Atomic Force Microscopy combined with Fluorescence Lifetime Imaging Microscopy (AFM + FLIM): a powerful approach to explore the structure and dynamics of biological membranes

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Summary

Atomic force microscopy (AFM) and fluorescence lifetime imaging microscopy (FLIM) are two powerful techniques that are often used in the biophysical community as a means of characterising biological membranes. On its own AFM can report on the high-resolution 3-D structure and mechanical properties of biological samples adhered to solid supports. FLIM provides information on the position and local environment of fluorescent pigments within samples. When used in combination, these two methodologies can provide a range of opportunities for investigating biological membranes where the position and local interactions between molecules may result in changes to fluorescence, or, in the example of light-regulated processes, changes to membrane/protein functionality. Here we present an application of combined AFM and FLIM to characterise the structure and photophysics of light-harvesting membranes, as well as using video-speed FLIM measurements to investigate membrane dynamics. This approach may be applied to a wide range of biological samples.

Introduction to Atomic Force Microscopy and Fluorescence Microscopy

Atomic Force Microscopy (AFM) is a powerful technique for recording 3-D maps of surfaces with nanoscale resolution (Santos and Carvalho, 2019). Samples are typically adhered onto a planar support surface, such as mica, glass or silicon, and then an ultra-sharp probe is used to generate a map of the differences in height (a topograph). In addition to recording XYZ images, the instrument can be used to measure the mechanical properties of a sample, for example, to see how the elastic modulus or deformability of a surface varies in different locations. The advantages of the AFM technique are: a relatively high spatial resolution (~1 nm laterally and ~0.1 nm in the Z-plane), the excellent signal-to-noise (so averaging is not required) and the ability to assess samples in a hydrated state and in real time (so sample fixation or freezing is not required). These features may explain why AFM has seen increasing use in the biological sciences over the last decade, with growing recognition that it can be used to observe the structure and mechanics of key biomolecules under relatively natural conditions (**Figure 1A**). This has included insightful studies of whole cells (Müller and Dufrêne, 2011), lipid membranes (Connell et al., 2013), proteins (Kumar et al., 2017), and polysaccharides (Chen et al., 2016).

However, AFM has its limitations which include: a relatively slow image acquisition speed (typically minutes), a lack of specific chemical recognition and a lower resolution than X-ray crystallography or cryo-electron microscopy. Each of these aspects are being addressed in ongoing research around the world, e.g., video-speed scanning (Ando, 2019), super-resolution analysis methods (Heath et al., 2021), and the use of functionalised AFM probes for chemical recognition (Vasilev et al., 2019) but these approaches are not straightforward. Fluorescence microscopy can be a complementary tool that offsets some of these limitations (**Figure 1B**). Standard fluorescence microscopes can record images very quickly (typically seconds) and allow

