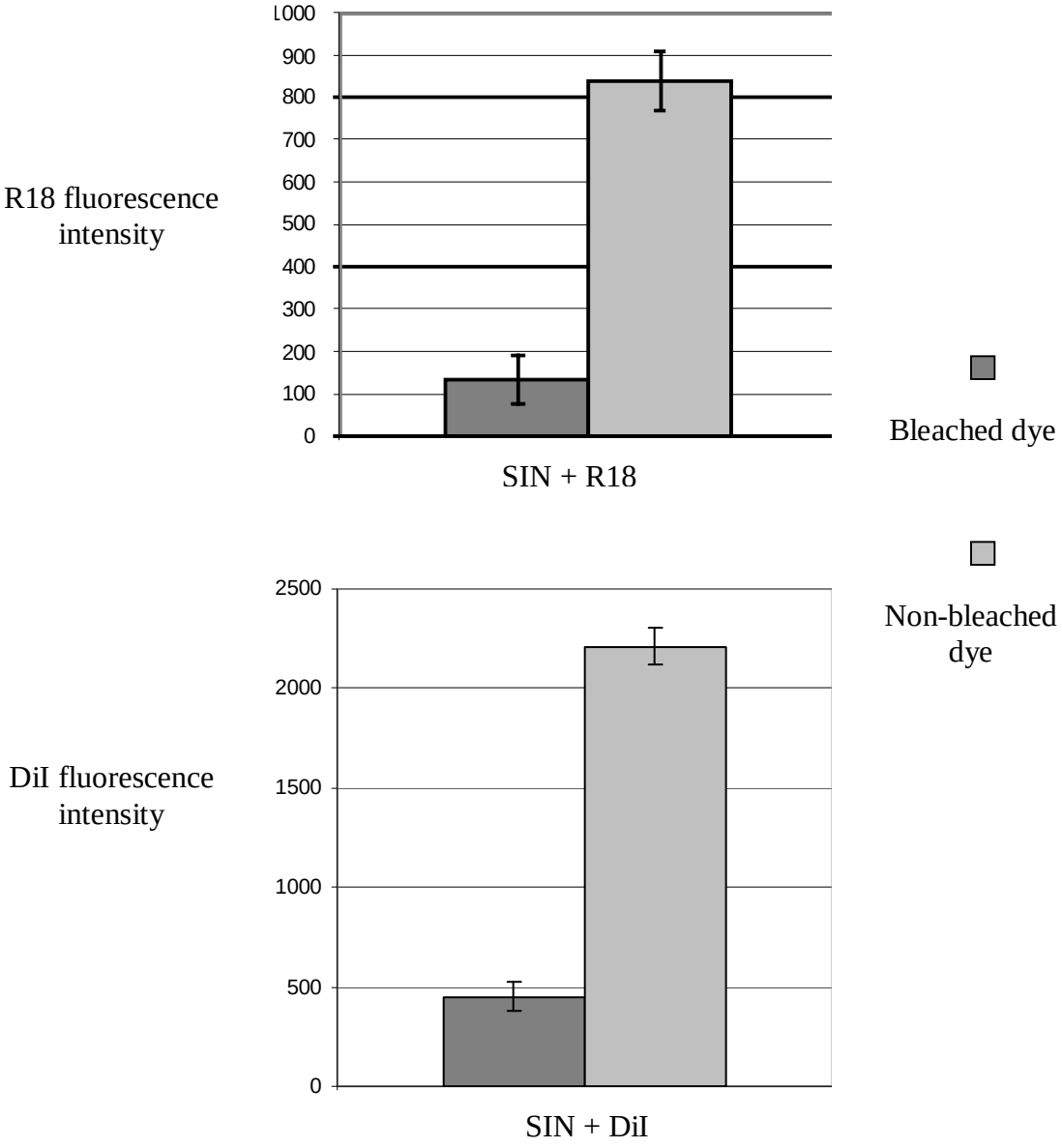

Figure 11. Dequenched intensity of bleached and non-bleached dye. The prebleaching decreased the intensity of R18 and DiI in Sindbis virus by 83%±3% and 80%±3%, respectively, verifying photobleaching. The error bars represent ±1 standard deviation of the final intensity.

