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Imaging mitochondrial matrix viscosity in live cells via Fluorescence Lifetime Imaging (FLIM) of fluorescent molecular rotors

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ABSTRACT

Here we use the combination of fluorescent molecular rotors (FMR) and fluorescence lifetime imaging (FLIM) to image matrix viscosity in live cells. We use non-cancerous, epithelial human cornea (HCE) cells as a model system. We find that mitochondrial matrix viscosity varies between individual cells and even between individual organelles, showcasing the potential of viscosity imaging for cell biology purposes.

Keywords: Fluorescent molecular rotors, viscosity, mitochondria, fluorescence lifetime imaging

1. INTRODUCTION

Cellular and organellar viscosity have gathered increasing attention over the past five to ten years^{1,2,3,4}. For some time, it has been known that viscosity within cells is often changed by disease or age^{5,6}. This likely greatly affects diffusion-mediated reactions in general and diffusion-limited reactions specifically. But until recent advances in fluorescence microscopy it has been difficult to visualise these changes. This is mainly due to some existing techniques being non-imaging ensemble spectroscopy, which will average out any single-cell or single-organelle information.

The most established way to visually observe microviscosity – viscosity on the microscopic level – is by using time-resolved fluorescence anisotropy imaging (TR-FAIM)^{7,4}. In TR-FAIM, the rotational correlation time, which describes the tumbling of a dye in its microenvironment, is extracted for each pixel. This is proportional to viscosity. However, TR-FAIM requires polarisation-resolved data and high photon counts as well as specialist knowledge as commercial analysis software is not available. This may limit the use of the technique for some applications.

An alternative to TR-FAIM is to use the combination of fluorescence molecular rotors and fluorescence lifetime imaging (FMR-FLIM).⁸ In FMR-FLIM, a special dye molecule with a rotating unit reports on microviscosity through its fluorescence lifetime.⁹ In a fluid medium, the rotation in the molecule is unhindered and this gives access to a non-radiative pathway, resulting in low quantum yield and short fluorescence lifetime. Conversely, in a viscous environment, the rotation is hindered and so the molecule decays radiatively through fluorescence - see Figure 1. As a result, quantum yields are high and fluorescence lifetimes long. When FMR is used in conjunction with FLIM, an image with a fluorescence lifetime in each pixel is easily acquired, creating "viscosity maps".

From a practical perspective, this is great experimental simplification as it only requires a standard FLIM setup (no polarisation-resolved emission) and can be analysed using standard, commercial or open-source software. We believe this makes FMR-FLIM a more obvious choice for the non-specialist. However, to achieve

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Figure 1. A) A simplified Jablonski diagram (adopted from Levitt et al.) showing how rotation within the FMR enables non-radiative decay. When rotation is restricted, fluorescence is more statistically likely to take place. B) A diagram of the fundamental stop-watch principle behind time-correlated single photon counting FLIM. C) The chemical structure of the FMR used in this work, termed **FMR-1**. D) Normalised absorption and emission of **FMR-1**.

organelle targeting in FMR-FLIM, new FMRs must be developed, whereas existing targeted dyes can be used for TR-FAIM. Fortunately, the interest in FMR-FLIM has been followed by an increase in newly developed FMRs targeted to different cellular domains^{10,11,12}.

We recently reported one such FMR, targeted to the mitochondria.¹³ Changes in mitochondrial viscosity has been linked to a range of diseases, including most notably Alzheimer's⁶ and complex I deficiency (leading to Leigh syndrome).³ The mitochondria is also home to several diffusion-limited enzyme systems which may be disproportionately affected by changes in viscosity. Using two different FMRs, we constructed "viscosity maps" of both the mitochondrial matrix and membranes, and we showed that this can be used to follow viscosity changes on a single-organelle basis in HeLa cells.

HeLa cells enjoy widespread use, because they are a robust immortal cell line which is easy to cultivate, even for non-specialists. However, they are also cancerous and by many not considered a good representation of "normal" cell physiology. Therefore, we are extending our line of inquiry by studying more physiologically relevant systems, specifically non-cancerous, epithelial cells from the cornea, under physiological conditions (37°C and 5% CO₂). We find that mitochondrial matrix of HCE cells under these conditions is less viscous than the mitochondrial matrix of HeLa cells at room temperature, and slightly more varied between cells.