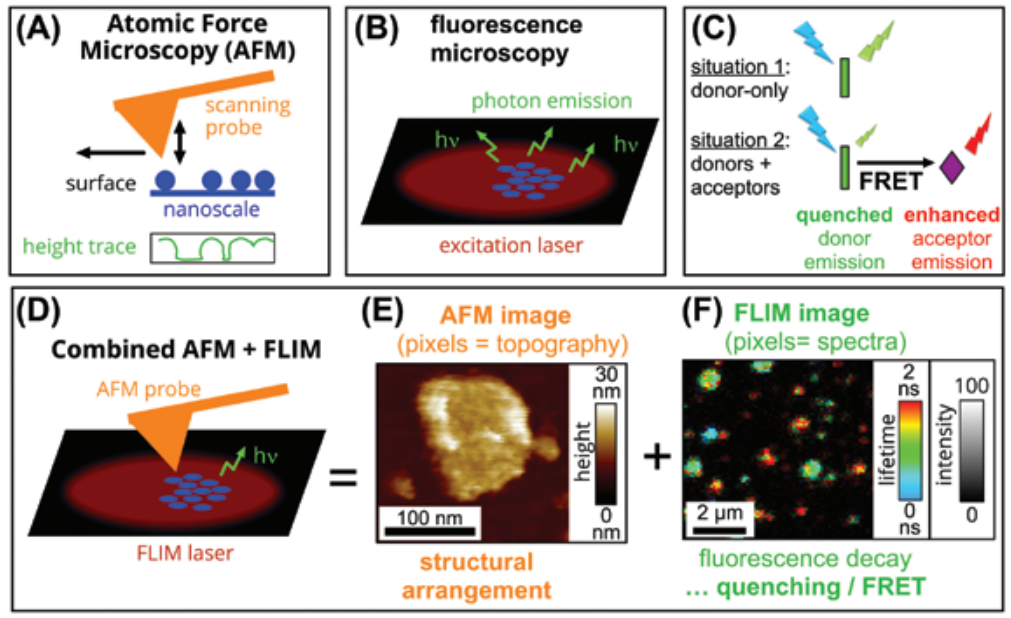

Figure 1. Comparison of AFM and FLIM methodologies. (A) Schematic of part of the Atomic Force Microscopy instrument. A sharp probe at the end of a flexible cantilever is scanned across the surface of a sample. Topographs (height maps) can be generated with nanometre-resolution. (B) Schematic showing laser excitation of a sample (a tightly-focussed laser spot might be 500 nm in diameter). If the sample contains pigments which can accept the energy then this causes fluorescence (emission of photons) which can be detected by a fluorescence microscope. (C) A cartoon of the principle behind Förster Resonance Energy Transfer (FRET). (D) Schematic of combined AFM and FLIM. (E) An example AFM topograph. (F) An example FLIM image, where each pixel has both a saturation (dark-to-bright) and a false colour (blue-to-red) scale representing the fluorescence intensity and lifetime, respectively.

us to observe dynamic biological processes, such as molecular diffusion or assembly processes. They can also verify the position of specific molecules because only the fluorescently-tagged (or naturally fluorescent) molecules will provide the fluorescence signal. Therefore, it can be a good strategy to use AFM and fluorescence microscopy in parallel as their strengths offset their weaknesses.

Fluorescence analysis using FRET and FLIM (Förster Resonance Energy Transfer and Fluorescence Lifetime Imaging Microscopy)

The fluorescence properties of pigments are sensitive to their local environment (e.g. pH, temperature, or the proximity of other molecules), and so fluorescence methods can also be exploited to reveal addition information beyond the structure of samples. A phenomenon known as Förster Resonance Energy Transfer (FRET), in which a donor pigment and an acceptor pigment may interact if they are spectrally (i.e., energetically) similar, is commonly used to assess protein-protein interactions or molecular distances. Energy transfer will occur when the donor and acceptors are in close proximity (**Figure 1C**) and causes the donor fluorescence intensity to reduce, termed donor "quenching", and the acceptor fluorescence intensity to increase due to receiving additional energy. The average distance between donor and acceptor molecules can be calculated very accurately based on Förster theory if these distances range from 1-10 nanometres, leading some researchers to refer to FRET as a "molecular ruler" (Roy et al., 2008). Alternatively, certain pigments are known to "self-quench" each other in a concentration-dependent manner and we can exploit this in experiments where a change in molecular concentration tells us something about the system of interest. For example, we can assess the stability of polymeric membranes that are designed to encapsulate drugs by including self-quenching carboxyfluorescein dye molecules (Seneviratne et al., 2020). If the polymer membranes burst then we observe a quantifiable fluorescence increase as the carboxyfluorescein is released and becomes de-quenched. Thus fluorescence quenching (via FRET or concentration-quenching) can be a useful tool for various experiments related to molecular interactions.

Quenching can be quantified via changes to the fluorescence intensity but the disadvantage here is that it can be challenging to distinguish between a reduced fluorescence caused by FRET or simply a decrease in the concentration of the fluorophore. Alternatively, FRET and other types of quenching are also manifested as an increased rate of decay by fluorescence because the excited electronic states of donor molecules now have additional pathways for decay (e.g., both transfer and fluorescence). This can be measured with time-resolved fluorescence (TRF) spectroscopy, which employs specialised detectors and timing electronics that act as a picosecond-accuracy stopwatch to measure the length of time between photon absorption and re-emission by fluorescence. Measuring millions of single-photon events in this manner allows us to calculate the "fluorescence lifetime" of a pigment which, in turn, can be used to quantify the quenching. Thus, a decreased fluorescence lifetime will reveal FRET independently of the pigment concentration and often more accurately than the fluorescence intensity change. Fluorescence Lifetime Imaging Microscopy (FLIM) is a powerful technique which acquires fluorescence images where every pixel contains both the intensity and lifetime information (**Figure 1F**) (Trautmann et al., 2013). FLIM has been used to determine how molecular interactions vary across an image, for example, proteins interacting in different parts of a cancer cell (Provenzano et al., 2009).

