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Establishment of Photo-Activated Localization Microscopy (PALM) for Imaging Signaling Complexes on the Surfaces of Cells

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ABSTRACT

Photoactivated Localization Microscopy (PALM) permits visualization of single molecules inside cells at ~10 times the resolution of existing optical microscopes. The 10-50 nanometer resolution afforded by PALM is ideally suited to studying the organization of receptor signal transduction occurring on the surfaces of cells. Initial efforts presented here focused on cells expressing Fc receptor fused to photoactivatable green fluorescent protein (paGFP). A small fraction of the paGFP molecules were photoactivated using a 405 nm laser pulse, resulting in single molecule spots that could then be imaged using 488 nm laser excitation until they photobleached. Both lasers illuminated the sample through the objective lens using Total Internal Reflection (TIR) excitation which allowed for selective imaging of molecules within about 200 nm of the cell/coverglass interface. Iterations of alternating laser excitations results in thousands of images of single molecules. These images were analyzed using the ImageJ plugin, QuickPALM, which performs a least squares fit of a two dimensional Gaussian to each diffraction-limited (~250 nm wide) fluorescent spot captured in the PALM images (Henriques). This process recovers the most probable position to within ~40 nm accuracy of each paGFP molecule. This information is used to create the super resolution image of the cell. Ongoing efforts are aimed at establishing two-color PALM to determine the distribution of signaling receptors relative to key sub-resolution regulators of signaling. A better understanding of the nanoscale

molecular activities within cells will allow for more in depth studies into many different fields including biophysics, molecular biology, and medicine.

INTRODUCTION

Photoactivated localization microscopy (PALM) was developed by Eric Betzig and Harald Hess to surpass the diffraction resolution limit of an optical microscope. The Rayleigh criteria defines the diffraction limit as: $R=0.61\lambda/NA$ or ~ 200 nm (Hess). PALM is capable of resolution on the order of tens of nanometers (Betzig). PALM utilizes photoactivatable proteins in order to sequentially localize single molecules with very high precision, and, ultimately, overcomes this limit. Detection of single fluorophores is achieved using two different lasers, the activation beam and the readout beam. The activation beam causes the photoactivatable fluorophores such as paGFP or pamCherry to convert from a dark state to a fluorescent state and the readout beam is used to generate the fluorescence needed for imaging and to bleach the activated molecules. The goal is to only activate one molecule in each diffraction limited area (or the area of the point spread function) (Henriques). We used the Fc-γ Receptor (FcγRIIA) fused to paGFP and the transferrin receptor fused to pamCherry to establish the PALM method in our lab.

METHODS

In order to perform PALM, cells must first be transfected with plasmid DNA encoding a photoactivatable protein. The photoactivatable protein we first used was photoactivatable Green Fluorescent Protein (paGFP). We fused this protein to the Fc receptor and expressed it in Cos-7 cells. The second photoactivatable protein we used was pamCherry fused to transferrin receptor and expressed in Cos-7 cells. Once efficient transfection was achieved, and it was determined that the molecules were indeed photoactivatable after being fused the Fc receptor or transferrin receptor, it was necessary to fix the cells. The cells were fixed using chemical crosslinking in 4% paraformaldehyde for seven minutes. This was done in a dark room with a green light, so that the molecules would not be photo-activated prematurely. Once the sample was prepared, it was necessary to find a cell expressing the photoactivatable proteins. When searching for cells transfected with paGFP, it was discovered that the 488 nm laser activates some of the molecules. In order to image single

molecules, it was necessary to prebleach the sample with the readout laser. After the molecules that were activated were bleached away by the readout laser, we started to see single molecules being activated and subsequently bleached away. This step was not necessary for pamCherry. Once a cell had been found and any activated molecules bleached, the PALM acquisition could be made using cycles of activation with 405 nm excitation and imaging with either 490 nm excitation (paGFP) or 561nm excitation (pamCherry). An example of the single molecule signatures observed for mCherry are shown in Figure 1.