2. MATERIALS AND METHODS

HCE cell sample preparation

HCE cells, generously gifted by Struan Bourke from King's College London and Min S Chang from Vanderbilt University,¹⁴ were cultured in a 12.5 mL petri dish (Thermo Fisher Scientific) in DMEM with 10% FBS, 1% 1X non-essential amino acid, 1 mM sodium-pyruvate and 0.1% penicillin/streptomycin at 37°C in a Hela Cell 150 incubator from Thermo Electron Corporation with 5% CO₂. A few days before imaging, a sterile and coated 8-well plate (Ibidi) was prepared. Cells were harvested using 3 mL 1X Trypsin/EDTA and 7 mL complete DMEM. On the day of imaging, cells were washed three times with fluorescence imaging medium (FluoroBrite DMEM, Thermo Fisher Scientific), before staining with **FMR-1** (well concentration 1 μ M). Cells were incubated for 30 min. They were imaged in a microscope stage-top incubator (Digital Pixel Cell Viability and Microscopy Systems) at 37°C with a 5% CO₂ gas inlet.

Fluorescent molecular rotors

The FMR used in this paper is a BODIPY-based FMR with a triphenylphosphonium (TPP⁺) moiety attached to the rotating unit, referred to as **FMR-1**. The construction of the calibration plot (based on fluorescence lifetime spectroscopy in glycerol-methanol mixtures of increasing viscosity) has been described elsewhere,¹³ as has the synthesis of **FMR-1**.

However, it will be useful to revisit the fundamental principle behind FMRs. To use FMRs as viscosity reporters, one must convert between fluorescence lifetime and viscosity through a calibration plot. For most

practical purposes, a simple empirical correlation between the two is sufficient. In general, FMR papers describe the relationship between fluorescence quantum yield and viscosity through the Förster-Hoffman equation,¹⁵ based on the Debye-Stokes-Einstein theory of diffusion^{8,11} :

$$\phi_f = z\eta^\alpha \quad (1)$$

In the original Förster-Hoffman paper, α is found to be $\frac{2}{3}$.¹⁶ In its log-log form, this gives a straight-line plot. This fit Förster and Hoffman's data on Crystal Violet for three orders of magnitude, with some deviation at higher viscosities. A lifetime version of the equation can be derived as follows:

$$\phi_f = \frac{k_r}{k_r + k_{nr}} = \tau \times k_r \quad (2)$$

$$\tau = \frac{z}{k_r} \eta^\alpha \quad (3)$$

$$\log \tau = \log \frac{z}{k_r} + \alpha \times \log \eta \quad (4)$$

The final equation yields a straight line, and a similar equation may be established for fluorescence intensity. It should be noted that the linear relationship only holds within an intermediate viscosity range which will vary depending on the FMR. This is roughly between 15 and 1000 cP for BODIPY FMRS in methanol/glycerol mixtures.⁸ Different linear regions may be fitted to account for this.

Fluorescence Lifetime Imaging Microscopy

In this paper, we use FLIM based on time-correlated single photon counting (TCSPC). In TCSPC, a pulsed laser is used to irradiate the sample, and each resultant fluorescence photon is then counted and added to a time bin histogram. By fitting this histogram, the lifetime can be extracted according to the following equation:

$$I_t = \alpha \times e^{-t/\tau} \quad (5)$$

Where τ is the fluorescence lifetime¹⁷ . The histogram may give bi-, or even tri-exponential decay based on the number of microenvironments probed by the dye. In TCSPC FLIM, histograms are collected in each pixel of the image, and during analysis, an overlay of the intensity image and a false colour map based on the lifetime value of each pixel is constructed. This is then the FLIM image where, in the case of FMR-FLIM, viscosity provides the contrast.