This article looks at the opportunities offered by using AFM and FLIM in correlation. This includes taking images on separate samples and correlating two different datasets and the more challenging task of spatially-correlated AFM+FLIM (**Figure 1D-F**). The complementarity of the nanoscale resolution of AFM and the ability to quantify changes in photophysical properties by FLIM is an enticing combination.

Case study: understanding photosynthesis using AFM and FLIM

Our research group is interested in understanding the molecular mechanisms of photosynthesis in plants and other organisms. In plants, the primary reactions of photosynthesis take place within organelles called chloroplasts. Contained within the chloroplast is a complex system of bio-membranes called "thylakoids" (**Figure 2A**), which are the location of solar energy absorption, energy transfer and the initial conversion of energy to a chemical state. Decades of research have revealed the overall architecture of the thylakoid membranes, atomic resolution structures of the proteins embedded within these membranes, and information about the bioenergetic pathways (Blankenship, 2021). We know that thylakoids are made up of interconnected membranes which form stacked disc-like arrangements, called grana, and single layered regions, called stromal lamellae. The thylakoid membranes are packed full of "light-harvesting" (LH) and "photosystem" (PS) membrane proteins which contain chlorophyll and other pigments for energy absorption and transfer (Johnson et al., 2014). However, there are still gaps in our knowledge of this important process. Specifically, we do not know the precise photophysical (energy transfer) pathways and their timescales of occurrence within individual LH proteins. There are also questions about how the overall membrane system adapts to changes in light intensity. Recent research suggests that exposure to elevated light intensities can trigger dynamic rearrangements of LH proteins and can trigger changes in the energetic balance of the system (Johnson and Wientjes, 2020). One way to understand more about the structure of these systems is using AFM and FLIM.

In recent research, we correlated the optical and structural properties of thylakoids extracted from plants by a combination of FLIM and AFM measurements (Meredith et al., 2021). We obtained FLIM images of extracted thylakoids adhered to glass coverslips, where the fluorescence signal is from the natural fluorescence of chlorophylls that are within LH proteins. These images show that