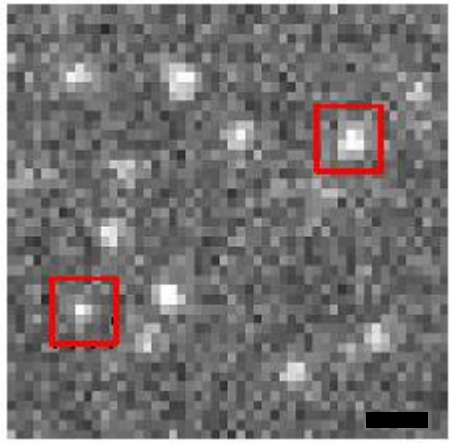

Figure 1 Raw PALM data for transferrin receptor-pamCherry. PALM was used to capture single transferrin receptors fused with pamCherry in the image. Two of these single molecules can be seen in the red boxes above. A least squares fit as a 2D Gaussian was used to localize the molecules, using the ImageJ plugin *QuickPALM*. The photoactivatable molecules were activated with a 405 nm laser and imaged with a 488 nm laser. Thousands of images must be taken so that enough of these single molecules can be localized to create a high resolution image. The high resolution allows for the study of the distribution of single molecules in a cell. The black scale bar is 1 µm.

The readout beam and activation beam were turned on alternately, in order to activate the molecules, image them, and then bleach them. In order to recover enough information to reconstruct a high resolution PALM image, around five to ten thousand images needed to be obtained. This is because the ideal image reconstruction would require complete sampling of the paGFP molecules. Furthermore, since the signal of a single molecule is

very low, it was necessary to use an Electron Multiplying Charge Couple Device (EMCCD) camera. While paGFP required full EM gain for optimal imaing, the pamCherry molecule was much brighter and did not require full EM-gain. Also the camera was set at -70° C in order to obtain the best signal-to-noise ratio and limit the dark noise. The microscope setup can be seen in **Figure 2.**

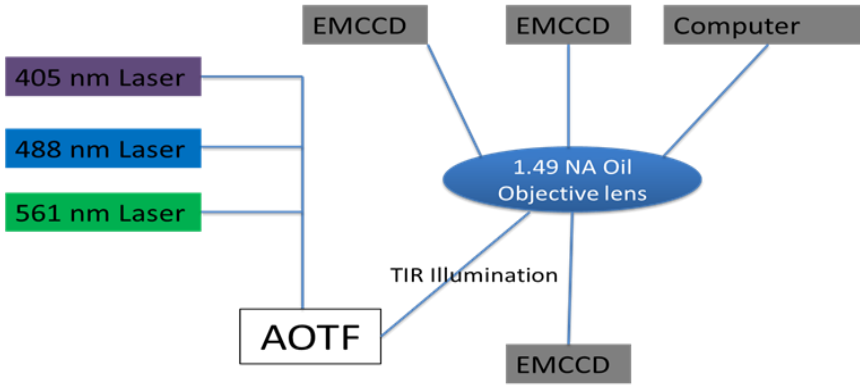

Figure 2 Microscope Setup for PALM Imaging

The lasers used for the experiment are controlled by the AOTF. It is then possible to alternate lasers using the computer control with very fine precision and timing. The power output and exposure time for the lasers can all be controlled robotically. The entire system, made by Till Photonics, is controlled via computer.

RESULTS

The first thing observed from this research was the issue of imaging single molecules. Not only was it a challenge to image something as small as molecules, it was also a problem to turn on molecules one at a time. Optimization of these methods required that we determine how long each laser needed to be on, and at what power (**Figure 3**). When the activation beam was turned on for too long or there was too much laser power, too many molecules would be activated. When the laser was turned on for too little time or without enough power, an insufficient number of molecules would be activated. The time period and power of the readout laser required optimization as well. At high laser power and long

exposure time, the molecules would bleach too rapidly, but insufficient exposure time would result in excess signal and the image would become saturated with photoactivated molecules.

