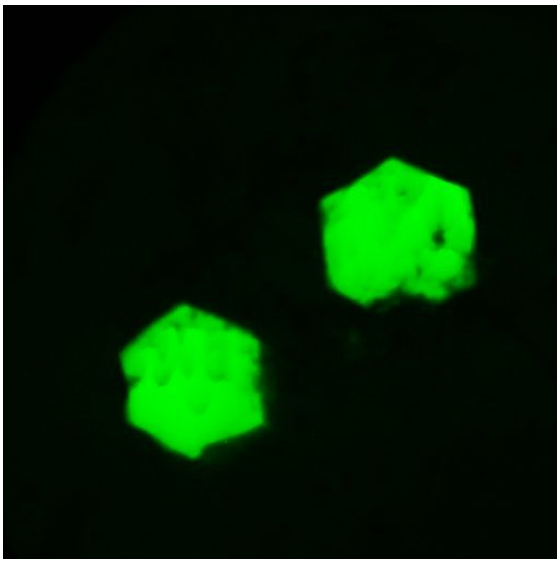
ssDNA Experimental Variations

Control Experiment (Tuesday, June 25, 2019)

The purpose of this experiment was to show that fluorescent single-stranded DNA could be loaded into a CJ Greg protein crystal. However, since the single-stranded DNA does not have an attached thiol, it would not covalently bond to the crystal and therefore could be easily washed out with ATP. This experiment would serve as the control for the future covalent installment of single-stranded DNA with a thiol attached.

The crosslinked CJ Greg crystals were looped into 100 µL Ellman's Reagent (4 mg of Ellman's in 1 mL of Reaction Buffer and allowed to sit over a weekend. On Monday, the crystals were looped into a solution of 5 mM TCEP in 10 mM Tris HCl and allowed to sit for approximately 6 hours before being transferred into 1000 µL of 10 mM Tris HCl overnight to wash. On Tuesday morning, two crystals were looped onto a chip with a well containing 2 µL of 1xTE Buffer. Images prior to DNA loading were taken with the confocal. 25 µM of ssDNA (8 bases fluorescently labeled with FAM) was loaded into the well (total of 10 µL of the solution in the well: 7.5 µL 1x TE Buffer, 2.5 µL 100 µM ssDNA). A 30 minute time-lapse was taken, which showed the crystal gradually gaining fluorescence over the course of 30 minutes.

At approximately 1 pm, the fluorescent crystals were looped into a well of 100 µL of 1x TE Buffer for a wash. At approximately 3:45 pm, the crystals were looped back onto the chip in 5 µL of 1x TE Buffer. At 4 pm, the crystals were imaged again.



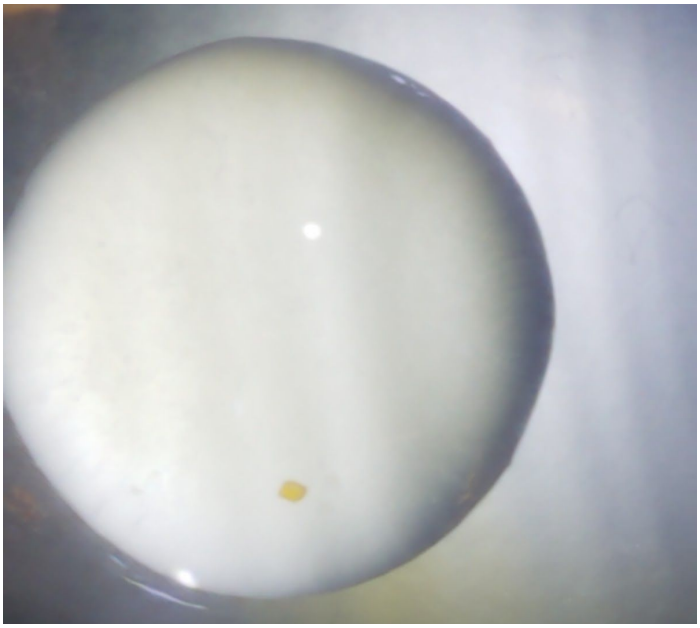
The crystals maintained fluorescence after being washed in 1x TE Buffer.