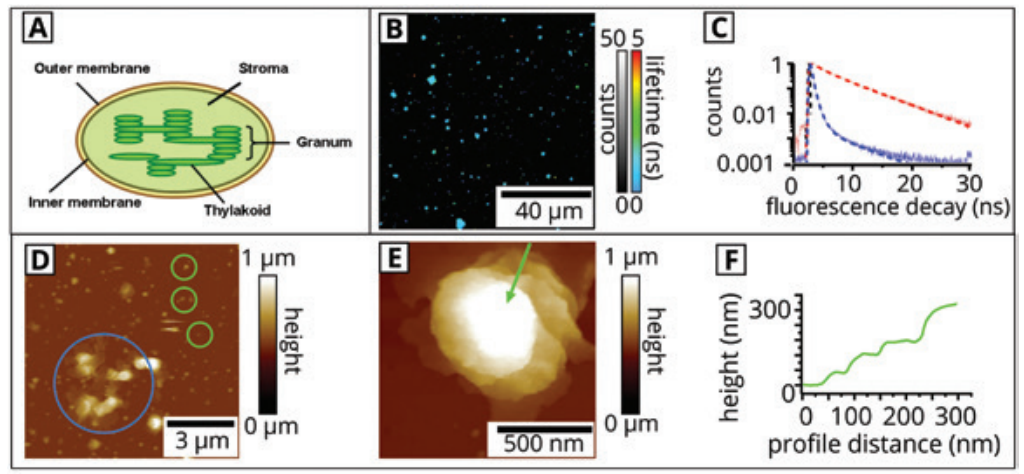

Figure 2. Investigation of the thylakoid membranes extracted from chloroplasts. (A) Cartoon of a chloroplast. (B) FLIM image of extracted thylakoid membranes adhered onto a hydrophilic glass surface. (C) Normalised fluorescence decay curves showing data from thylakoids (blue) versus isolated LH proteins (red). (D) AFM image of a similar sample as in (B). The topograph shows small, adhered membrane patches (ringed green) and also larger multilayered structures (ringed blue). (E) A zoomed-in topograph of a multilayered thylakoid extract. (F) A height profile drawn along the green line in panel (E), showing the multilayer steps of the membrane. Panel (A) image credit: Wikimedia Commons (public domain). Panels (B)-(F) are adapted from Meredith et al. 2021, and is used and licensed under CC BY 4.0.

thylakoids appear as distinct objects, small spots of fluorescence often below 1 micron in size (**Figure 2B**). These fluorescent spots all appear to have similar, quite short fluorescence lifetimes of ≈0.5 ns (*blue*). Note, all FLIM images have a colour scale with fluorescence lifetime represented from *blue* (short lifetime) to *red* (long lifetime) and an intensity scale representing the total counts in each pixel. We can quantify the fluorescence quenching by extracting the TRF data from the bright pixels within this FLIM image. The graph generated (**Figure 2C**) shows the exponential decay of the fluorescence signal that allows us to quantify a fluorescence lifetime of 0.4 ns. AFM images reveal that these objects have a heterogeneous size distribution. In a low-magnification field of view (**Figure 2D**), a variety of structures are observed from relatively compact assemblies (100–200 nm laterally and 10–100 nm in height, *ringed green*) to large microscale structures which contain distinct multilayers (3–4 µm laterally and up to 750 nm in height, *ringed blue*). Higher-magnification AFM images (**Figure 2E**) show multilayered structures and we can draw profiles (**Figure 2F**) to assess the increase in height over consecutive multilayers, finding stacks of ≈300 nm in height. LH and PS proteins are known to be 5-10 nm in height, so it is likely that even the smallest objects observed via AFM must consist of a few stacked protein-rich membranes, increasing up to tens of stacked membranes for the largest objects. The structures observed by AFM are consistent with the tightly stacked thylakoid membranes observed *in vivo* (e.g., in intact membranes by electron microscopy). The short fluorescence lifetime suggests that the LH proteins are in a "self-quenched" state. Together, this is a correlation of the nanoscale structure and fluorescence of these important biomembranes. The fact that such a heterogeneous and disordered distribution of randomly adhered membranes is found highlights the need for a method to promote the formation of large, homogeneous membrane structures that are suitable for quantitative studies.

