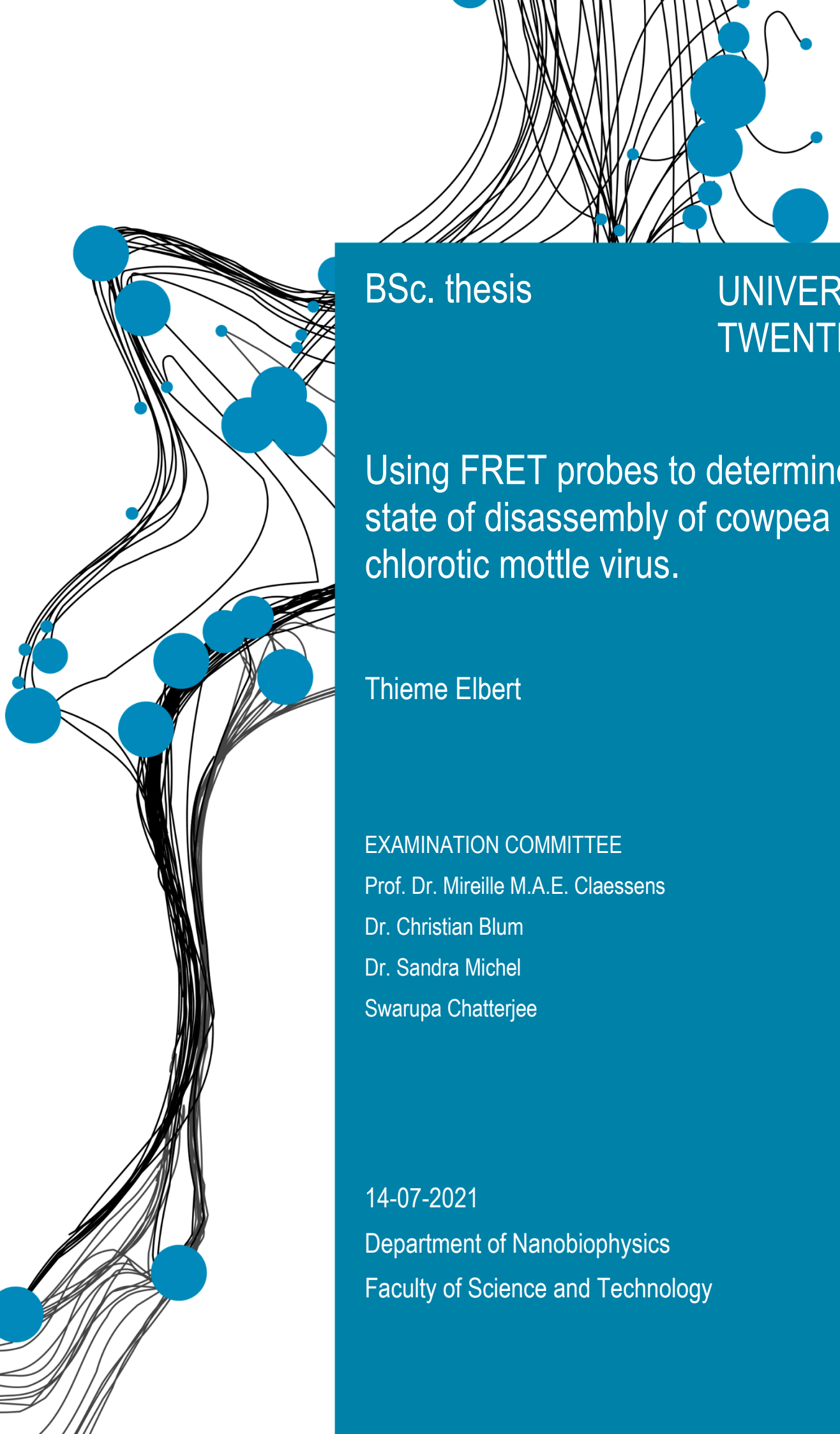

BSc. thesis

UNIVERSITY OF TWENTE.

Using FRET probes to determine state of disassembly of cowpea chlorotic mottle virus.

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Abstract

Many people in the world lack access to clean water and sanitation. Either it is too far away, or it is contaminated with pathogens and dirt. Many new ways to create clean water are being researched, one of them being cationic membranes, which can potentially disassemble virus capsids. A big problem in the development of new antiviral membranes is tracking their effectiveness. Virus particles are very small, and it is difficult to prove they are fully disassembled. In this report, a new method using FRET labeling to determine state of virus disassembly is researched. The FRET dyes were bound to CCMV, a model virus, which was then disassembled by adding either SDS or PEI. Using FCCS and emission spectra, the samples were analyzed. The results show this method is viable for determining state of disassembly of viruses, though more tests are needed to optimize the procedure.

Contents

Introduction

Clean drinking water is considered to be a human right by the United Nations[1], and was made one of the sustainable developmental goals[2] in 2015. The goal is to make clean water for both drinking and sanitation available to everyone on the planet by the year 2030. And even though progress is being made, the world is not on track to achieve this goal. Billions of people around the world lack a safely regulated source of clean drinking water, and lack access to safely managed sanitation services. Not only is there a lack of water overall, the water available is often contaminated with pathogens, which cause diseases and death to those who drink it. In poor countries, common water treatments, like adding chlorine, are often not an option, since it releases dangerous chemicals and requires big installations. New research is performed to find new ways to clean water, one of these ways is by using membranes[3].

Membranes

Normal filtering membranes are effective at removing dirt and other components from the water, though their pores are relatively big, causing pathogens to pass them with ease. Membranes with smaller pores[4] do exist, and are used for ultrafiltration, with very good results. However, these membranes cannot work on gravity alone, and need added pressure to push water through with an acceptable speed. In poor countries this is not ideal, since this method is expensive, and requires big installations to be worth it. Gravity filtration is the best option under those circumstances. Recently, cationic polymers[3], like Polyethylenimine (PEI), have been proven to work both antibacterial and antiviral, by destroying virus capsids. These polymers do not require small pores in order to remove viruses, and can be used with gravity filtration. These are potential candidates to make the drinking water safer, by removing the dangerous pathogens.

Virus capsid disassembly

The cationic membranes inactivate the virus by attacking the virus capsids[5]. Virus capsids[6] are supra molecular protein assemblies, which surround the genetic components. Once the capsid is destroyed, the genetic material will spill out, inactivating the ability to reproduce in host cells. One big problem with researching new antiviral membranes, is proving the membranes work. The virus particles are very small, between 20 and 500 nm, with the capsids being even smaller. Current methods which allow tracking of capsid disassembly, like NMR[7], are expensive, require expertise and are labor intensive. Easier and quicker tests are needed should the membranes be installed in actual water filtration facilities. In this report, a new way to track virus disassembly is explored, using fluorescent probes. These probes may help track the disassembly of the virus, by looking at an interesting phenomenon called FRET.

Förster resonance energy transfer

Förster resonance energy transfer (FRET), is an electrodynamic phenomenon[8] which occurs between 2 molecules. FRET occurs when these 2 molecules have overlapping emission spectra and are in close proximity of each other. After a donor molecule (D) gets excited by light, it transfers its energy nonradiatively to an acceptor (A) molecule in close proximity to the donor. The acceptor receives the energy, and in turn gets excited, and will release said energy through fluorescence, at a slightly higher wavelength. The requirement of close proximity for FRET to occur is exploited in this paper.