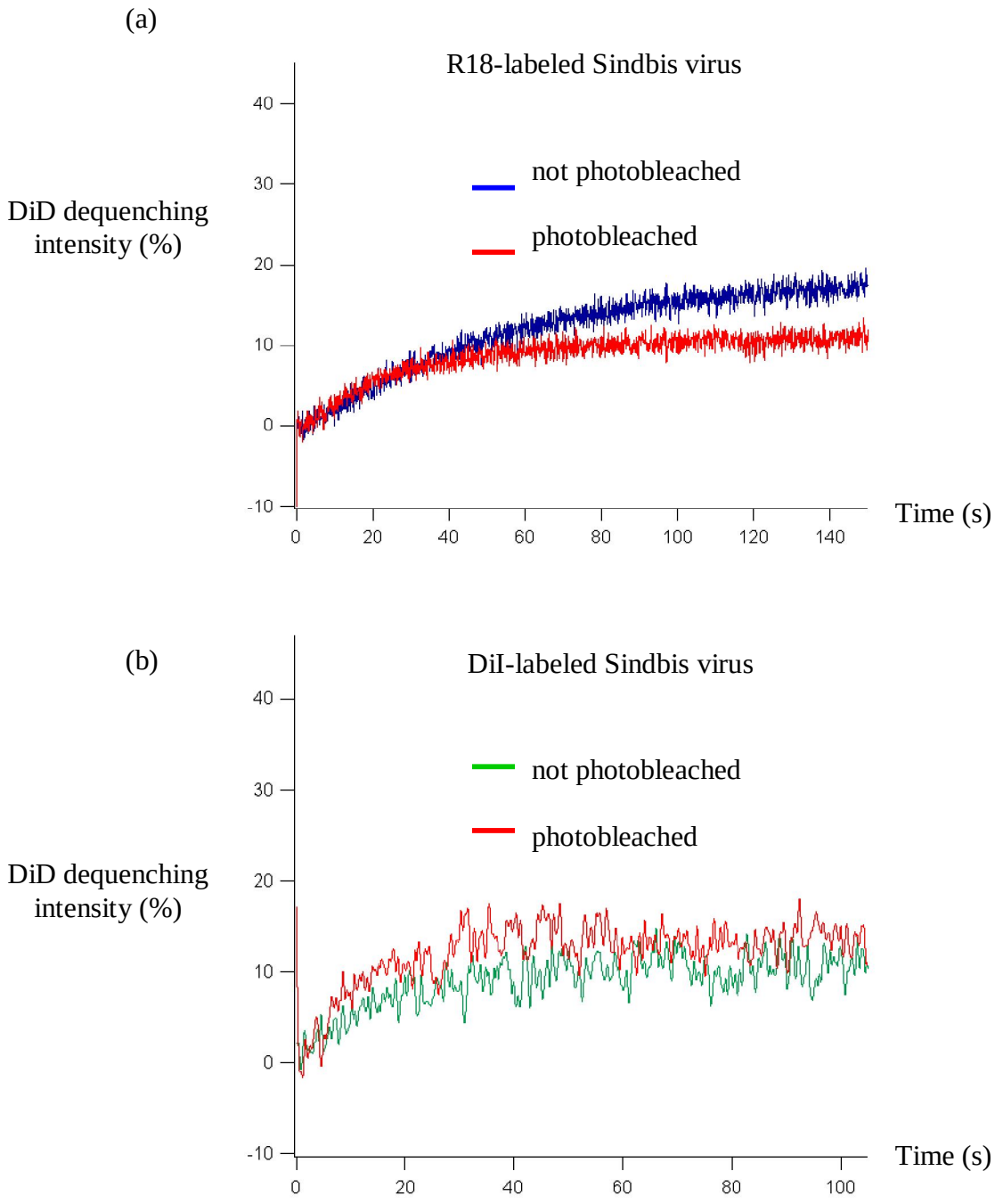

Figure 12. Effect of bleached fluorescent dye on low-pH triggere ndbis virus fusion. Fusion between DiD liver liposome and bleached/non-bleached Sindbis virus labeled with (a) R18, and (b) DiI. We conclude that prebleaching dye-labeled Sindbis virus did not eliminate low-pH triggered fusion to liposomes.