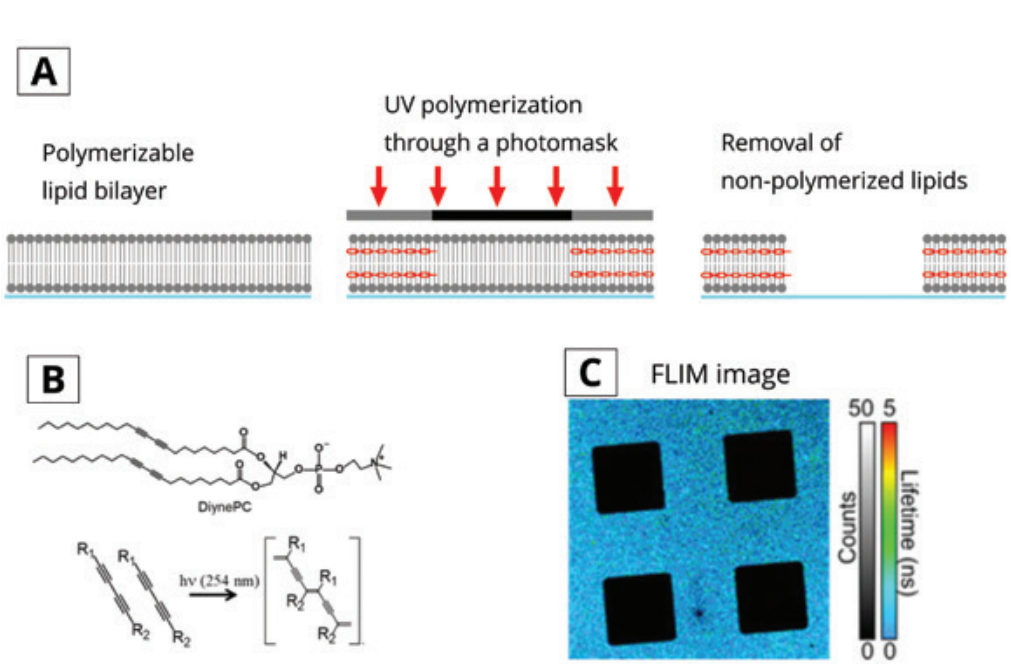

Figure 3. Microscale template patterns on glass coverslips for microscopy. (A) A schematic of the process of UV photolithography. By use of a photomask we induce crosslinking of special photo-active lipids in micro-patterns, in order to generate a grid-like pattern of the polmerized membranes. This acts as a template into which biological membranes will readily fuse. (B) The special lipids (DiynePC) used for photo-crosslinking into a stable form. (C) An example FLIM image of an empty polymerised template. This image represents an 80 × 80 µm region. This figure is adapted from Meredith et al. 2021, and is used and licensed under CC BY 4.0.

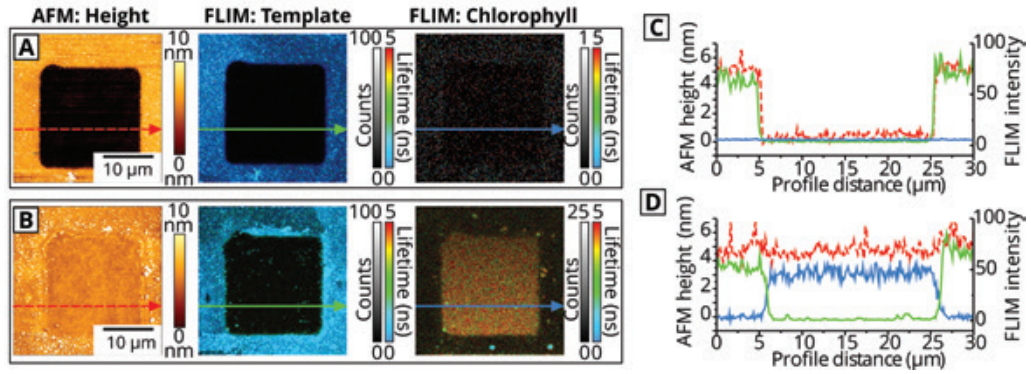

Figure 4. Analysis of "hybrid membranes" by correlated FLIM and AFM measurements. For (A) and (B): the left panel is an AFM topograph, the centre panel is the "Template FLIM channel" and the right panel is the "chlorophyll FLIM channel". (A) Correlated FLIM+AFM data showing a single square of the polymerised lipid "empty" template. (B) Correlated FLIM+AFM data showing a similar region as in (A), but after the corrals were "backfilled" with the extracted thylakoids and synthetic lipid vesicles to form the hybrid membrane. (C),(D) Profiles drawn across the region in (A) or (B), respectively, showing the AFM height (red), FLIM intensity from the template (green), and FLIM intensity from chlorophyll (blue). This figure is adapted from Meredith et al. 2021, and is used and licensed under CC BY 4.0.

A new micro-patterned template for assessing biomembranes

Next, we collaborated with a research team in Japan who produce microscale templates on glass surfaces that are suitable for stabilising biomembranes for more detailed FLIM/AFM studies (Yoneda et al., 2020). These templates take the form of a 2-D array pattern, comprised of empty boxes of 20 × 20 µm in size, for the membranes to adhere (**Figure 3**). Now, we can take our complex biological membrane of choice and deposit it into the template pattern. Natural membranes will fuse with the exposed edges of the template, so long as they are mixed with synthetic lipid membranes. It appears that the synthetic lipids support fusion and rearrangement of the membranes into a single layer that is confined to the corral (box) region. There are multiple benefits of generating a micro-array pattern of biomembranes, particularly, that they provide an obvious target in the coral regions and a stable, planar form of membrane that is amenable to microscopy.