At the same lighting conditions, the crystal appeared to have maintained fluorescence after being washed. Then 5 µL of 40 mM ATP in HEPES was loaded onto the chip. A 30 minute time-lapse was recorded. A distinct loss of fluorescence in the crystal was noticeable over the course of 30 minutes.

Covalent Installment Experimentation (Wednesday, July 3, 2019)

The purpose of this experiment was to show that ssDNA with a thiol could be covalently installed in a CJ Greg crystal via the formation of disulfide bonds.

At approximately 8:30 am, the crystals, which had been in 5 mM TCEP for approximately one week, were transferred to wash in a fresh well of 1x TE Buffer for 10 minutes. The crystals were washed two additional times, giving a total of three 10 minute washes in fresh 1x TE buffer. The loading chip was prepared by cleaning with DI water and sealing the bottom layer with tape. 4.5 µL of 1x TE Buffer was pipetted into the well of the chip. One large crystal was looped into the well of the chip. An initial image under the confocal was taken. Then, while the chip was still in place under the confocal, 1.5 µL of ssDNA with FAM fluorophore and a thiol attachment was pipetted into the well of the chip. Tweezers were used to delicately place a piece of tape over the chip and seal the well. A 2 hour timelapse was recorded. The crystal had a distinct bright gold color visible to the eye.



The crystal, visible to the eye, while incubating with 5 mM TCEP in 1x TE buffer.

It was then realized that covalent attachment would not likely happen because the thiol attached to the ssDNA comes with a protecting group from the DNA company IDT. The ssDNA should

have been reduced before loading (TCEP, then dialysis). A hypothesis was formed: the ssDNA that had loaded into the crystals could still be reduced with TCEP while in the crystal.

5 mM TCEP in 1x TE Buffer was prepared, and 100 µL of the mixture was pipetted into a clean well of a 9-well glass plate. The crystal that had been loaded with ssDNA was transferred to the well and sealed with tape for 2 hours. The crystal was washed twice in 100 µL of 1x TE for 30 minutes each. The crystal was transferred into the well of the chip with 3 µL of 1x TE for an initial image before adding 3 µL of 40 mM ATP in 1x TE Buffer, pH 7.5 (to make a total volume concentration of 20 mM). A 2 hour timelapse was recorded. The crystal maintained fluorescence throughout the timelapse.

Friday, 07/12/19: Preparing crystals for loading experiment

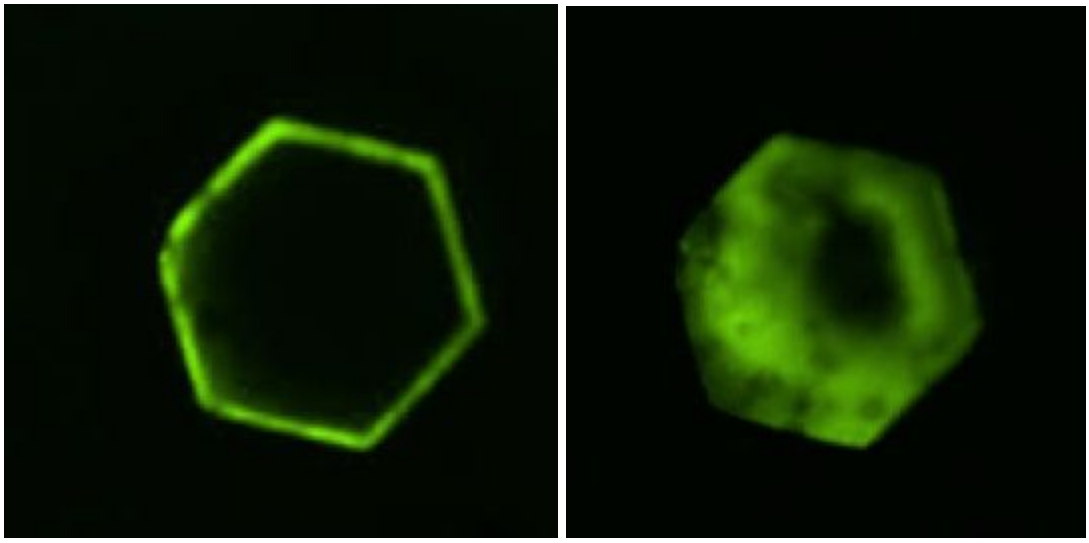
After an Ellman's Reagent experiment, two CJ Greg crystals (one full hexagonal crystal and one half "house" crystal) were placed in 100 µL of 5 mM TCEP in 1x TE in the well of a 9-well glass plate for 2 hours. The crystals were then transferred to another well containing 200 µL of 1x TE Buffer. The well was sealed with tape and allowed to sit over the weekend