For a clear visual of FRET, see figure 1. This figure shows Jablonski diagrams, and the intra- and intermolecular mechanisms present with FRET. In figure 1A, the diagram shows the absorption of a

photon, which excites a fluorophore to a higher energy state. When the fluorophore falls back to a lower energy level, it releases a photon of equal energy. In figure 1B, the donor loses its energy as well, but it is transferred nonradiatively to a different fluorophore, which is in close proximity, which in turn gets excited. After which, it can release the gained energy in the form of another photon. As seen in the Jablonski diagrams, the released energy of the acceptor is lower than the released energy of the donor, resulting in a higher wavelength of the emitted photon. Figure 1C shows the relation between the donor and acceptor, and the FRET efficiency. More FRET occurs when the fluorophores are close together.

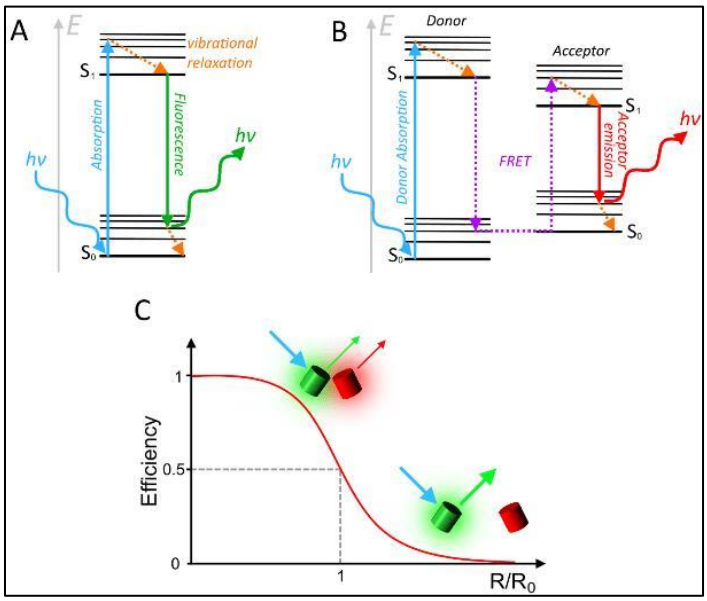

Figure 1: A visual representation of FRET[9]. Picture A shows normal fluorescence, when no FRET occurs. B shows when FRET occurs between a donor and acceptor molecule. C shows the FRET efficiency as a function of distance, showing the FRET reduces when the distance is increased between the donor and acceptor. R being the distance between the two molecules, and R0 being the theoretical maximum distance the dyes could be apart, which is around 10 nm[10].

The goal

There are 2 hypotheses I will address in this thesis:

1. labeled virus capsids which are still intact are capable of FRET. After virus capsid disassembly, fluorophores attached to the destroyed capsids are in further proximity, and are unable to perform FRET.
2. It is possible to prove virus disassembly resulting from the addition of SDS and PEI by using FRET emission spectra.

By labelling a model cowpea chlorotic mottle virus (CCMV) with FRET probes, the hypotheses are tested. The resulting viruses are then disassembled by using an anionic surfactant: sodium dodecyl sulfate (SDS). SDS is known to be able to disassemble virus capsids[11]. In order to prove the viruses are disassembled, a control group is needed to prove it actually works. If the method is proven to work, the labeled viruses will be exposed to a cationic polymer: Polyethylenimine (PEI). PEI is a potential cationic coating, which can disassemble viruses when added to a membrane[3]. By using PEI, the new method of testing potential membranes could be proven. By using emission spectra and Fluorescence cross correlation spectroscopy (FCCS), the results of SDS and PEI addition are analyzed and conclusions are drawn.

Methods and materials

All materials were purchased from Sigma Aldrich unless stated otherwise.

Creation and analysis of the labeled viruses

In order to test if the hypothesis is correct, model viruses must be acquired and labeled, before the disassembly can be proven. As a model virus, the cowpea chlorotic mottle virus (CCMV) is used, acquired following literature protocol[12]. The labeling is done using FRET probes, specifically Atto 488 (D) (ATTO-TEC GmbH), Atto 590(D) (ATTO-TEC GmbH)and Atto 647N(A) (ATTO-TEC GmbH). These dyes are the NHS ester variant, which will allow the dyes to bind with the virus capsids. 4 different samples were created, one being an Atto 488/647N sample, and 3 Atto 590/647N samples varying in number of fluorophores per virus or degree of labeling (DOL), and ratio of donor/acceptor. For a table with used quantities, see the results paragraph.

Labeling of the virus

Using a NanoDrop 1000 spectrometer (Thermo Scientific), the virus concentration of the stock was measured. After calibrating the nanodrop, 2µL was pipetted from the stock, and analyzed. The nanodrop gave the concentration of the virus present in the 2 µL sample. The concentration was then used to calculate the amount of virus stock necessary to create a virus sample of 400 µL, with a virus concentration between 0.5 and 1.5 mg/mL, 1 being ideal. The needed quantity of stock was added to a 2 mL Eppendorf tube, and diluted using Phosphate buffered saline (PBS) with a pH of 7.4 (Gibco). The nanodrop is used to determine the final concentration after dilution, which should be between 0.5 and 1.5 mg/mL. In addition, the ratio of 260/280 nm was noted. The 260/280 peaks are used to determine the purity of the sample, and tell something about the amounts of capsid proteins and RNA in the sample. The ratio should be below 1.7 for a good virus sample. If the ratio was above 1.7, the virus may have aggregated, and could have given invalid results and made analysis difficult.

Once the virus sample is ready, the dyes were added. The dyes were defrosted to room temperature, after which the desired quantities of both the donor and acceptor dye were added. For samples 2 and 3 (see results), certain dyes were diluted using dimethylsulfoxide (DMSO). DMSO was chosen instead of water, to prevent hydrolyzation and inactivation of the NHS esters. The dilution results in lower degrees of labeling (DOL). See the results paragraph for a table with used volumes.

Once the dyes were added, the sample was incubated for one hour, at 37 °C, at 450 rpm. The incubation allowed the labels to attach to the viruses.

45 minutes after the incubation started, 2 Zeba Spin Desalting Columns (Thermo Scientific) 2 mL were prepared according to protocol[13]. This means: The cap of the column was loosened, and the end was twisted off. The column was put in a 15 ml Eppendorf tube collection vat and marked with a permanent marker, to ensure the columns are spun the same way every time. The buffers in the columns were spun down using a centrifuge at 1000 RCF at 4°C for 2 minutes. After the initial buffer was gone, this was discarded. The Zeba columns were then washed by loading 1 mL of PBS, and spinning it down with the centrifuge. This step was repeated 3 times. After which, the columns were moved to new collection 15 mL Eppendorf tubes, and the virus sample, now done incubating, was added to the first column. This was spun down, to remove any excess dye. The recovered sample was then added to the second column, and spun down again, to clean the sample one more time. The sample is then stored in 4 2 mL Eppendorf tubes, each containing 100 µL. A fifth tube may be necessary, since a bit of PBS could have been present in the Zeba columns. The samples were stored in a fridge at 4 °C. The samples are now ready for analysis.

Absorption spectra analysis

The recovered sample of labeled viruses was analyzed using a UV-VIS spectrometer (Shimadzu UV-2600). First a blank of PBS was loaded for background absorption, after which the absorption of the samples were analyzed from 200 nm to 700 nm. Once the absorption spectra is obtained, the sample is recovered for later use.

The absorption spectra is used to determine the degree of labeling (DOL). The absorption at the highest point around 260 nm, the absorption at 280 nm exact and the absorption of the respective dyes at their peaks were obtained. Atto 590 has a peak around 603 nm, Atto 647N has a peak around 651 nm and Atto 488 has a peak around 491 nm.

Using the formula

$$DOL = \frac{Cdye}{Cvirus} = \frac{(\frac{Adye}{\varepsilon \text{dye}})}{(\frac{Avirus}{\varepsilon \text{Virus}})}$$

the degree of labeling (DOL) could be obtained.