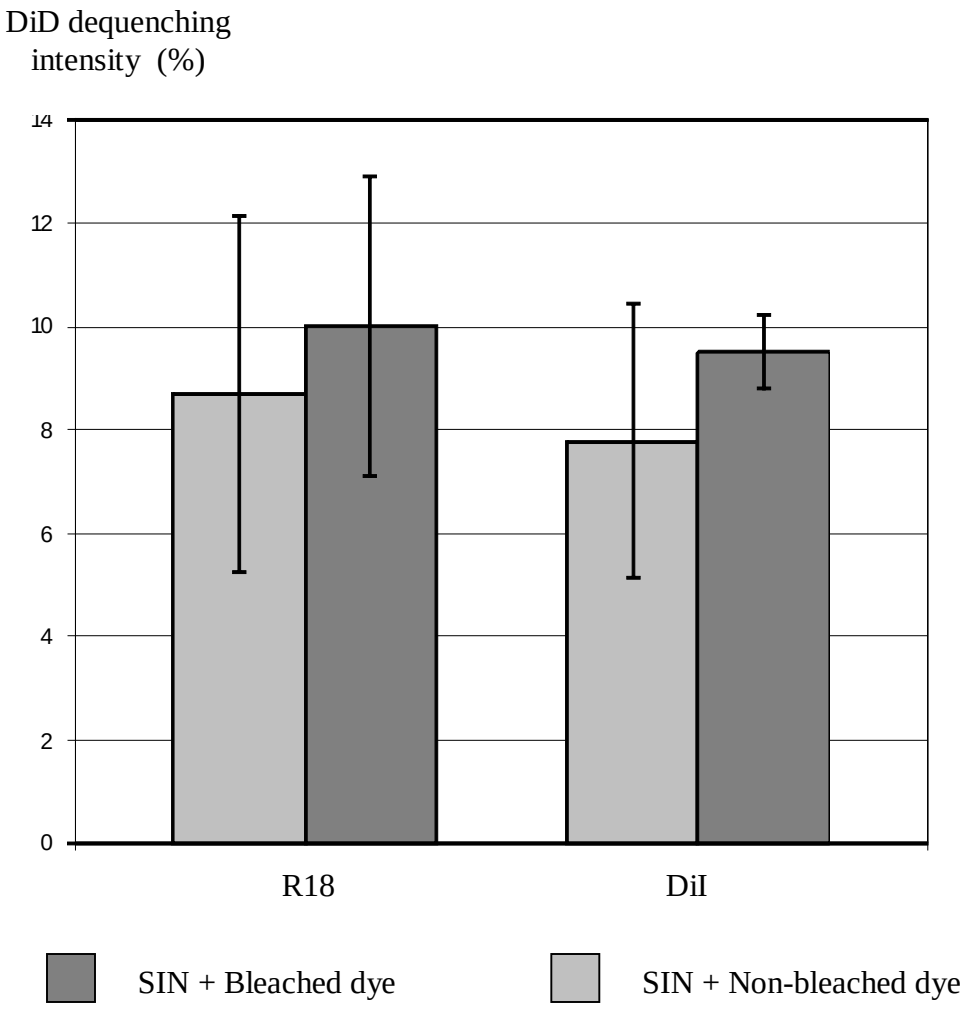

Figure 13. Prebleaching dye-labeled Sindbis virus does not eliminate membrane fusion. Bars show the average final dequenching value of DiD in liver extract liposome following low-pH triggered fusion experiments with dye labeled Sindbis virus. The left group is R18-labeled Sindbis virus. The right group is DiI-labeled Sindbis virus. The error bars represents ±1 standard deviation of the final dequenching values. We conclude no change outside the measurement error in fusion due to bleaching occurred.

