Super-Resolution Imaging of Mitochondria and Microtubules

Super-resolution microscopy using the TIRFM (total internal reflection fluorescence microscope) was employed to visualize and elucidate the interactions between mitochondria and microtubules inside the cell. The mitochondria were labeled with the enhanced yellow fluorescent protein (EYFP) through transfection, and the microtubules were labeled with the synthetic dye, Alexa Fluor 647, through immunostaining. The acquired dSTORM images show clear localization of mitochondria and microtubules in healthy cells and may suggest a possible interaction between the two organelles.

Introduction:

Super-resolution fluorescence microscopy is an imaging technique that can be used to visualize biological specimens in much greater detail than conventional light microscopes. In conventional microscopes, resolution, the ability to distinguish two points as separate, is limited by the diffraction of light. The wave-particle duality describes that light behaves both like a wave and a particle. Due to the wave-like characteristic of light, light diffracts slightly as it passes through the microscope objective onto the sample. In other words, rather than converging to a single desired point, light from the objective scatters around the centroid, forming a wider blurred sphere. The distribution of the intensity of light in the sphere is known as the point spread function (PSF). The diffraction and diffusion of light prevent the distinction of points that are less than a certain distance apart; this distance is called the diffraction limit. The diffraction limit of conventional light microscopes is around 200-300 nm.

Super-resolution microscopy works by using methods to counter the diffraction limit. There are two main types of super-resolution microscopy techniques: illumination-based (e.g. STED (stimulated microscopy depletion)) and single-molecule localization (e.g. PALM (photoactivated localization microscopy)/STORM (stochastic optical reconstruction microscopy)/dSTORM (direct stochastic optical reconstruction microscopy)).

STED increases image resolution by reducing the size of the PSF. In this technology, the fluorescent probes of the sample are first activated by an excitation laser pulse. A second laser pulse, a red-shifted STED ray, then
focuses on the same spot but with no intensity at the focal point. The fluorescent probes that are hit by the STED ray are immediately deactivated to their ground states. In this manner, the unfocused region around the focal point created by the diffraction of light is eliminated, leaving a sharp centroid point.

PALM, STORM, and dSTORM refer to the same general imaging technique, in which single molecules are imaged and localized, but they were named differently by different developers. Specifically, PALM is commonly associated with using fluorescent proteins as probes, while STORM and dSTORM usually use synthetic dyes. In the single-molecule localization technique, the position of a single fluorophore can be determined to great precision. Specifically, the laser light does not activate all the fluorescent probes in a sample at once but only a small subset at any one time. After the probes are activated, they are imaged and localized. Then the fluorophores in that subset are deactivated or photobleached, allowing the next random subset to be activated, imaged, and localized. By activating the fluorescent probes stochastically, this technology is able to temporally separate points that would otherwise be spatially indistinguishable due to the diffraction limit. This type of microscopy can achieve a 2D resolution that is better than 20 nm, compared to around 200 nm of conventional light microscopes.

In order to maximize the advantage of the PALM/STORM/dSTORM technology, it is beneficial to use bright fluorescent probes, ones that will detect and emit many protons while in their activated state. In the case of photoswitching fluorescent probes, whose emission can be switched on and off, it is advisable to use probes with balanced on and off durations, so that only a small subset is switched on at each time. Ideally, the probes should also have a high contrast difference in emission between the on and off states, so that autofluorescence from the off states can be distinguished from the fluorescence from the on states.

This project utilizes the dSTORM technology. dSTORM differs slightly from STORM in that a special buffer for the cell sample is needed to be prepared. The buffer contains ingredients such as glucose oxidase and catalase, so that cycles of oxidation and reduction are induced. Glucose oxidase catalyzes the oxidation of glucose, another ingredient of the buffer, to hydrogen peroxide; in this reaction, oxygen is consumed. Catalase catalyzes the reduction of hydrogen peroxide to water and oxygen; here, oxygen is returned to the buffer solution. These cycles of oxidation and reduction are crucial for the photoswitching effect. Specifically, when a fluorescent probe is switched on by laser light, the molecule is excited from the singlet state to the triplet state. The presence of oxygen represses the triplet state, thus switching off fluorescence. Subsequently, at low oxygen concentrations, some fluorescent probes are excited to the triplet state.

To illustrate the difference in resolution between conventional and super-resolution microscopy techniques, two target molecules in the cell, mitochondria and microtubules, are selected, labeled with fluorescence, and visualized under both the confocal laser
scanning microscope and the TIRFM (total internal reflection fluorescence microscope). In this project, super-resolution imaging is especially helpful in visualizing microtubules because it is able to achieve a resolution of around 50 nm, matching the diameter of a microtubule, when conventional microscopy stops at around 500 nm.

The mitochondrion produces ATP (adenosine triphosphate) and is the site of cellular respiration. As the mitochondria have to provide energy to maintain cellular function, they exist in great quantities and are interspersed throughout the cytosol. Mitochondria are known to move around in the cytosol; however, the mechanism of their movement is unclear. Microtubules are part of the cytoskeleton; they are responsible for maintaining cell structure and intracellular transport by tubulin assembly and disassembly. It was discovered using indirect experimental approaches that microtubules may be responsible for the movement of mitochondria. This project aims to achieve a better visualization of the interaction between microtubules and mitochondria using super-resolution imaging.