Monday, 07/15/19: Loading experiment-TCEP vs. NO TCEP

The purpose of this experiment was to determine whether or not TCEP was required to deprotect the thiols of the ssDNA from IDT when covalently installing thiolated ssDNA. A hypothesis was formed that thiol-disulfide exchange would occur between the protecting disulfide bond in the ssDNA and the exposed thiol in the crystal. The ssDNA would be covalently installed in both crystals and the ssDNA would not be displaced in the presence of ATP (i.e. the crystal would maintain fluorescence). One full hexagonal crystal and one "house" crystal were selected for the experiment for easy distinction when in the same well.

In the morning, both crystals were washed twice in 200 µL of 1x TE Buffer twice for 30 minutes each. The crystals were a clear color. The black plastic loading chip was cleaned by rinsing with RO water and drying using the pressurized air hose under the hood. A piece of clear plastic tape was used to create a sealed base at the bottom of the chip.

In the confocal room, 4.5 µL of 1x TE Buffer was pipetted into the well of the chip. The crystals were positioned in the well of the chip and an initial image was taken at 5% intensity 488 nm. With the chip still positioned under the confocal, 1.5 µL of ssDNA (CG CTG GCG with 5' FAM and 3' thiol) was pipetted using a 2.5 µL pipette. Tweezers were used to adhere a small piece of tape to seal the well. A 2 hour timelapse was recorded. During the timelapse, fluorescence increased in the crystal. The fluorescence loaded into the crystal from the outside edges of the flat hexagon and worked its way inwards. The loading process was performed separately for each crystal. The full crystal was loaded first, followed by the larger house crystal. Both crystals exhibited a large increase in fluorescence--indicating that the ssDNA did enter the crystal.



CJ Greg crystal initial image at zero minutes of timelapse (left) and final image after 2 hour timelapse (right). Images were taken every five minutes for the two-hour duration.

After loading for two hours, the hexagonal crystal was placed in 100 µL of 5 mM TCEP in 1x TE for two hours before being transferred to 100 µL of 1x TE for overnight storage. The house crystal was immediately transferred to 100 µL of 1x TE after the loading period for overnight storage.

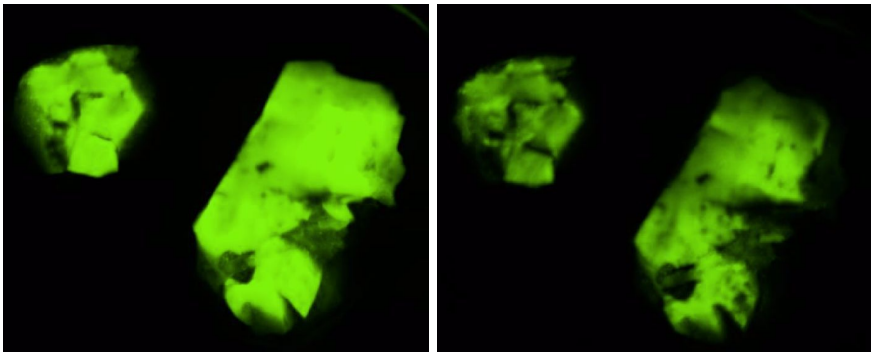
Tuesday, 07/16/19: Unloading and quenching experiment

In the morning, both crystals were washed twice in 100 µL of 1x TE for 30 minutes each. The two crystals were then placed in the same well of a clean plastic loading chip containing 3 µL of 1x TE Buffer. An initial image was taken with the confocal to show that both crystals were fluorescent after being washed. A 10 µL pipette was used to add 3 µL of 40 mM ATP in 1x TE (for a total concentration of 20 mM ATP) to the well of the chip. Tweezers were used to fix a piece of tape to seal the well and a two hour timelapse was recorded. Both crystals maintained fluorescence during this time, suggesting that a wash with TCEP is not required for the covalent installation of ssDNA.