Cdye being the concentration of the dye, Cvirus being the concentration of the virus. Adye is the absorbance of the dye at their peak, Avirus being the measured absorbance of the virus, εdye being the molar extinction coefficient which is given with their respective dye, and εvirus also being the molar extinction coefficient of CCMV, which is 5.87 $M^{-1}cm^{-1}$[14].

In order to calculate the virus concentration, the absorbance must be corrected, by using the formula $Absorbance260corrected = A260 - CF260 * Adye1 - CF260 * Adye2$

A260 is the absorbance at 260 nm, CF being a constant which is a property of the dye which is used, and Adye1 being the absorbance at the peak of dye 1. Adye 2 is the measured absorbance at the peak of dye 2.

Using the law of Lambert-Beer, $A = C\varepsilon \mathrm{L}$ or $C = \frac{A}{\varepsilon \mathrm{L}}$, the concentration of the dyes and virus can be calculated. L is the pathlength of the cuvette used with the UV-VIS spectrometer, and ε is the molar extinction coefficient of the dye or the virus. A is the corrected absorbance of the virus, or the absorbance of the dyes at their respective peaks.

The concentration of the virus must divided by the molecular weight of $4.6 * 10\text{^}6$ g/mol. With these formulae and constants, the DOL for both the dyes were calculable.

Emission spectrum analysis

The emission of the dyes was measured by using a spectrofluorometer (FluoroMax 4, Horiba-Jobin). The samples were excited twice. The first excitation was at the wavelength of the donor dye (590 nm), and detected between 600 and 800 nm. This allowed the donor to start emitting fluorescence, and in the case of FRET, also allow the donor to emit fluorescence. The second measurement was exciting the sample at the wavelength of the acceptor (630 nm), and detecting the emission between the wavelengths of 640 and 800 nm. This way, only the acceptor would get excited and detected. Should no FRET be present, the second measurement can prove the absence of FRET is caused by the DOL or disassembly of the viruses, and not by an absence of acceptors.

SDS

The labeled viruses were exposed to sodium dodecyl sulfate (SDS), to destroy the virus capsids. Dilutions were created in 2 mL Eppendorf tubes, each containing a constant of 5 µL of labeled virus, and a varying amount of SDS and PBS, resulting in a final volume of 100 µL, with different concentrations of SDS. The SDS concentrations were chosen based on previous research[15]. The critical micelle concentration of SDS is 0.0011 mol/L[16], and should not be crossed to prevent the formation of micelles.

Fluorescence cross-correlation spectroscopy

As an extra test, and a way to determine the state of disassembly of a labeled virus without FRET, a PicoQuant MicroTime 200 confocal microscope system was used, capable of Fluorescence Cross-Correlation Spectroscopy (FCCS). The device used 2 lasers, both of the lasers exciting one of the dyes each. By using cross correlation, it is possible to determine how much the virus has disassembled. If the cross correlation is high, it means a virus particle containing both dyes is in focus. If cross correlation is low, it means single dye pieces are in focus, from destroyed viruses. For the 488/674N sample, the 483 nm and the 636 nm lasers were used to excite the dyes. For the 590/647 sample, the 560 nm and 636 nm lasers were used.

PEI

As a potential molecule with antiviral properties which could coat membranes for antiviral properties, 0.3% Polyethylenimine (PEI) was added in different quantities to the labeled viruses to further analyze their disassembly. The PEI is branched and has an average mass of 25 kDa. PEI dilutions were made the same way as the SDS, by adding 5 µL of labeled viruses to 2 mL Eppendorf tubes, and a varying amount of PBS and PEI to reach a final volume of 100 µL, with different concentrations of PEI.

As a final test, the FRET efficiency was calculated. The following formula was used:

$$FRET\ efficiency = \frac{Apeak}{(Dpeak + Apeak)}$$

Where Apeak is the measured maximum emission intensity and Dpeak is the measured donor peak intensity.

With this formula, the FRET efficiencies of every PEI concentration were calculated, and can be found in figure 13 in the results and discussion section.

Results and discussion

Created samples

4 samples of labeled viruses were created, with different amounts of donor (D) and acceptor (A) dyes, resulting in different properties. The samples absorption spectra were analyzed with a UV-VIS spectrometer (Shimadzu UV-2600), and the calculated properties are shown below in table 1.

table 1: The 4 created labeled viruses, with varying amounts of added dye. Certain dyes were diluted 5x using DMSO, to reduce the corresponding DOL and resulting ratio. The used dyes have a concentration of 1 mg/mL and different molarities based on their molecular weight.

Sample	Added Atto 590 (D) (µL)	Added Atto 488 (D) (µL)	Atto Atto 647N (A) (µL)	DOL Atto 590	DOL Atto 488	DOL Atto 647N	Ratio Donor/Acceptor
1	0.5	-	0,2	13.2	-	7.62	2:1
2	0.6 (5x diluted)	-	0.2 (5x diluted)	4.8	-	3.0	1.6:1
3	1.5	-	0.2 (5x diluted)	21.4	-	5.15	4.2:1
4	-	0.4	0.2	-	7.9	5.3	1.5:1

The table shows the increase and decrease of DOL is not linear with the added dyes. After diluting the dyes 5x, the expectation was the DOL should decrease with a factor of 5, which is not the case.

The samples were then analyzed using a spectrofluorometer (FluoroMax 4, Horiba-Jobin), to which samples were able to perform FRET. The resulting emission spectra are shown in figure 2.

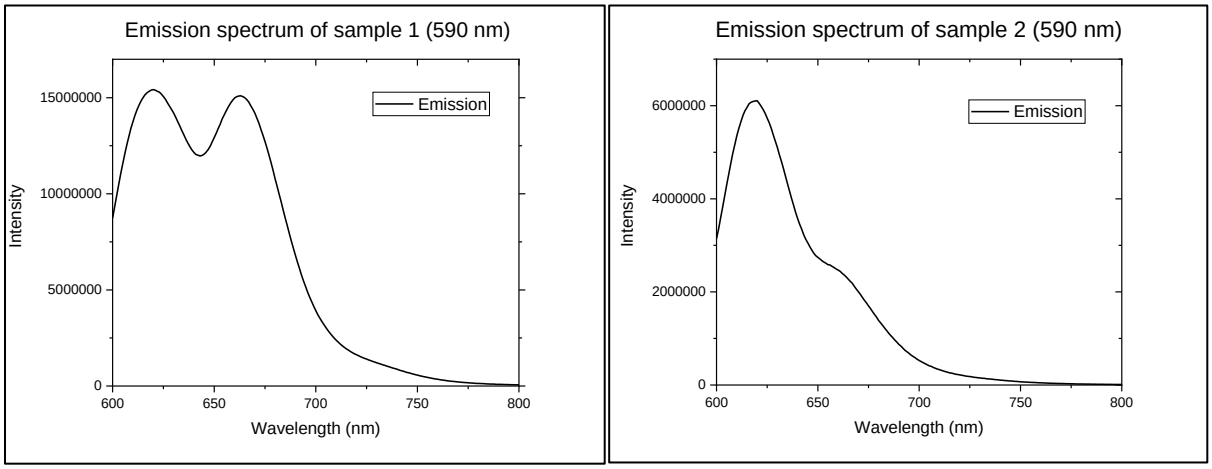

Figure 2: Emission spectra of samples 1 (left) and 2 (right). The graphs show emission spectra after excitation at 590 nm. Sample 1 shows clear signs of FRET, with a clear acceptor peak present. Sample 2 shows FRET to a lesser degree, with a smaller acceptor peak present.