**Materials and Methods:**

We cultured U2OS cells, a type of human osteosarcoma cell, in high glucose medium, D-MEM (Dulbecco’s Modified Eagle Medium). Growth factors were added to the medium to promote cell division.

To visualize mitochondria:

We inserted EYFP (enhanced yellow fluorescent protein) into pre-prepared plasmids containing an ampicillin resistance gene and TOMM20 (translocase of outer mitochondrial membrane 22 homolog), a gene that encodes for a protein that localizes to the outer membrane of mitochondria. We amplified the EYFP insert sequence by PCR (polymerase chain reaction). Then we cut both the insert and vector by the restriction enzymes, EcoRI and XhoI. The sizes of the vector and insert were verified though gel electrophoresis. The expected sizes of the vector and insert were 5900 base pairs and 720 base pairs respectively.

We then ligated the vectors and inserts with DNA ligase. To amplify the completed plasmids, we transformed them into E. coli (Escherichia coli), which were spread on agar plates containing the antibiotic ampicillin. We left the cells to grow overnight at 37ºC. We also performed a negative control, in which only the vector and not the insert was transformed into the bacteria. After the overnight incubation, based on the differences in the appearance of the quantity of colonies between the negative control and sample plates, we selected some colonies at random from the sample plate to check if they contained the desired plasmid DNA, which should be inserted with EYFP. For example, if there was a major difference in colony quantity between the two plates, fewer colonies from the sample plate might need to be tested, as there would be a greater probability that at least one selected colony would contain the desired plasmid.

To amplify and isolate the plasmid DNA of the selected colonies, we transferred the E. coli from those colonies to a liquid media and incubated the samples overnight at 37ºC.
After sufficient plasmids were accumulated, we extracted the plasmids from E. coli through ethanol precipitation. Then we cut a small amount of the extracted DNA from each colony by the same restriction enzymes, EcoR I and XhoI, so that we could verify the fragment sizes by gel electrophoresis. We saw two bands, one for the vector at 5900 base pairs and another for the insert at 720 base pairs.

We introduced the plasmid DNA from the correct colony into U2OS cells through lipofection. In this procedure, we added Plus Reagent, Lipofectamine and transfection media to the plasmid DNA. Then we incubated the mixture at room temperature to allow for maximum formation of liposomes.

The Plus Reagent works to optimize efficiency of the liposome formation. The ingredients of Plus Reagent or its mechanism unknown, but perhaps the reagent contains a further aqueous solution which acts to drive the hydrophobic components into closer proximity to each other, thus increasing efficiency.

After incubation, we added the mixture to a culture of U2OS cells with a cell density of approximately 80% relative to the area of the plate. Then we incubated the sample at 37°C. During this second period of incubation, the liposomes would merge with the U2OS plasma membrane as they both have a similar hydrophobic lipid bilayer structure. Upon merging, the contents (plasmid DNA) enclosed by the liposomes would be released into the interior of the cell.

The size of the nuclear pore is small enough to allow the plasmid DNA to diffuse through. In the cell nucleus, the plasmid DNA undergoes transcription, in which a complementary mRNA strand is written from the DNA template. In the cytosol, mRNA strand is translated to protein. The TOMM20 protein localizes to the outer membrane of mitochondria, bringing along the fluorescent tag, EYFP. In this way, EYFP is able to attach to mitochondria. The remains of the liposomes are degraded by the cells.

To visualize microtubules:
After the mitochondria tag was introduced into the cells through lipofection, we fixed the cells by 4% PFA (paraformaldehyde). As antibodies are on average too large to penetrate cell membranes, we permeabilized the cells using Triton X-100. After permeabilization, we blocked the non-specific binding sites on the cover slip and waited for one hour. Then we added the primary antibody, anti-beta-antibody, which had been diluted 100 times. After adding the primary antibody, we incubated the sample at room temperature for an hour on a mechanical rocker. Afterwards, we washed the cover slip three times with blocking buffer to eliminate excess primary antibodies that did not bind. We then added the secondary antibody, anti-rabbit antibody conjugated to the synthetic dye, Alexa Fluor 647. The secondary antibody had been diluted 2000 times. The secondary antibody binds to the primary antibody, labeling the microtubule structure. We then incubated the sample for one hour at room temperature on a mechanical rocker. Afterwards, we washed the slide three times with blocking buffer. Finally, we added 1.5 mL of blocking buffer to keep the cells moisturized until imaging.
Preparation for imaging on the confocal laser scanning microscope:
We added 20 μL of mounting solution on a microscope slide. We then dropped the cover slip with the cells side facing down onto the slide. We dried the slide at 4°C for a few hours.

Preparation for imaging on the TIRF microscope:
A buffer was needed to create an O$_2$ free or at least a very low [O$_2$] environment for the cells. The buffer was prepared by adding 100 mM of BME (2-mercaptoethanol), 10 mM of TRIS (tris(hydroxymethyl)aminomethane), 10% glucose, 160 μL of glucose oxidase and 40 μL of catalase to a beaker containing 20 mL of water.