The crystals were removed from the chip and washed twice in 100 µL of 1x TE Buffer for 30 minutes each.

The crystals were then placed in a clean loading chip with 4.5 µL of 1x TE Buffer and an initial image was taken. A 2.5 µL pipette was used to add 1.5 µL of 100 µM ssDNA (CGC CAG CG with 3' DAB) to the well. The chip was sealed with tape under the confocal microscope and a two hour timelapse was taken with images every 5 minutes at 5% intensity 488 nm. The

timelapse was stopped after 45 minutes as a distinct decrease in fluorescence could be seen. The crystals remained in the chip overnight and a final image was taken in the morning, where the crystals had little to no fluorescence.



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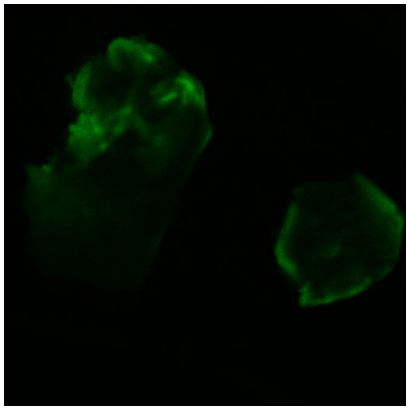
A quenching effect was seen as the fluorescence decreased within a 45-minute timelapse. On the left are the crystals at zero time of the timelapse. The image on the right is after 45 minutes.



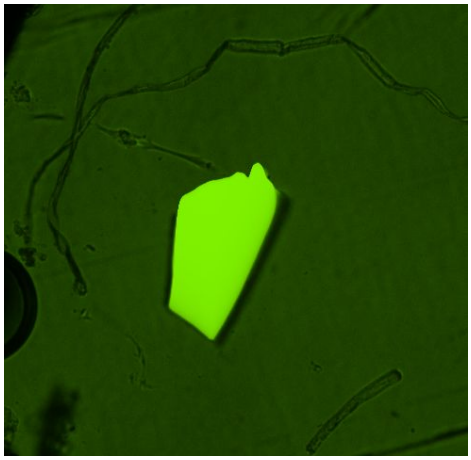
The quenching effect was much more apparent after the crystals were allowed to incubate with quenching strands overnight.

Wednesday, 07/17/19: Quick loading and unloading experiment

There were two main goals of this experiment. The first was to see if the loading/unloading process for covalent installment of ssDNA could be shortened. After determining in the previous experiment that the deprotection of the disulfide bonds in the ssDNA was unecessary, it was proposed that a strand could be loaded for two hours, washed for 1 hour, and then unloaded. The second goal of this experiment was to further provide evidence of covalent installment by displacing the crystal-ssDNA disulfide bond with a combination of ATP and TCEP. It was hypothesized that while ATP alone could not displace the covalent bonds formed between the crystal and the ssDNA, a combination of TCEP and ATP simultaneously could reduce disulfide bonds and “push” the ssDNA out of the crystal.

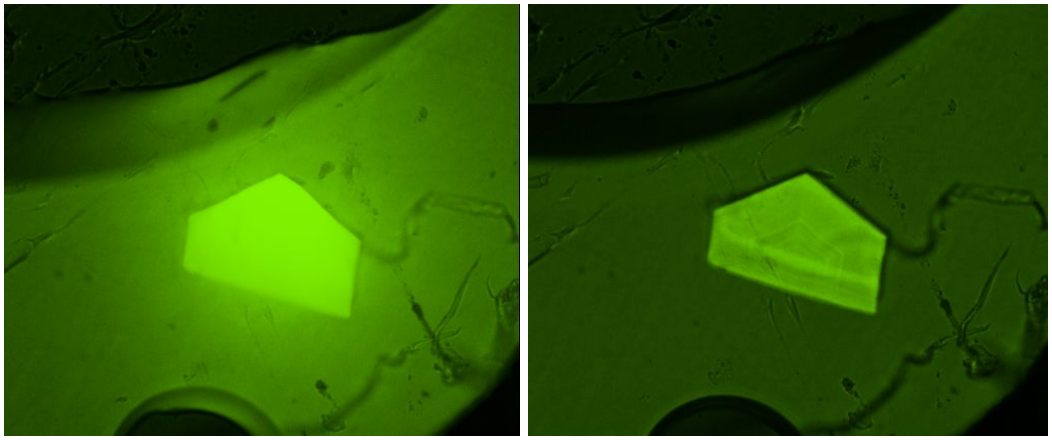
One CJ Greg crystal was washed twice in 100 µL of 1x TE Buffer for 30 minutes each. The crystal was then placed in the well of a plastic loading chip with 4.5 µL of 1x TE Buffer and an initial image was taken under the confocal at 5% intensity, 488 nm. A 2.5 µL pipette was used to add 1.5 µL of ssDNA (CG CTG GCG with 5' FAM and 3' thiol) to the well of the chip. Tweezers were used to gently seal the well with clear tape under the confocal. A two hour timelapse was recorded. The crystal was a bright gold color visible to the eye after the two hour loading period. The crystal was then washed twice in 100 µL of 1x TE Buffer for 30 minutes each.