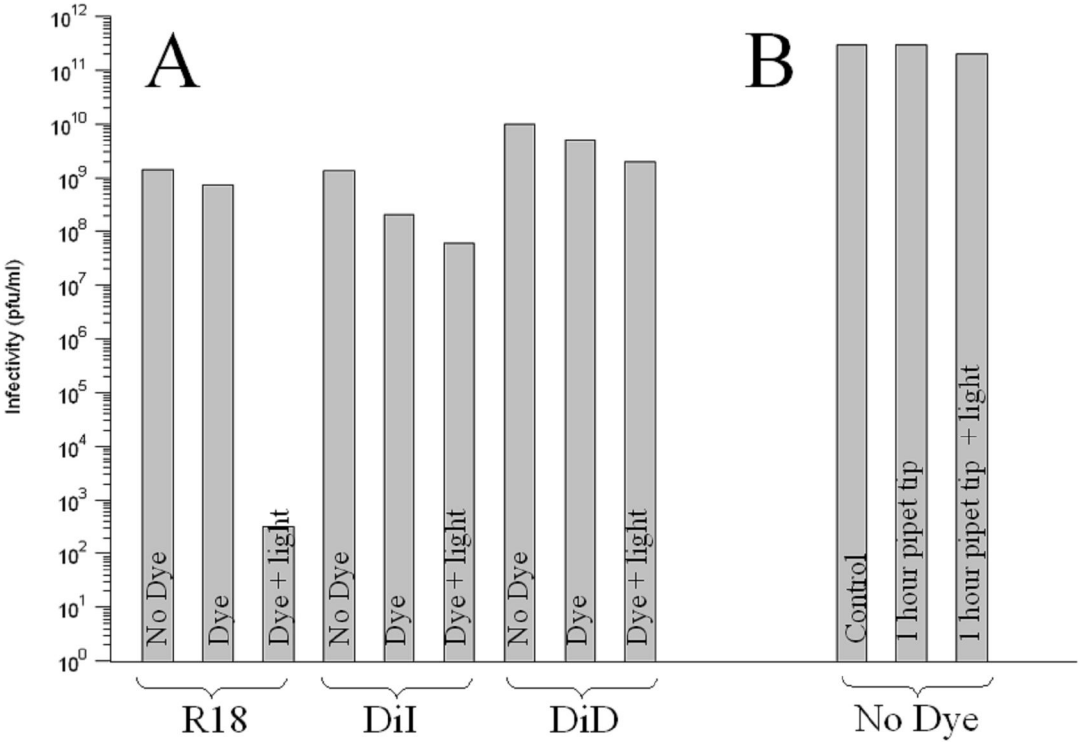

Figure 14. Plaque assay results. A) Shows infectivity of SIN under various labeling conditions while B) displays control experiments. Infectivity is reported as plaque forming unit/ml or pfu/ml. Adding dyes and photobleaching decreased infectivity less than a factor of 10, while only SIN labeled with R18 **and** photobleached led to dramatic loss in infectivity by 5-6 orders of magnitude. B) Also shows no loss of infectivity in SIN contained in a pipet tip and/or exposed to laser in the absent of fluorescent dyes, both for the same amount of time as the bleaching process. We conclude that only Sindbis virus labeled with R18 and photobleached was inactivated.

3.IV Protein gel analysis

We searched for damage to viral proteins following the photobleaching process using SDS-PAGE. Virus samples were mixed with SDS loading buffer (0.5M dithiothreitol (DTT) or 5% mercaotoethanol (750mM), 10% sodium dodecyl sulfate (SDS), 85% HBS) and separated with a phastgel SDS-PAGE instrument. Gels were silver stained for protein visualization. The gel in Figure 15 shows bands of protein around the 45kDa molecular weight marker in agreement with the molecular weight of E1 (47.4kDa) and E2 (46.9kDa) viral proteins. No evidence of protein damage to E1 or E2 due to cross-linking or degradation were observed. The capsid protein (29.4kDa) was notably absent in almost all of the samples except in the most concentrated sample of non-labeled Sindbis virus. When visible, the capsid band was always much less intense than the E1 or E2 bands. Because the E1/E2 bands in the photobleached samples were significantly lighter than the control concentrated virus, we believe the capsid bands in the dye-labeled samples were too faint to observe.