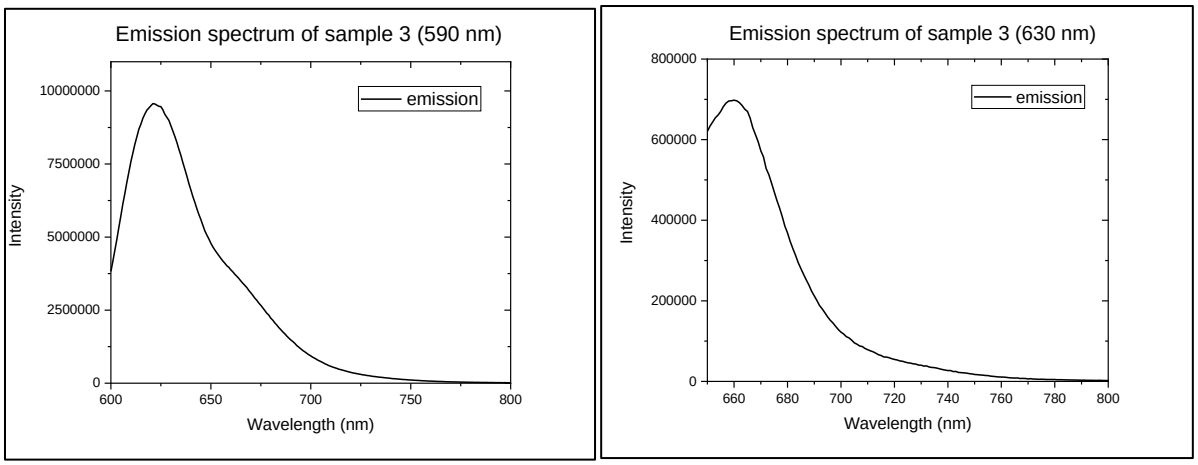

Figure 3: The emission spectra of sample 3. Left is the resulting emission spectrum after excitation at 590 nm. This sample shows almost no FRET. Only a very small acceptor peak is visible. The right graph is the result of excitation at 630 nm, where only the acceptor is excited. The peak proves acceptors are present on the virus.

The emission spectra show different degrees of FRET when the samples are excited at the donor wavelength (590 nm). Sample 1 has a very clear acceptor peak, and is the sample with the most FRET. Sample 2 shows significantly less FRET, but a peak is still present. Sample 3 has even less FRET, and the peak is almost invisible. In order to prove acceptors are present, sample 3 was excited at 630 nm as well (figure 3). This figure proves acceptors are present on sample 3, though they are not capable releasing much energy through FRET, if any at all. The probes could be too far apart for FRET to occur.

SDS addition

Sample 1 shows the most FRET, and was the best candidate for disassembly with the SDS. The SDS was added in different concentrations, the control being 0 mM SDS.

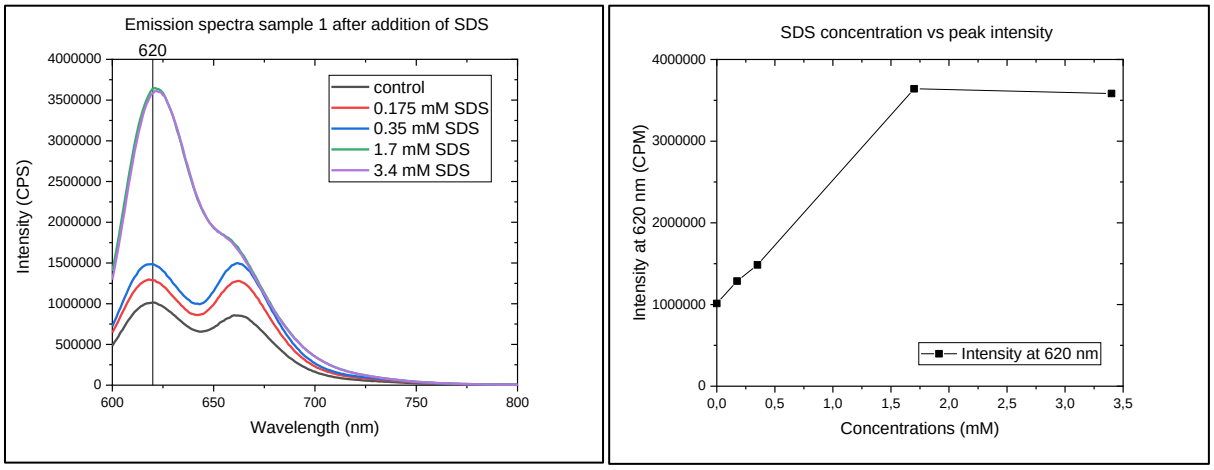

Figure 4: Result of SDS addition to sample 1. The left picture shows the emission spectra recorded for constant CCMV concentration upon addition of SDS, while the right shows the peak intensity at 620 nm, graphed against the different SDS concentrations.

Figure 4 clearly shows the donor peak rises with increasing concentration of SDS. Once a molarity of 1.7 mM is achieved, the peak no longer rises. This means all viruses have been disassembled by the SDS. The acceptor peaks have risen as well, which was unexpected. With normal FRET situations, the

donor peak should have risen, while the acceptor peak should have fallen. This phenomenon could be a result of emission quenching. The more SDS is added, the more dyes are dequenched. This could result in an increase in intensity. There is also a big gap in between the lines, which should be explored further.

In order to fill the gap between 0.35 mM and 1.7 mM SDS, new dilutions were created and analyzed. This measurement was done one month after the initial measurements of figure 4, resulting in degradation of the sample. In order to analyze how much the sample has degraded, a new experiment was performed. The control sample data of figure 4 was put next to a new control sample. The degradation of the sample is shown is figure 5.

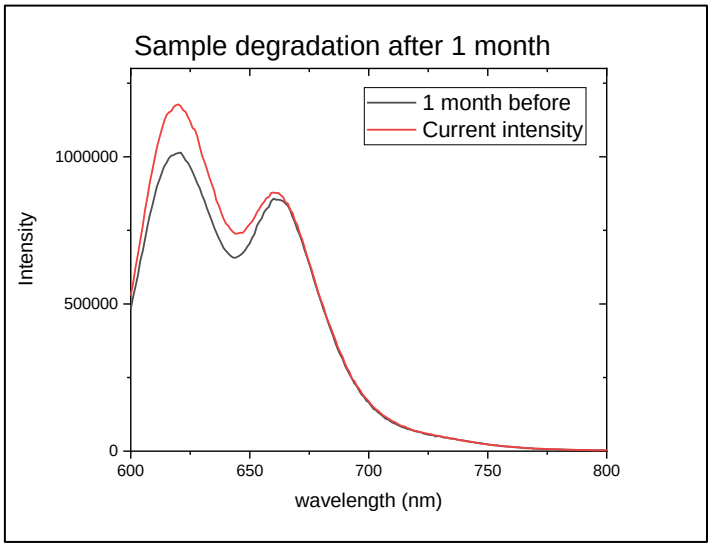

Figure 5: the comparison of the virus samples. The black line shows the virus intensity of the control sample from figure 4, which was acquired one month before this measurement. The red line shows the intensity of a different Eppendorf tube (see method), from the same sample. This sample has been stored in a fridge for a month, without further usage. The sample was prepared the same way as the control sample in figure 4. It shows the intensity has gone up over the course of one month.

It is clear the intensity profile has changed. The results of figure 4 and the following SDS concentration measurements cannot be combined for this reason. However, the FRET is still present, which means the sample is still viable. The new concentrations are graphed below in figure 6.