To validate this approach and learn more about photosynthesis, we utilised the same thylakoid membranes described in the last section to produce micro-array patterned membranes (Meredith et al., 2021). Extracted thylakoid membranes and synthetic lipid vesicles were incubated with a template pattern and after 20 minutes a stable "hybrid membrane" was generated. To determine the hybrid membrane structure and correlate this to the fluorescence properties, an instrument combining AFM with FLIM was used to record nanoscale topography maps spatially correlated to multi-channel fluorescence data. The AFM image and height profiles (**Figure 4A** and **4C** *red line*) revealed that the "empty" template pattern of polymerised lipids was the expected ~4.8 nm in height and this aligned with the fluorescence intensity of the template from the partner FLIM image (**Figure 4C** *green line*). After the formation of the hybrid membranes, FLIM images show that there was largely homogeneous chlorophyll fluorescence within the square corral regions with no resolvable defects at this scale (**Figure 4B**). The increase in the chlorophyll fluorescence intensity (**Figure 4D** *blue line*) corresponded with a small change in the AFM height of just 0.2 nm (**Figure 4D** *red line*). In summary, the FLIM images revealed clear array patterns where the vast majority of chlorophyll fluorescence is localised within the square corral regions defined by the template. These patterned hybrid membranes were highly reproducible, with similar dimensions and fluorescence intensity across multiple preparations.

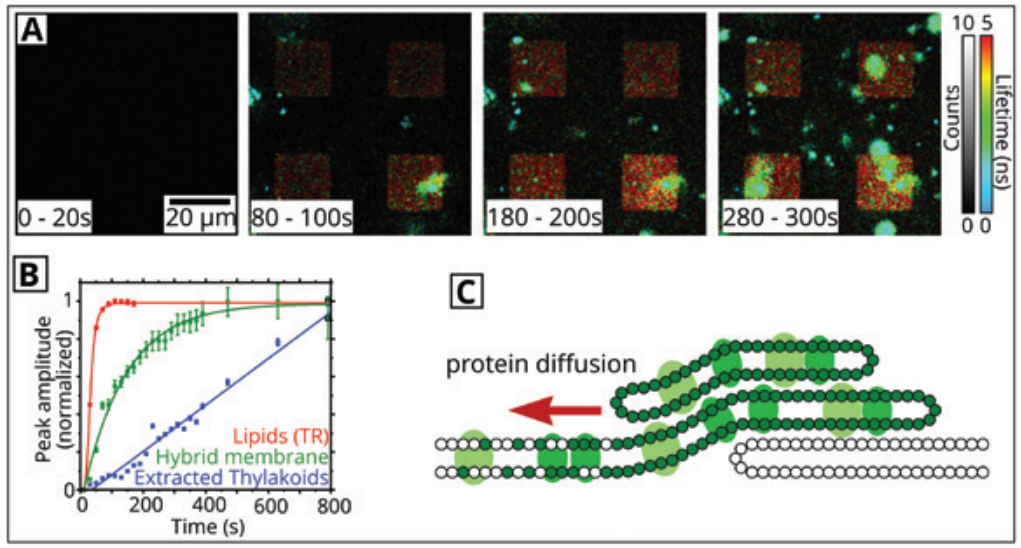

Figure 5. Analysis of the dynamic assembly processes occurring during hybrid membrane formation. (A) Time-lapse series of FLIM images during the formation of hybrid membranes. Each panel shows a 20 s acquisition during the real-time membrane assembly. (B) Analysis of the kinetics of hybrid membrane assembly as tracked by the fluorescence signal from lipids (red), photosynthetic proteins accumulating into hybrid membranes (green), and extracted thylakoids which adhere non-specifically (blue). (C) Cartoon of the self-assembly of hybrid membranes. This figure is adapted from Meredith et al. 2021, and is used and licensed under CC BY 4.0.

AFM topographs reveal that the membrane structure is mostly flat and homogeneous across widespread areas. Thus, the average measured thickness of the hybrid membrane was inferred to be 4.6 nm. The precise spatial correlation between chlorophyll fluorescence and the topography of the deposited membrane demonstrates that the LH and PS proteins are present specifically within the corral regions.