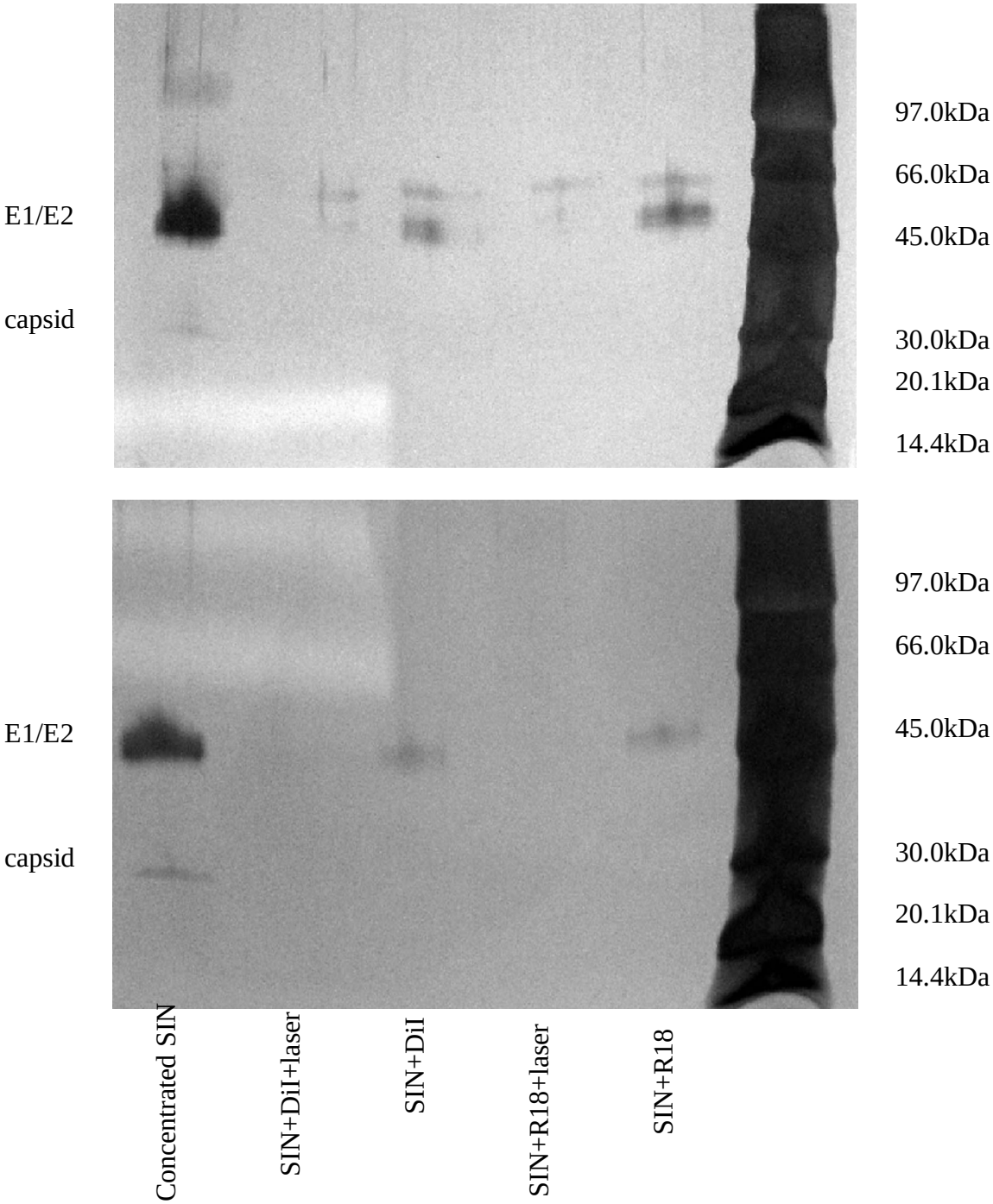

Figure 15. Protein gels. Two repeat SDS-PAGE gels (10-15% gradient) show that while there are no evidence of protein cross-linking, the protein capsid can only be seen in concentrated SIN samples.

3.V RNA gel analysis

We investigated possible damage to Sindbis virus RNA following the photobleaching process using formaldehyde gel. RNA was extracted from bleached and unbleached samples of Sindbis virus by cesium chloride precipitation, and subsequently radiolabeled [32]. The labeled RNA samples were separated on a denaturing agarose gel (1% agarose, 2.2M formaldehyde) in standard buffer and imaged with a phosophoimager. The RNA gel shown in figure 16 revealed possible degradation to the photobleached R18-labeled Sindbis virus (red box). We, however, were unable to duplicate the same result in a repeat experiment (Figure 17), where the RNA of the photobleached R18-labeled Sindbis virus was absent entirely.

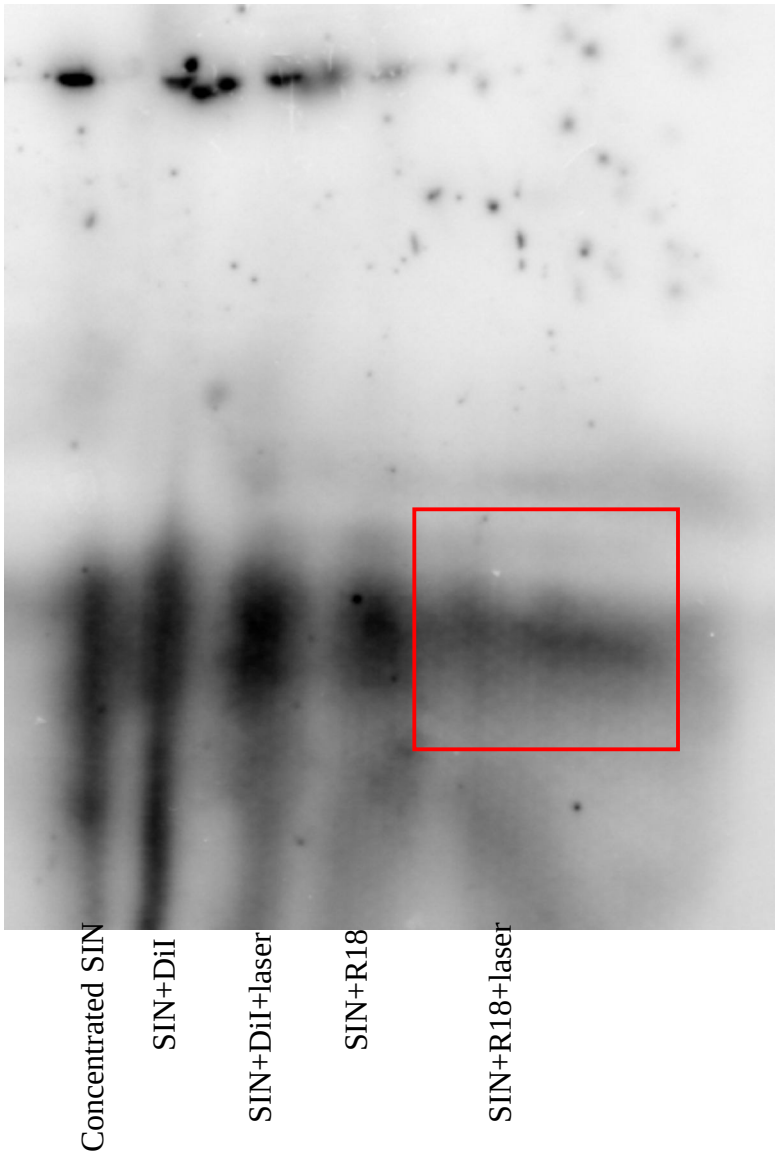

Figure 16. RNA gel result. This figure shows degradation of photobleached R18-labeled Sindbis virus (red box) in comparison to other samples. Note that the shape of the band is smeared horizontally. We attribute the smear to sample being drop in the adjacent wells. We verify that the total amount of RNA has decreased through employing a software to evaluate the total intensity of each sample by integrating over the area of interest.