The crystal was then placed in the well of a clean loading chip with 3 µL of 1x TE Buffer. An initial image was taken under the confocal microscope to show that the crystal maintained fluorescence after being washed. A 10 µL pipette was used to add 3 µL of 40 mM ATP to the well (for a total concentration of 20 mM ATP in the well). Tweezers were used to seal the well with tape and a one hour timelapse was recorded. The crystal did not decrease in fluorescence during the one hour period.



The crystal maintained fluorescence for one hour when incubated with ATP alone.

The crystal was then transferred to a separate loading chip with 3 µL of 40 mM ATP in 1x TE Buffer. An initial image was taken before 3 µL of 50 mM TCEP was pipetted into the well (total concentrations in well: 20 mM ATP, 25 mM TCEP). The well was sealed with tape and a two hour time-lapse was recorded. Over the first 30 minutes, there was a decrease in the fluorescence of the crystal.



After 30 minutes, the fluorescence in the crystal had decreased during incubation with both ATP and TCEP. The image on the left is a time zero, while the image on the right was taken at 30 minutes.

Monday, July 22, 2019: Loading with Magnesium Sulfate TE Buffer

The purpose of this experiment was to attempt the covalent installment of ssDNA CJ Greg crystal in the presence of a slightly different buffer. CJ Greg crystals are known to have 12-13 nm pores that lie perpendicular to the face of the hexagonal crystals. There or smaller pores approximately 2 nm in length that run along the sides of the hexagonal crystal. Previous experiments indicate that the ssDNA has been entering the crystal through the sides, suggesting that the ssDNA prefers the smaller pores. One hypothesis was that there is some sort of electronegative presence. One idea was to attempt a loading experiment with a buffer that contains ions.

A buffer of 100 μM Magnesium Sulfate in 1x TE Buffer was prepared. To make 50 mL of the solution, 0.012037 grams of Magnesium Sulfate was weighed in a tared 50 mL tube and 1x TE Buffer was added until the fill line.

One crystal was washed twice in 100 μL of 100 μM Magnesium Sulfate 1x TE Buffer for 30 minutes each. The crystal was then placed into the well of a plastic loading chip containing 4.5 μL of 100 μM Magnesium Sulfate 1x TE Buffer. An initial image was taken using the confocal microscope at 5% intensity, 488 nm excitation. A 2.5 μL pipette was used to add 1.5 μL of ssDNA (CG CTG GCG with 5' FAM and 3' thiol) to the well. The well was sealed with tape and a two hour time-lapse was recorded. It was noticeable that loading (i.e. the gain of fluorescence in the crystal) was much slower than usual loading in 1x TE Buffer. After loading for two hours, the crystal was washed twice in 100 μL of 100 μM Magnesium Sulfate 1x TE Buffer for 30 minutes each. The crystal was then placed in the well of a loading chip with 3 100 μL of 100 μM Magnesium Sulfate 1x TE Buffer. An initial image was taken at 5% intensity, 488 nm excitation wavelength to show that the crystal maintained fluorescence after being washed. A 10 μL pipette was used to add 3 μL of 40 mM ATP to the well before sealing the well with tape. A two hour time lapse was recorded, during which the crystal maintained fluorescence. Results from

this experiment suggest that there is no difference in loading pattern in the presence of magnesium sulfate, but possibly a difference in the rate at which ssDNA can enter and exit the crystal.