Results and discussion

We have extended our previous work on mitochondrial molecular rotors in human cancer cell to a non-cancerous, epithelial human cell line at physiological temperature and CO₂. The results can be seen in Figure 3. The FMR was uptaken by the cells and showed the characteristic staining of the mitochondria. The fluorescence intensity decay was fitted to a bi-exponential model, which is common for biological environments. The average lifetime was roughly 1.9 ns, in line with what we would expect based on the temperature. This shows that FMR-FLIM is also a viable option for cell biologists who are studying specific biological systems, rather than model systems.

We wanted to explore whether we could draw any conclusions about the physiological baseline of mitochondrial matrix viscosity. Regarding this second ambition, we note that as expected the viscosity of HCE mitochondrial matrix at 37° is significantly lower compared to that of HeLa cells at room temperature (just over half a nanosecond). This is likely to be due to the change of temperature rather than cell line, as temperature and viscosity is inherently linked. There may of course be a specific cell line component which would need teasing out by conducting experiments of HeLa cells at the same temperature.

Figure 2. A) Representative single decays of **FMR-1** from different solutions of increasing glycerol content and hence viscosity, clearly showing how the lifetime increases with increasing viscosity. B) The calibration plot between FMR lifetime and bulk viscosity, constructed from solutions with increasing glycerol content. C) A series of control experiments, exploring potential polarity, ionic strength and temperature effects on **FMR-1** lifetime.

Figure 3. A) The intensity and FLIM image of a group of **FMR-1**-stained HCE cells, followed by a representative decay with noise-weighted residuals and a lifetime histogram. B) A series of other FLIM images of HCE cells.

Figure 4. Going from left to right, representative intensity and FLIM images of HeLa cells, followed by HCE cells. On the far right is the average lifetime distribution of the two images on the left, showing a clear shift to lower lifetime for HCE and a slightly wider histogram.

More interestingly, there seems to be a greater level of cellular variation in the HCE patches (see Figure 4). The average lifetime standard deviation for the HCE images is generally higher. The standard deviation for the HeLa images was on average 202.6 ± 76.5 ps (averaged over five FLIM image lifetime histograms) whereas the standard deviation for the HCE images on average was 477.8 ± 142.2 ps (averaged over six FLIM image lifetime histograms).

However, looking at the average lifetime distributions is only part of the answer. Lifetime distributions are taken over the whole image, meaning that each pixel with a fitted photon histogram (i.e. excluding those which fail to reach a set photon threshold) is counted. But biology is not made of pixels. Rather, we must inspect the images themselves to make biological sense of this spread.

Judging from the HCE FLIM images, the variation of lifetimes stems both from clear differences *between* cells and *within* cells. Consider the left-most image of Figure 3B: some cells are visibly 'bluer' than others, i.e. their average lifetimes are longer and their viscosity higher. Compare this to the right-most image of Figure 3B, where several cells appear to contain both very 'red' (i.e. more fluid) and more 'green' (i.e. less fluid) regions. One might conclude that the viscosity variation may arise both on a cellular *and* on an organellar level.

Whether this is due to a specific property of the HCE cells or simply the result of more physiological conditions will need further investigation. As HCE cells are epithelial and so grow in patches, part of the variation could potentially be due to effects of the larger structure/patch, but this is not obvious from the images collected so far. To fully explain these results, a greater understanding of what drives viscosity changes in biological lumen and matrices is needed, an area of research which is active and on-going^{2,3}.

What can be concluded, however, is that this type of rich information can only be accessed with imaging. Alternative non-imaging methods, such as fluorescence anisotropy spectroscopy, fluorescence correlation spectroscopy or fluorescence recovery after photobleaching, would provide ensemble information, resulting in an averaging out of any single-cell or single-organellar information.

Conclusion

We have used a recently developed mitochondrial-targeted FMR-FLIM system to study viscosity in the mitochondrial matrix under physiologically relevant conditions. Comparing FLIM images of epithelial HCE cells imaged at physiological temperature to images of room-temperature HeLa cells, we found a lower average viscosity in the HCE cells and a general larger spread, with both between and within cell variation. This shows the clear

advantage of viscosity imaging. FMR-FLIM is an easier alternative to TR-FAIM which should find widespread use within cellular biology.

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