But what do we learn about the biological system? Again, the FLIM data reveals important information about the photophysical behaviour, which informs us about the energy balance of the LH proteins. In these hybrid membranes, we find that the average fluorescence lifetime of the chlorophyll signal is approx. 4.0 ns, which is similar to the value known to represent isolated LH proteins in an entirely "non-quenched" state. This long lifetime is in stark contrast to the short lifetime found for thylakoids (0.5 ns). The long average lifetime suggests that the proteins became spaced-out within hybrid membranes so that the protein-protein interactions found in the natural thylakoids are relatively rare. It is interesting that this nanotechnological platform appears to present the natural photosynthetic proteins in a diluted form compared to natural membranes but, yet, maintains their activity for further analysis.

Observing dynamic changes with FLIM: watching membrane assembly

FLIM measurements have the advantage that it is possible to acquire images in rapid succession and to acquire "video-speed" images to investigate changes to samples in real time. For our photosynthetic model, we wanted to watch hybrid membranes assembling in order to increase our understanding of the changes in photophysics of the incorporated proteins and the occurrence of micro/nanoscale topographical features. In a biophysical context, one may also wish to understand more about the inherent diffusivity of these proteins. Therefore, the kinetics of protein insertion into the hybrid membranes were studied in real time by acquiring a time-lapse series of FLIM images (**Figure 5A**) (Meredith et al., 2021).

Inside corral regions, there was a gradual accumulation of chlorophyll fluorescence characterised by a predominantly long fluorescence lifetime (**Figure 5A**, *red* colour). Whereas, across the image various globular particles with short fluorescence lifetimes became more numerous over time (**Figure 5A**, large *blue*/*green* spots), presumably representing extracted thylakoids that had not merged with the synthetic lipid bilayers. We found that lipids assemble most quickly, with a more gradual accumulation of LH proteins, and that both eventually saturate as the corral area was filled (*red* vs *green* curve, **Figure 5B**). Whereas, non-specifically adhered thylakoid membranes continued to slowly adhere over time (*blue* line, **Figure 5B**). Considering the kinetic and visual data on the assembly process overall, we hypothesised that extracted thylakoids adhere on top of the nascent lipid bilayer and act as reservoirs from which photosynthetic proteins undergo diffusion down a concentration gradient into the spreading hybrid membrane, as proposed in the cartoon in **Figure 5C**. Random Brownian motion in 2-D is expected to lead to an overall migration of membrane proteins from a high concentration in the thylakoids to a lower concentration in the hybrid membranes, tending toward a lower energy state of dynamic equilibrium. Watching this dynamic assembly process is interesting from a photosynthesis viewpoint as it bears similarities to the dynamic protein rearrangements which may occur in natural thylakoids (Johnson and Wientjes, 2020). We imagine that other researchers could use time-lapse FLIM measurements to obtain interesting information about the assembly of alternative biomembranes and the migration of other fluorescent biomolecules.

Concluding remarks

Overall, the use of FLIM allowed us to quantify the chlorophyll fluorescence decay rates towards understanding more about the energy transfer and energy dissipative properties of LH proteins, whilst AFM provided nanoscale spatial information about the membrane structures. These micro-array patterns allowed us to assess a natural mixture of LH and PS proteins the within a model membrane system and, in future, may allow us to perform measurements of low-concentration LH proteins diffusing within these corrals. In the process of this research, we are improving our understanding of the molecular basis of photosynthesis and the potential for nanotechnology that exploits light harvesting components.

For other biological systems, we anticipate that the combination of FLIM and AFM could be used correlate changes in the conformation of membrane proteins to their arrangements. FLIM could be useful for researchers who already perform FRET measurements on their own biomolecules-of-interest (i.e., a protein that is tagged with FRET donor and acceptor pigments). One could imagine various different experimental configurations, for example, FLIM-FRET assessment of (i) single proteins tethered onto glass coverslips, (ii) protein-protein or protein-lipid interactions for transmembrane proteins within surface-supported lipid bilayers, or (iii) various tagged biomolecules within whole cells. AFM can then provide complementary structural information, for example, of (i) single-protein positions, (iii) nanoscale protein arrangements within a membrane, or (iii) cellular mechanics. Micro-array patterns have their own applications, for example, in the development of biosensors (Bally et al., 2010) where pattern-recognition technology may allow more streamlined and automated computer-based analysis.

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