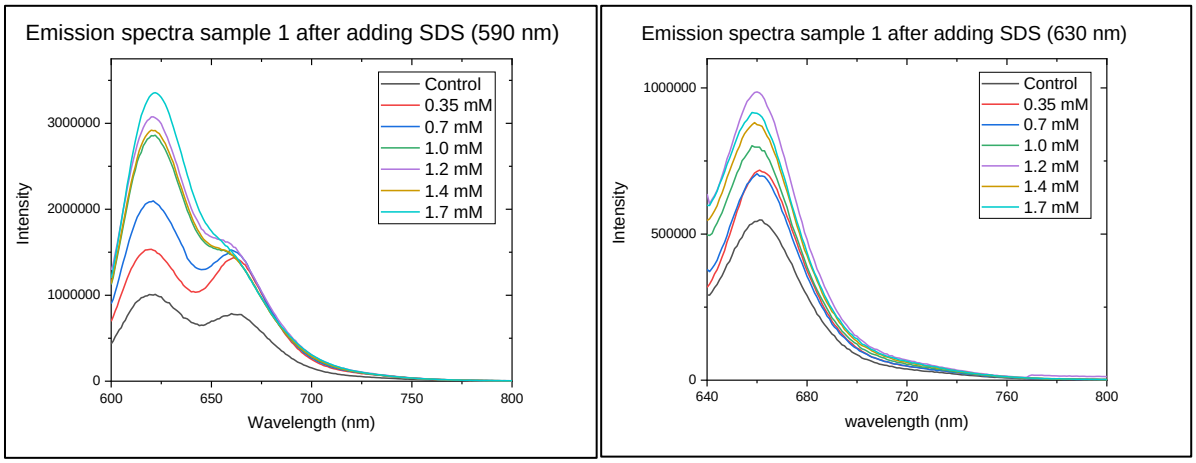

Figure 6: Further analysis of the SDS addition to sample 1. The graphs show the emission spectra of the samples, when excited at different wavelengths. The left figure shows excitation at 590 nm, and the right figure shows excitation at 630 nm.

In order to get a clearer picture, the highest peaks of the FRET donor and acceptor were graphed against the SDS concentrations. This is shown in figure 7 below.

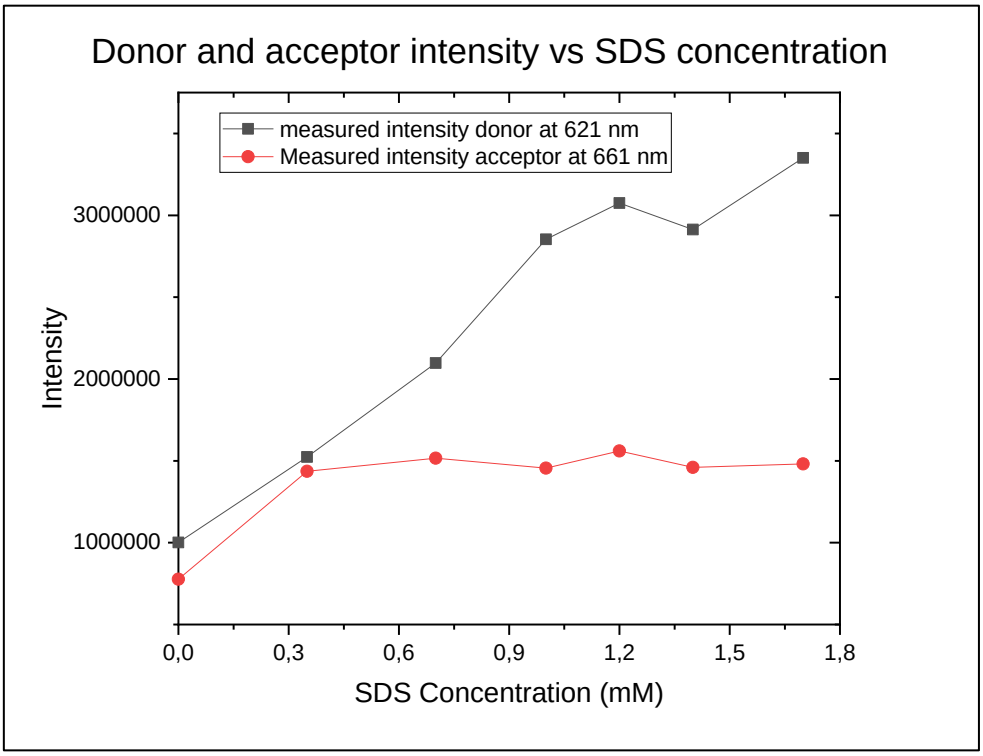

Figure 7: The donor and acceptor peak intensity of sample 1 graphed against the SDS concentration. The donor peak was highest at 621 nm, and the acceptor peak was highest at 661 nm.

The plot shows the donor intensity increases roughly linear with the addition of SDS, which was as expected from figure 4. The acceptor increases in intensity as well, which is not expected. As stated in the introduction, when the FRET is reduced, the donor peak should rise, while the acceptor peak should fall. In this case, both the donor and acceptor peak rise. The acceptor peak rising from 0 to 0.4 mM could be due to the dequenching[15] of the acceptor probes. Sample 1 has a very high DOL on the viruses. The acceptor probes could have formed dark aggregates, which lowers the measured intensity. Once the virus particles are disassembled, the acceptor probes are no longer aggregated, and are able to give full intensity. However, it does not explain why the acceptor intensity remains somewhat constant afterwards. Once more SDS starts attacking the labeled viruses, more capsids should start falling apart and the distance between fluorophores will increase, which should result in a decrease of acceptor intensity. Figure 7 shows this is not the case.

Fluorescence cross correlation spectroscopy sample 4

Due to the close emission spectra, the Atto 590/647N samples are not suitable for FCCS in the available setup. The fluorescence of Atto 590 is detected by both the red and green detector, and invalidates the results. As a solution, sample 4 was created, which will remove any spectral leakage, since the emission spectra are too far apart. This also means sample 4 is unable to perform FRET, since there is no overlap of emission spectra, and cannot be used for further FRET emission spectrum analysis.

The samples were loaded in the microscope of a PicoQuant MicroTime 200 confocal microscope, and excited for 20 seconds. This was repeated 5 times per concentration. The data was analyzed using SymphoTime 64 software. The software was able to perform fluorescence cross correlation spectroscopy (FCCS) on the data.

The resulting FCCS graphs are shown below in figure 8, and show one of the 20 second measurements for each concentration. The other measurements, which acted as controls, are left out, as they are in line with these following results. The green line shows the measured cross correlation, while the grey lines are the autocorrelation. For the control sample, the cross correlation starts at 0.5. The cross correlation decreases with the addition of more SDS. The samples of 0.17 and 0.34 mM SDS show the cross correlation of the virus particles has decreased to 0.2. The FCCS curves are shown in figure 8.

