Investigating the liquid phase separation of Rubisco and linker proteins *in vitro*

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Most of the oxygen we breathe finds its origins in marine algal photosynthesis. These algae organise their Rubisco, the enzyme responsible for capturing carbon dioxide and releasing oxygen, into a liquid droplet called the 'pyrenoid'. The algae then concentrate carbon dioxide into the liquid, and the reactions can run faster. The pyrenoid improves the efficiency of the oxygen-producing reaction by around 60% compared to the same reaction in plant leaves. Understanding the phase-separation process will help scientists introduce these carbon concentration mechanisms into plants, with the hope of increasing crop yields to help with food security. However, for these liquid droplets to form at all, Rubisco requires a counterpart 'linker' protein. We don't know what minimal properties of a linker protein are needed for pyrenoids to function this way. And why a liquid? What are the resulting properties of the droplet that drive this incredible boost in photosynthetic output?
I created pyrenoid-like droplets by mixing these proteins outside the confines of a cell. I used a specialised microscope to measure the droplet refractive index (the amount that light is slowed down in a material) as a starting point for estimating the amounts of Rubisco and linker concentrated inside the droplets.

What was the aim of your project?

Liquid phase separation is a fascinating strategy that cells borrow from physics to speed up vital reactions. I aimed to take CO2-fixing enzyme and linker from Chlorella algae, to mix them to get droplets, and to correlate the droplet size, fluorescence (labelled protein) and refractive index (total protein) with composition.

How did you address the aim?

The project started with micromolar amounts of purified Rubisco and linker proteins. I mixed them

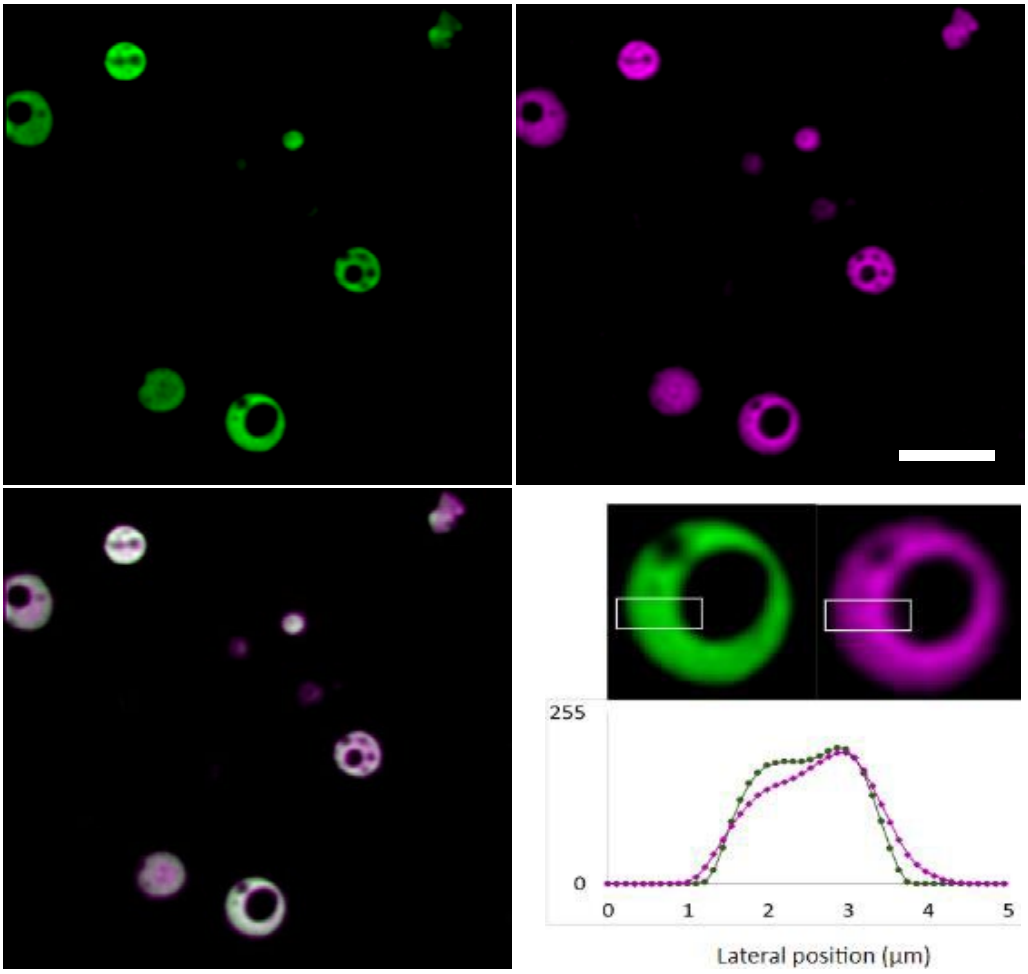


Figure 1. Liquid droplets of protein show inclusions of the dilute buffer phase when agitated. Widefield epifluorescence image (green) and refractive index map (magenta) and merged overlay (left). Registered line profiles show good correlation (right). Scale bar: 5 µm.

at specific ratios. After confirming the presence of droplets in brightfield, I then put the droplet mixtures in a Tomocube microscope, which performs label-free holotomographic reconstruction of the sample volume. I converted the image of optical phase shift to an estimated map of the refractive index of the droplets, using a calibration with two well-defined samples: first, the neat buffer without protein, and second, polystyrene microbeads in glycerol. While the peaks in refractive index were distinct from the background, artifacts of the reconstruction were problematic as some droplets were very small. With high-precision coverslips, these artifacts became more manageable. However, I still needed a way to confirm which signals were droplets. I doped the mixtures with 5% or 100% GFP-tagged linker protein so that I could observe the droplets under epifluorescence and compare with the maps of refractive index. I wrote macros in FIJI to determine fluorescence thresholds that correctly segmented droplets from surrounding buffer. This eliminated remaining artifacts of the tomographic reconstruction. I could then map the refractive index inside the droplets, which I then used as a proxy for the local protein concentration.