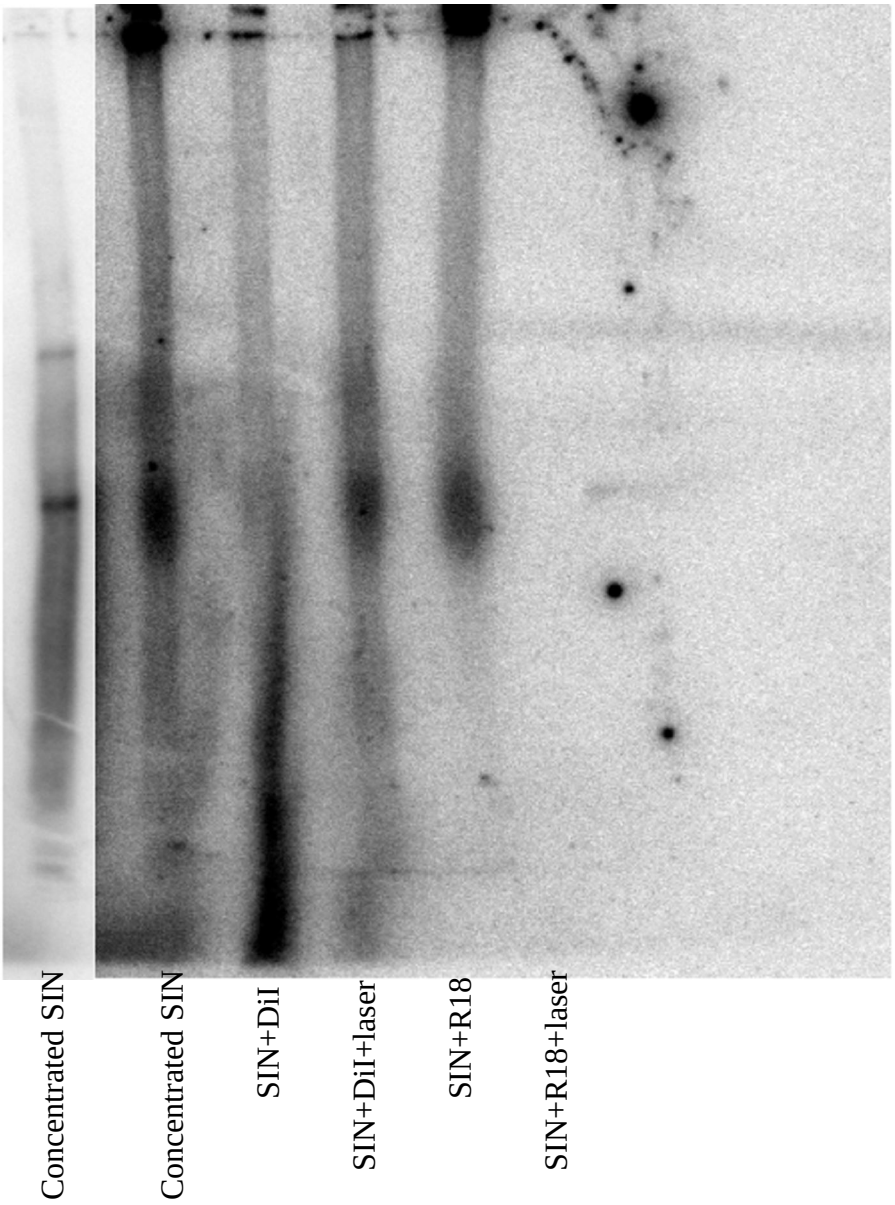

Figure 17. Repeat RNA gel experiment. This figure shows our sample along with another control sample (SIN) from the Brown lab (far left). Note that the photobleached R18-labeled Sindbis virus sample is missing entirely from the gel. We did not draw definite conclusion of RNA degradation due to different outcome in repeated experiment.

DISCUSSION

Studies of photoinactivation of viruses have been driven in part by potential applications in clinical medicine. Photoinactivation describes the phenomenon by which pathogens become noninfectious after exposure to light. Photoinactivation of both enveloped and nonenveloped viruses has been extensively studied [33-41]. While studies of nonenveloped viruses have pointed toward damaged-genetic materials resulting from interactions with photo-exposed dyes as the main reason for inactivation [33, 35], the inactivation processes of enveloped viruses are less clear. For example, Lenard et al. has reported that cross-linking, or damage to the fusion proteins, can be observed after light exposure of mixtures of influenza virus and rose bengal, a fluorescence dye. In that work, a hemolysis assay indicated that the loss of infectivity was due to the inability of the photo treated dye virus to fuse [39]. Other studies have shown that the ability of influenza, vesicular stomatitis virus (VSV), and herpes simplex virus (HSV) to bind to host cells was vastly reduced as a result from damage to envelope proteins [40, 42, 43]. Moor et al. have also demonstrated that it is possible to inhibit membrane fusion of VSV with photodynamic treatment while preserving virus-receptor binding [44].