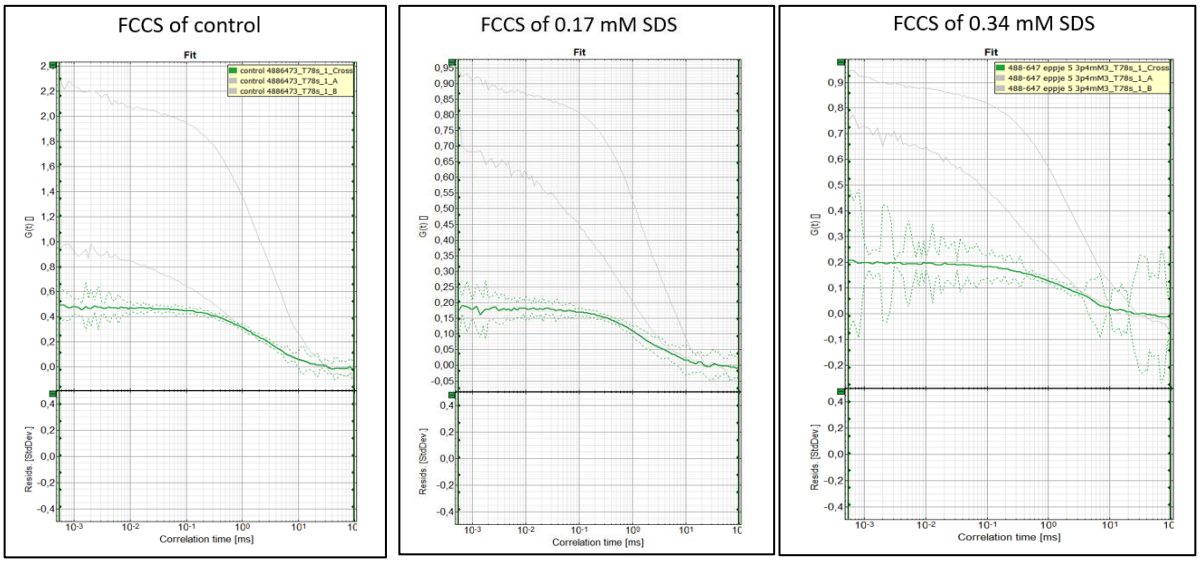

Figure 8: The FCCS data of sample 4. The left picture shows the control sample, without SDS present. The concentration of the SDS increases from left to right. The cross correlation starts at 0.5 for the intact viruses, and has decreased to 0.2 after the addtion of low SDS concentrations. The cross correlation is not 0, which means the viruses are not completely disassembled.

Figure 8 shows the cross correlation is lower when a low amount of SDS is present. The cross correlation is not 0, which means there is still some cross correlation between the virus particles. This is only possible when the fluophores are still on the same particle, meaning the capsids have not been completely destroyed. In order to fully destroy the virus capsids, more SDS is added to raise the concentration. The result of this addition is shown in figure 9.

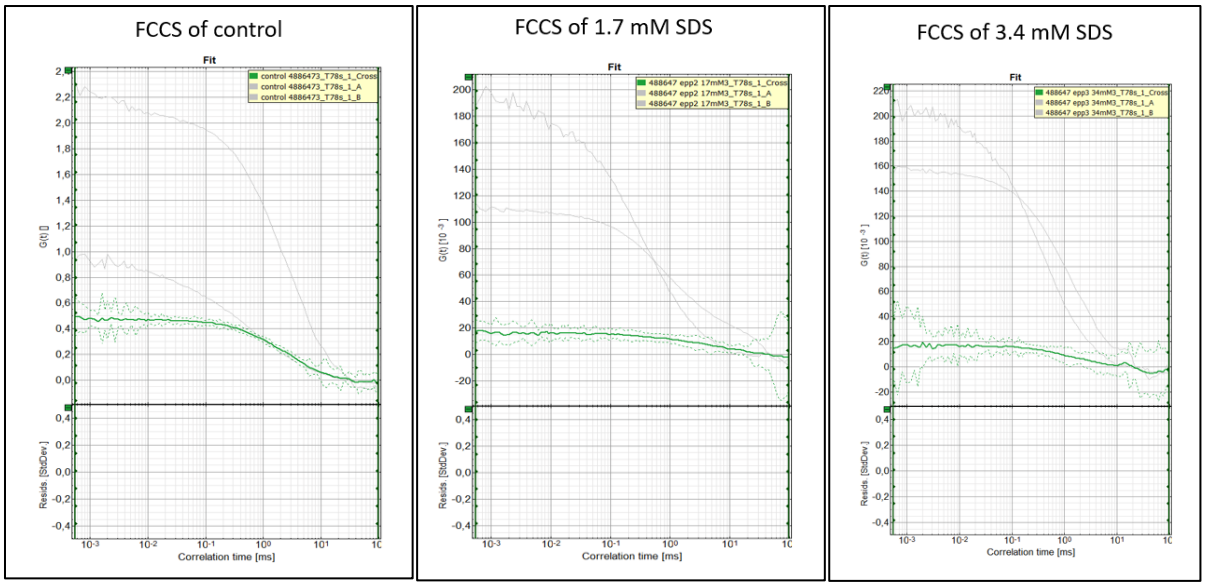

Figure 9: The FCCS data of sample 4, with higher concentrations of SDS present. The left picture shows the control sample, without SDS present. Note: the y-axis has changed for middle and right figures. The control is the same as in figure 8. The SDS concentration of figure 8 was multiplied by a factor of 10. The cross correlation of the resulting samples is 0.02, which is very close to 0.

After the addition of a higher concentration of SDS, the cross correlation has fallen down to 0.02. This shows the virus no longer has cross correlation, and has fallen apart completely. Figure 9 also shows the virus was disassembled with 1.7 mM SDS. The 3.4 mM was not able to destoy the capsids any further. These results are in line with figure 4, where the virus was completely disassembled after the addition of 1.7 mM SDS.

Addition of PEI to sample 4

The addition of PEI to sample 4 was done after the SDS measurements confirmed the virus fell apart. The PEI was added in different quantities, resulting in different concentrations. The PEI was first added to sample 4, and measured 5 times for 20 seconds. The resulting concentrations and FCCS curves can be seen below in figure 10. 1 FCCS curve of each PEI concentration is shown, the other 4 graphs acted as a control to remove experimental errors. These are in line with these results, and are left out.