What did you find out?

Although the fluorescent signal was weak, the relative lack of tagged linker outside the droplets meant a very low fluorescent background as well. I

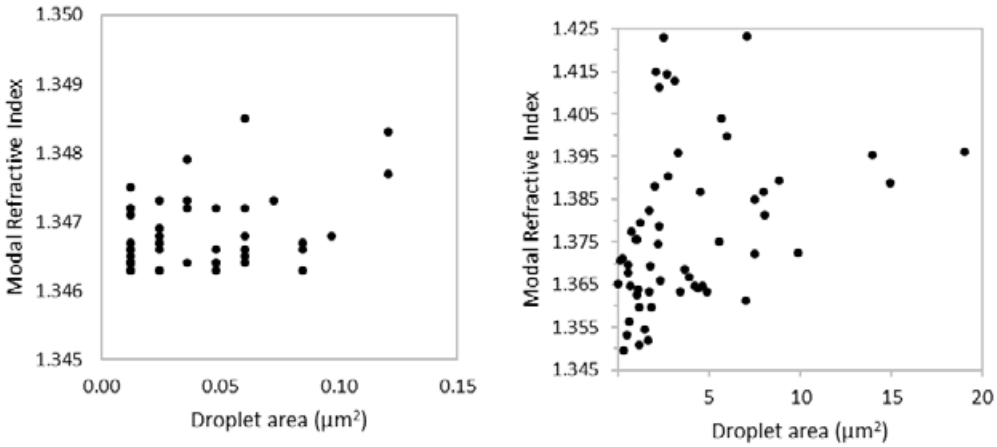


Figure 2. The modal refractive index measured inside each droplet against its cross-section size, using (left) 5% GFP-tagged + 95% native linker, or (right) using 100% GFP-tagged linker.

was able to segment each droplet in the fluorescence channel and estimate its projected size. After I registered the fluorescent and refractive index images, I was able to confirm that they matched up with good overlap (Figure 1, lower left). I could then identify which areas of the images corresponded to the same droplet and look at correlated data across droplet populations.

A key question was whether droplets produced with 100% GFP-tagged linker had the same properties, or if the fluorescent tag somehow perturbed the phase separation process. I observed that droplets formed using 5% GFP-tagged linker (Figure 2, left) were many times smaller than those with 100% GFP-tagged linker (Figure 2, right). This suggests that tags encourage phase separation, probably by increasing the attractive interactions of the linker with Rubisco. When agitating the 100% tagged mixture with a pipette, the regular droplets formed beautiful double emulsions (Figure 1). Hopefully, the reluctance of these droplets to fuse might tell us something about their structure or surface properties in future work.

No strong correlation was found between droplet size and refractive index, at least for very small droplets with 5% GFP-tagged linker (Figure 2, left). However, using 100% GFP-tagged linker to generate a larger range of sizes (Figure 2, right), I found the average refractive index rapidly increased to a high 'plateau' for droplets larger than a micron. This might be explained by a thin surface layer that is lower in refractive index than the droplet core, though this isn't clear from the line profiles (Figure 1, lower right). Inside the pyrenoid, the Rubisco enzyme is expected from reaction models to a hundredfold more concentrated than in the buffer. In agreement with this, I measure a refractive index range that corresponds to a Rubisco enrichment of between 100- to 400-fold inside the droplets.

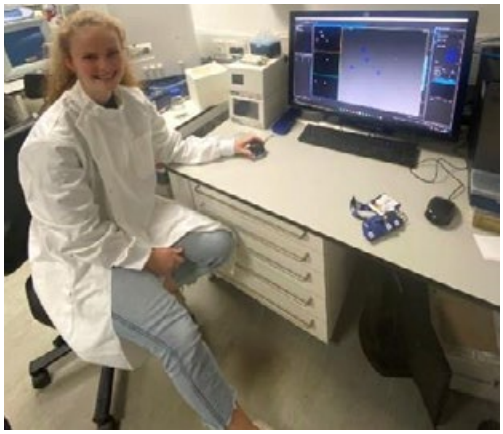
What did you learn from participating in this project?

During my project I have received training on a number of different microscopes as well as the Tomocube, including Slimfield (single molecule tracking in the Leake group at York), Zeiss LSM confocal and epifluorescence microscopes. The Tomocube had a difficult learning curve, but I became more proficient towards the end of my project. I was able to acquire some good images and it helped with my general understanding of research microscopy and scientific instruments. I have a background in biochemistry and hadn't realised that such a large component of microscopy at this level is downstream image analysis, which I

applied through use of FIJI software to compare images and put numbers to different measures. I have also experienced training in the wet lab such as preparing the protein mixtures, mounting different slides, and preparing bead samples for calibration of different microscopes.

How has this project affected your long-term goals?

While I am undecided what path I want to pursue in the future, as there are so many options, this project has definitely shown me that research is a very rewarding line of work. During research many different challenges are encountered - my progress was quite limited by the short timespan of the project and the purified protein available. Yet time is spent finding different solutions and ways around these obstacles. It is also a very friendly environment as part of a research team; everyone shares their findings and helps each other out with their different projects, all working to reach one final result. Doing this project has confirmed that I enjoy interdisciplinary science so that is definitely something I would wish to continue in future work.



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