While many studies correlated viral photoinactivation with the loss of fusion, other studies have shown that inactivation in enveloped virus could be attributed to damage to the viral genome. Abe and Wagner, for example, suggested that loss of fusion is less correlated with loss of infectivity in VSV when compared to RNA damage [45].

In addition, it has been demonstrated that photodynamic treatments may lead to reduction in RNA polymerase activity [41, 44].

While most photoinactivating agents tested for clinical photodynamic therapies are water-soluble dyes, which can be easily added to blood or other biological samples, we have instead investigated inactivation through photobleaching of hydrophobic dyes that spontaneously integrate into the viral envelope. Although not widely examined for clinical applications, these dyes have been exploited for studies of viral membrane fusion [46] and recently for several single virus particle tracking studies in living cells [4, 13, 14].

We tested two dyes with absorptions in the range of green light, R18 and DiI. Fusion of Sindbis virus labeled by these dyes was assessed by dequenching of a separate lipophilic fluorescent dye with the absorption range of red light, DiD, incorporated into liposomes that were not exposed to light. Past studies have shown that simply labeling influenza with R18 can lead to a decrease in fusion and infectivity [34]. Furthermore, evidence has pointed toward photosensitizer labeling reducing virus binding efficiency even without light exposure [44]. However, we found that labeling Sindbis virus with R18 and DiI does not prohibit fusion (Figure 7), while the infectivity of labeled Sindbis virus, although systematically reduced, was within the error range of the plaque assay. We also found that membrane fusion was unaffected by the extensive bleaching of R18 or DiI in the virus membrane. Surprisingly, we found that the bleaching of R18-labeled Sindbis virus reduced the infectivity of the virus by at least 5 orders of magnitude,

whereas no loss of infectivity was detected following extensive bleaching of DiI or DiD labeled Sindbis virus.

The particularly simple structure of Sindbis virus [47] limits possible hypotheses about the mechanism of inactivation due to R18 bleaching that we have observed. Sindbis virus, an enveloped virus, contains a single strand, positive sense RNA genome that is infectious when transfected directly into cells. The genome is encased within a capsid formed from a single species of protein that is contained within a lipid membrane envelope. The outer protein shell is composed of E1:E2 dimers, which are organized into trimers. Photoinactivation could potentially attack either the cell entry apparatus (the viral envelope, the cell receptor binding motifs, the fusion activity of the surface proteins) or the capsid of Sindbis particles (either cross-linking the capsid protein, crosslinking RNA, or damaging RNA). Our observation that membrane fusion is unaltered by bleaching R18 rules out dye photodestruction mediated damage to the fusion capacity of the virus fusion proteins.

Our ethanolic injection method for labeling the virus membrane with R18 or DiI initially incorporates the lipophilic dyes into the outer leaflet of the viral envelope. The fusion results shown in Figure 10 indicate that incorporation of fluorescent dyes led to no significant reduction in the ability of Sindbis virus to fuse, indicating that the virus envelope was not extensively damaged. While SDS-PAGE analysis revealed no visible sign of cross-linking between the E1 and E2, which constitute the viral envelope, the absence of capsid protein detectablility in our SDS-PAGE even in the control sample

prevented us from reaching definite conclusions about damage to the capsid proteins (Figure 15).

Past studies of enveloped viruses indicated that photosensitizers, which are incorporated into the virus envelope, could cause damage to the enveloped proteins by two types of photoinactivation. Type I inactivation is mediated by the release of free-radicals from the photosensitizers after photon excitation [44] while type II is mediated by singlet oxygen generated by energy transfer to oxygen in the environment [37, 44]. It was assumed, in those studies, that the incorporated photosensitizers remained on the viral membrane and thus damage was limited to the viral envelope. Hydrophobic acyl chains (one in case of R18 and two chains for DiI and DiD (Figure 4)) anchor the dyes we tested to the viral envelope/lipid bilayers, it is not clear if the R18 and DiI headgroups remained segregated only to the outer leaflet of the virus particle after initial incorporation. If the dyes on the headgroup could gain access to the capsid in the interior of the virus, it would be more likely for reactive photobleched byproducts to damage the RNA.