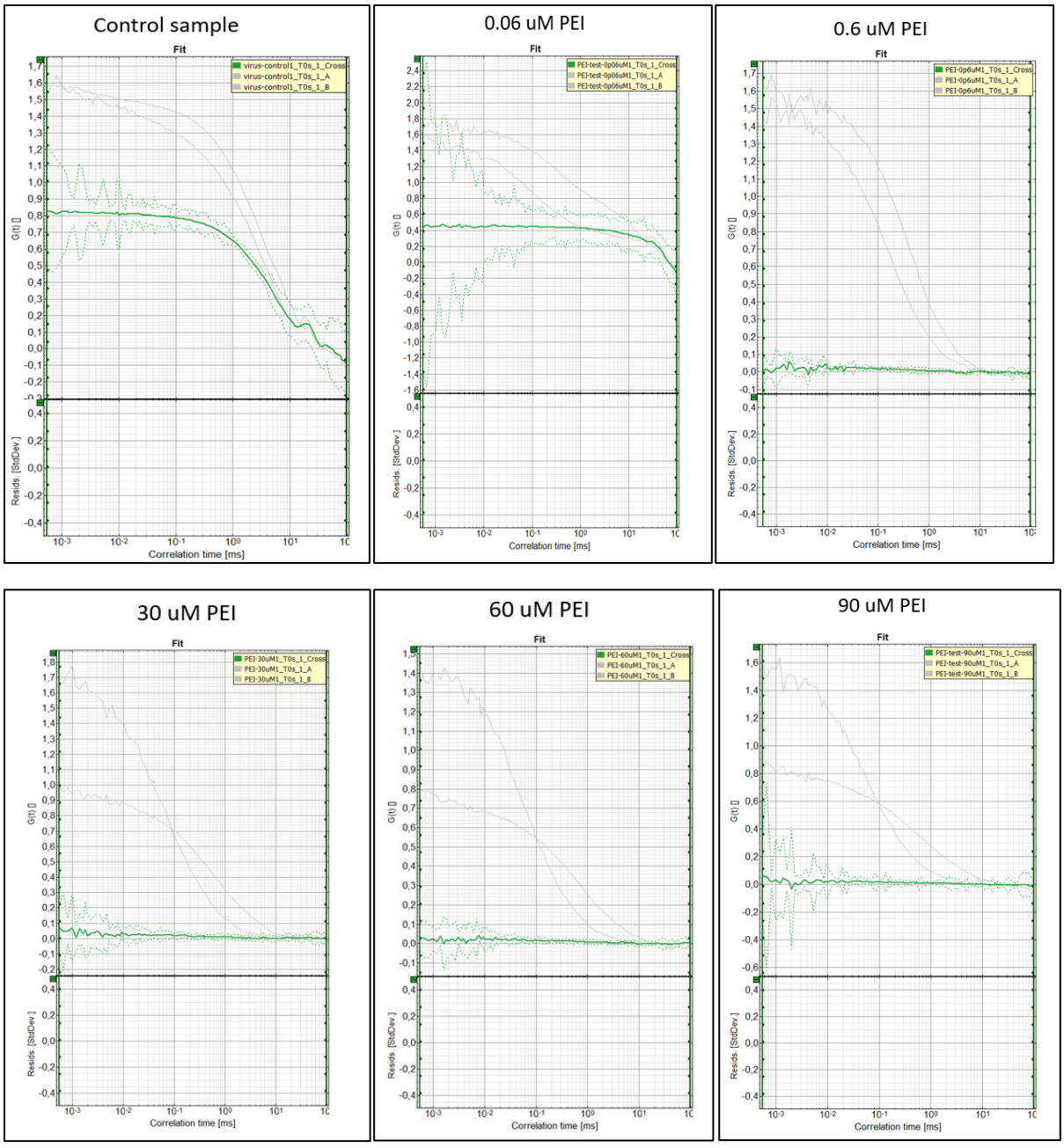

Figure 10: The FCCS curves of sample 4 after addition of varying concentrations of PEI (see titles). The graphs show the virus is no longer intact, after the addition of PEI. At 0.06 µM, the viruses are falling apart, but are not yet completely disassembled. After the 0.6 µM, the cross correlation falls to zero, indicating the virus fell apart.

The graphs show a cross correlation of 0.8 for the control sample without PEI. At a PEI concentration of 0.06 µM, the cross correlation dropped to 0.5, which means the particles are falling apart. Some viruses are still intact, or intact enough for the system to detect donor and acceptor fluorophores simultaneously. Not enough PEI is present to completely destroy the virus particles at a concentration of 0.06 µM. At 0.6 µM, the cross correlation has dropped to 0. The low cross correlation shows the labels attached to the virus no longer get detected together, which is only possible if the virus particles are completely disassembled. This should mean the higher concentrations of PEI are able to disassemble the virus as well.

This is not the case however, as a sample of 6 µM PEI was also measured with FCCS. These results are not in line with the rest of the results, and are looked at in further detail in figure 11. In figure 11, multiple FCCS curves of the same concentration are shown, which were made chronologically.

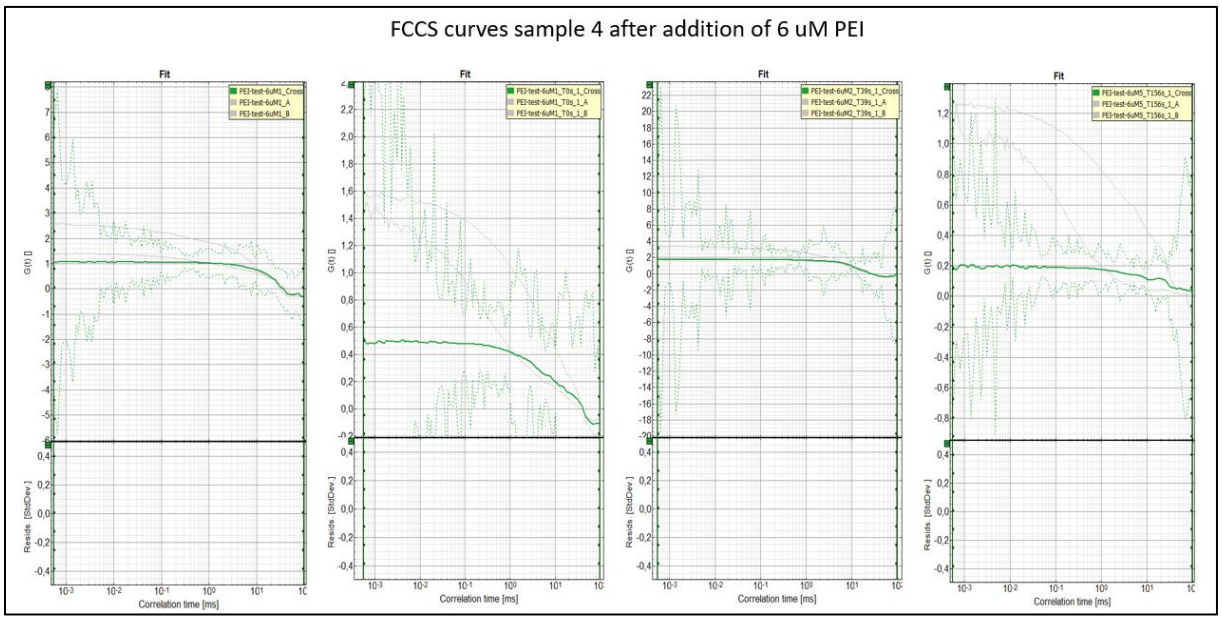

Figure 11: The FCCS curves of sample 4, with a concentration of 6 µM, made chronologically. The graphs show the viruses do not have 1 state of disassembly, but multiple. The cross correlation varies between 0.2 and 2.

The 5 measurements of 20 seconds were not consistent, and showed cross correlations between 0.2 and 2. A cross correlation of 2 would mean more fluorophores are detected together than with the control sample, which does not make sense. The FCCS curves show the 6 µM sample contains different states of disassembly of the virus. In order to verify this was not an experimental error, a new sample was prepared with the same molarity of PEI, and the experiment was performed again. The resulting graphs can be found in figure 12.

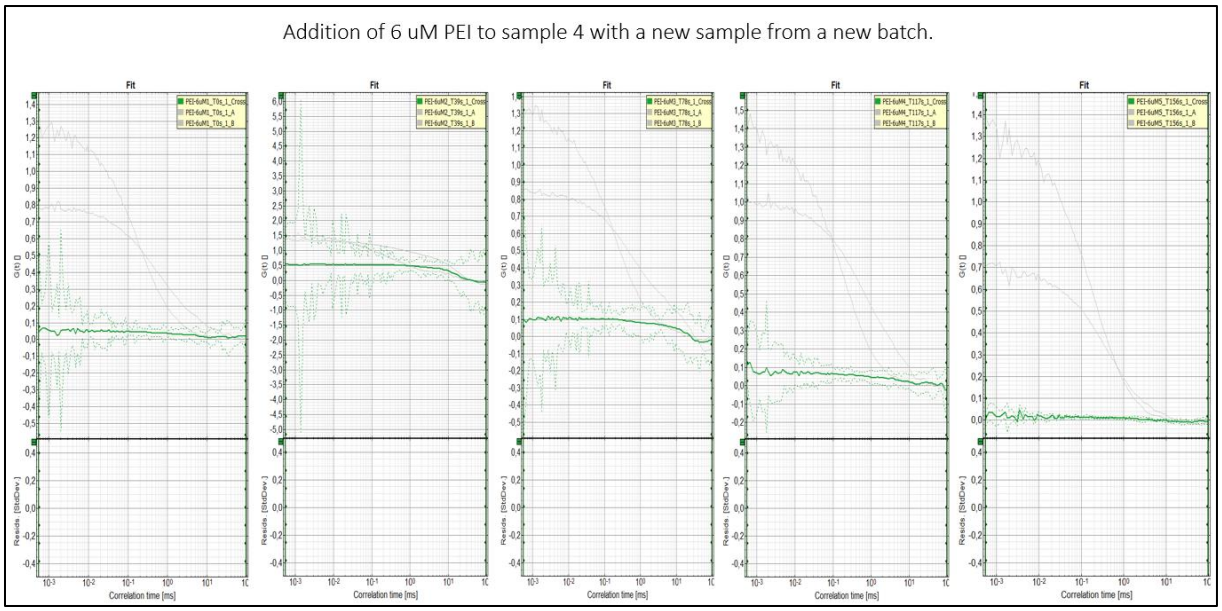

Figure 12: The FCCS curves of a new sample, with a concentration of 6 µM PEI, made chronologically.