After we added the ingredients, we waited an hour for the deprivation of O$_2$ in the buffer. Afterwards, we added 25 mM of HEPES to the solution to increase the pH and slow acidification. We also added KOH (potassium hydroxide) to the solution as necessary to raise the pH to 8.

After the buffer was prepared, we sealed the beaker with plastic wrap. At the commencement of TIRF imaging, we fixed the cover slip on a microscope slide holder and added 1 mL of the buffer solution on top of the cover slip.

As the pH of the buffer was gradually decreasing and the concentration of oxygen was increasing, the time that could be spent on sample visualization was limited. About an hour after the buffer was made, the pH would have dropped to a level that was not suitable for the photoswitching effect.
Results:

**Figure 1.** Images taken on the confocal laser scanning microscope showing mitochondria and microtubules labeling in U2OS cells. The acquisition conditions were 100X objective lens magnification and 1.4 objective N.A (numerical aperture). The blue channel shows the nucleus as labeled by Hoechst 33342. Excitation wavelength was 405 nm and emission wavelength was 461 nm. The green channel shows mitochondria as labeled by EYFP. Excitation wavelength was 515 nm and emission wavelength was 527 nm. The red channel shows microtubules as labeled by Alexa Fluor 647. Excitation wavelength was 635 nm and emission wavelength was 668 nm.
**Figure 2.** dSTORM image taken on the TIRFM (total internal reflection fluorescence microscope) under 200X magnification showing the structure of microtubules as labeled by Alexa Fluor 647. Excitation wavelength was 647 nm and emission wavelength was 665 nm.

**Figure 3.** Images showing the structure of mitochondria as labeled by EYFP. The top image is a dSTORM image taken on the TIRFM under 200X magnification. Excitation wavelength was 514 nm and emission wavelength was 527 nm. The bottom image is what would have been of the same image on conventional light microscopes.
Figure 4. dSTORM dual-color image showing labeled mitochondria and microtubules in one U2OS cell taken on the TIRFM. The cell sample was first excited by a lower energy light with wavelength of 647 nm to switch on Alexa Fluor 647 fluorescent fluorophores. Then the sample was excited by a higher energy light with wavelength of 514 nm to switch on EYFP fluorescent fluorophores. The green channel shows mitochondria structure, and the red channel shows microtubules structure.

Figure 5. A more magnified image showing mitochondria and microtubules interaction.
Discussion:

Super-resolution technology allows us to visualize specimens smaller than the diffraction limit with clear resolution. While conventional fluorescence microscopes can view specimens with a best resolution of about 500 nm, super-resolution technology can reach 50 nm. In this project, super-resolution imaging is especially relevant because the diameter of a microtubule is about 50 nm. From the dual-color dSTORM images of mitochondria and microtubules, we recognize a close relationship between the two organelles. They have apparent spatial proximity to each other; this may suggest some kind of physical or chemical linkage. Mitochondria may be anchored in the cytosol by microtubules, for example.

In this experiment, we used immunostaining and transfection to label the target organelles. Both methods have advantages and disadvantages. For example, immunostaining can only be used on fixed cells and transfection can only be used on live cells. Due to the large size of antibodies (about 7 nm), antibodies are unable to diffuse through the plasma membrane. As a result, the cell must be fixed and permeabilized. On the other hand, as transfection requires the cell to undergo transcription and translation, transfection only works on live cells. Although immunostaining using antibodies requires dead cells, it is 100% effective. In other words, all the target molecules in every cell will be tagged with the antibodies. However, due to the large size of antibodies, the fluorescent labeling is not very specific. When using two layers of antibodies like in this project, the labeling is even more unspecific. Conversely, transfection is not 100% effective; only about 20% of cells will express the fluorescence. The level of expression among cells also varies. Therefore, it is beneficial when doing transfection to use samples with high cell densities. However, compared to immunostaining, transfection results in more specific labeling.

With regards to imaging techniques, it is evident from the single-color images of mitochondria that dSTORM using the TIRFM achieves much higher resolution than conventional microscopy. However, the z-axis on conventional microscopes is adjustable, while it is restricted to a 100 – 150 nm region immediately above the slide on the TIRFM. Between dSTORM/STORM/PALM and STED, the former has better performance in spatial resolution while the latter is better at temporal resolution, or live-cell imaging.

Using super-resolution technology, we have acquired clear images of the localization of mitochondria and microtubules in healthy cells. Further work may need to be done in order to understand the mechanism and biological significance of the interaction. We could perform an experiment with apoptotic cells, as the mitochondria get fragmented and move toward the nucleus during apoptosis. This experiment would allow us to detect any changes in relative position of the mitochondria-microtubule interaction before and after mitochondrial movement. In another experiment, we could add nocodazole to inhibit microtubule polymerization and see if mitochondrial movement is affected. Similarly, we could use chemicals to inhibit the assembly of other cytoskeleton elements, such as
microfilaments, to eliminate or bring in the possibility of other cytoskeleton elements being involved in the process of mitochondrial movement.

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