There are disagreements in literature about the ability of R18 and DiI to flip-flop and translocate to the opposite leaflet of lipid membranes (Figure 16) [48]. Some studies have found DiI does not flip spontaneously between leaflets [49], while others have found the opposite [50, 51]. R18 is claimed not to spontaneously flip-flop in some experiments [50]. Both R18 and DiI can be forced to switch leaflets of a lipid bilayer by the application of an external electrical potential across the membrane [52, 53]. In studies of SNARE driven membrane fusion, the existence of hemifused intermediates

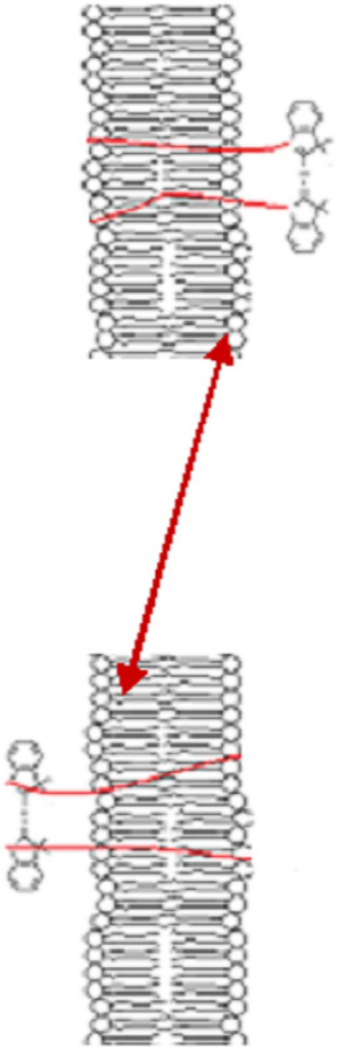

Figure 18. Dye translocation. Fluorescence dye headgroups (DiI in illustration above) may translocate (shown in red) across the viral membrane. Notice that R18 only has on acyl chain while DiI and DiD have two (Figure 4). It is possible that the number of acyl chain affects the translocation ability of the headgroups. Note that the size of the fluorescence dye is not drawn to scale.

lasting for tens of seconds were concluded based upon the lack of mixing between DiI and DiD present on the inner leaflet of fusing liposomes, suggesting that flip-flop is slower than 10 sec for these dyes [8]. If R18 and DiI can flip-flop on lipid bilayers, then the dye headgroups can gain access to the capsid region allowing byproducts from photobleaching the chance to damage the viral genome.

Although our studies did not explicitly investigate the translocating ability of R18 and DiI across viral envelope or lipid bilayers, our results did show significant loss in infectivity of bleached R18-labeled Sindbis virus in stark contrast to bleached DiI while no significant change in membrane fusion has been observed. Since our protein gel analysis revealed no apparent cross-linking within the E1/E2 protein envelope and viral fusion was not altered by photobleaching, we suggest that the loss of infectivity can be attributed to damage of the viral genome. However, as previously stated, we were not able to verify our hypothesis due to the inability of our SDS-PAGE analysis to visualize capsid protein. Attempts to detect possible RNA degradation were equally inconclusive due to the inconsistency of the results, which prevented us from drawing any definite conclusion. It is certain, however, that in order to damage the genome, byproducts from photobleaching must access the interior of the viral membrane. The different structures of R18 and DiI may affect their propensities to flip-flop in the viral membrane.

Alternatively, differences in the chemical products resulting from photobleaching of the rhodamine fluorophore present in R18 [54] compared to the products resulting from bleaching carbocyanine dyes [55] may be responsible for the different inactivation we observe between R18 and DiI. We also point out that the similarity in the chemical

structure of the DiI and DiD headgroups supports this hypothesis. Overall, our results motivate further study of the propensity for these dyes to flip-flop in membranes.

CONCLUSION AND FUTURE DIRECTIONS

We have found that Sindbis virus, a prototypical enveloped virus with type 2 fusion proteins, can be photoinactivated by photobleaching the lipophilic dye, R18, incorporated in its membrane, although similar bleaching of carbocyanine dyes (DiI and DiD) does not cause inactivation. For all of these dyes, we observed no noticeable decrease in low pH triggered membrane fusion in a cuvette based liposome fusion assay. Our findings suggest the possibility that different lipophilic fluorophores have very distinct propensities for flip-flop translocation between the inner and outer leaflet of the viral envelope. These dyes provide a new dimension for applications of photodynamic therapy for the treatment of enveloped viruses. Furthermore, our findings validate the use of fluorescence dequenching signals from enveloped viruses to report membrane fusion during time-lapse single particle tracking studies of Sindbis virus infecting living cells to examine the role of membrane fusion in cell entry, despite the fact that photobleaching of R18 ultimately leads to viral inactiviation. Use of DiI or DiD avoids this complication for Sindbis virus and also raises the question of the relative abilities of R18, DiI, and DiD to spontaneously translocate lipid bilayer membranes. Although results from protein and RNA gel analysis have not yielded conclusive results, further studies and comparisons of viral proteins and RNA before and after photo exposure should eventually yield convincing results regarding the actual inactivation process.

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