The results of figure 12 were more consistent, with cross correlations between 0 and 0.5, although still not consistent like the other concentrations. By having a varying cross-correlation over time, the 6 µM remains out of line in comparison to the other concentrations.

The concentrations of 30 µM and higher show a cross correlation of zero, meaning only disassembled viruses remain in the analyzed samples.

Emission spectra of sample 1 after PEI addition.

The previous results from figure 10 show the sample falls apart at a PEI concentration of 0.6 µM PEI, then at a concentration of 6 µM PEI, the virus is in different states of disassembly, and at higher concentrations the virus is shown to be completely disassembled. Using this knowledge, a final experiment was conducted, using sample 1. The PEI concentration range was changed to 0.06-0.6 uM, since previous experiments showed 0.6 µM PEI was able to completely disassemble the labeled viruses. Different concentrations in this range were created, and sample 1 was added to these concentrations. The resulting emission spectra are shown in figure 13.

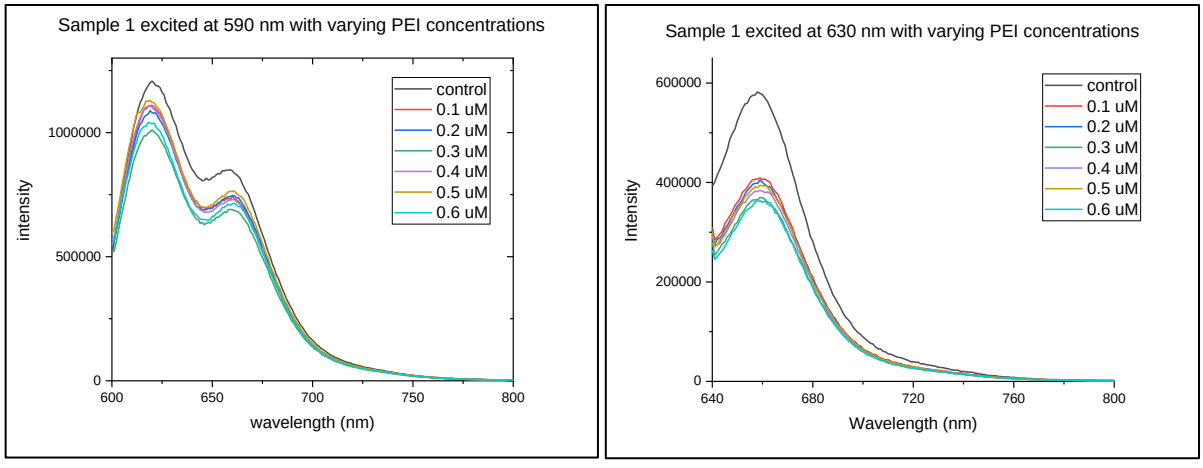

Figure 13: Emission spectra of sample 1 at varying concentrations of PEI present in the sample. The left figure shows the emission after excitation at 590 nm, and the right shows the emission after excitation at 630 nm.

The emission spectra shown in figure 13 are not very clear, so they are plotted again in figure 14, which show the peak intensities of the donor and acceptor against the different PEI concentrations.

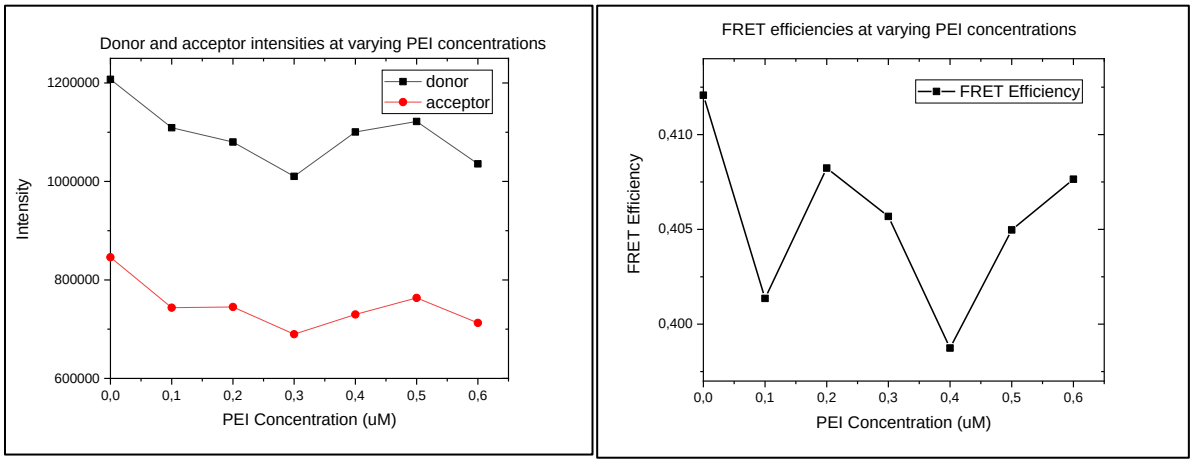

Figure 14: Results of the emission spectrum analysis. The left figure shows the peak intensities of both the donor and acceptor at varying PEI concentrations, and the right figure shows the corresponding FRET efficiency.

The emission spectra have unexpected results. The FCCS figures which were acquired earlier showed the viruses were disassembled at a PEI concentration of 0.6 µM. Figure 13A shows that by addition of PEI, the intensities drop. In theory, the donor should have risen, while only the acceptor drops. The graph does not show this however, and shows the donor and acceptor follow the same path. In order to prove the FRET stays the same, the FRET efficiency was calculated. The efficiency is shown in figure 14. The graph shows the efficiency is roughly constant. This is not expected to happen. With an increase in PEI concentration, the viruses should disassemble, resulting in a loss of FRET efficiency. More research should be conducted as to why this is happening.

Conclusion

The goal was to test a new method of tracking virus disassembly, and test it with current research methods. Results show the FRET labeling of viruses is an effective way to track virus disassembly after the addition of SDS, though the addition of PEI generated some unexpected results with the emission spectra. In order to conclude if the goal was reached, the hypotheses must be tested.

The first hypothesis was:

1. Labeled virus capsids which are still intact are capable of FRET. After virus capsid disassembly, fluorophores attached to the destroyed capsids are in further proximity, and are unable to perform FRET.

The hypothesis cannot be fully accepted. Figure 4 shows the donor intensity rises the more the viruses are disassembled. However, the acceptor peak rises too, which means something else is happening with the labeled viruses. Since the acceptor peak does not go down, the hypothesis is rejected.

2. It is possible to prove virus disassembly resulting from the addition of SDS and PEI by using FRET emission spectra.

This hypothesis is rejected. It is possible with SDS to prove the virus has disassembled. However, the emission spectra of the labeled viruses treated with PEI shown in figure 13 and 14 give unexpected results, which are not in line with the hypothesis, resulting in a rejection.

In conclusion: The goal was not reached. Virus disassembly by SDS was proven using FCCS and emission spectroscopy. However, with the PEI addition, no clear conclusions can be drawn. More tests need to be done in order to fully test this new method of virus disassembly tracking.

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