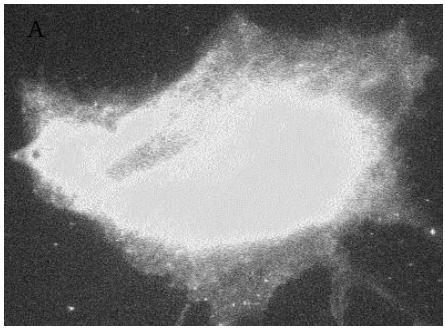

Figure 3

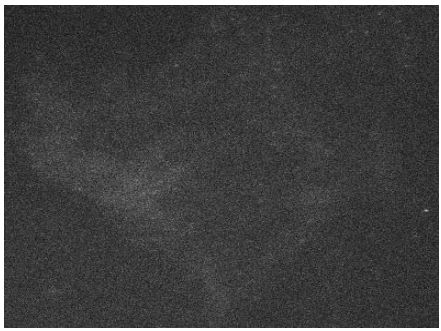

Figure 4

Laser power and exposure time must be optimized to activate and bleach the correct number of fluorophores during PALM. Determining the amount of exposure time and laser power to image single molecules proved to be a difficult task. Figure 3 shows an example of too much exposure time and laser power which would saturate the detector. Figure 4 shows not enough laser power and exposure time of the activation laser, which leads to low signal. The correct balance of laser power and exposure time of the readout laser was equally difficult to determine.

Many trials were performed in order to address the data collection issues. Eventually, the correct amount of laser power and exposure time for the different photoactivatable

molecules was determined. When the right amount of laser power and exposure time was determined, useful data started to be obtained. Single molecules were then able to be resolved. For the two different photoactivatable molecules used in the experiments, different settings for all aspects of microscope set up were required (**Figure 5 and Figure 6**).

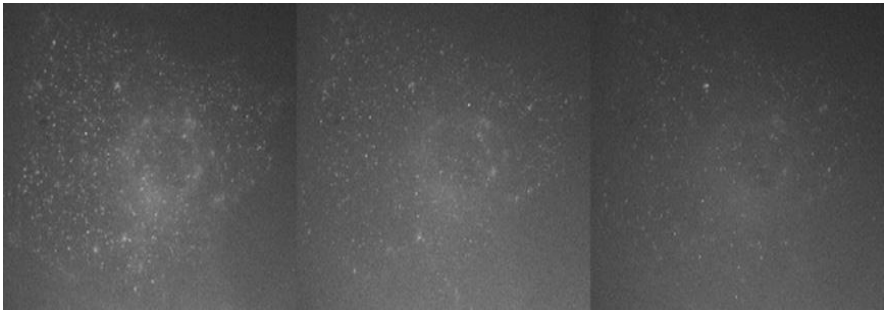

Figure 5 Raw PALM data for FcR-paGFP

Isolated single molecule emissions can be observed as single spots in these images. The activation laser was on for 20 ms while the readout laser was on for 500 ms. The three images above are 50 cycles apart. These molecules were localized and used to reconstruct a PALM image of the cell. When taking PALM data, thousands of images like the ones above are captured in order to localize as many of the photactivatable molecules as possible. A problem with the paGFP was the contrast ratio, because the noise was close to the level of the signal. The poor contrast ratio of paGFP can be seen when comparing the fluorescence of single molecules compared to dim fluorescence from the inactivated molecules in the rest of the cell.

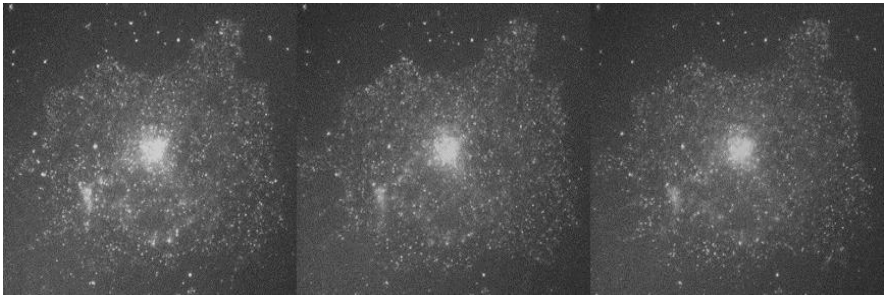

Figure 6 Raw data for transferrin-pamCherry

The three images were selected from a 5000 image stack used to create a PALM image of this Cos 7 cell. The pamCherry molecule was much brighter than the paGFP, resulting in better contrast than for paGFP. The pamCherry molecules were activated easier than the paGFP using the 405 nm activation laser.

Once single molecules were captured, the data needed to be analyzed. An ImageJ plugin, QuickPALM, was implemented (Henriques). QuickPALM determines the most probable position of each molecule using a least squares fit of a two dimensional Gaussian of each fluorescent spot captured in the PALM image. This process then recovers the most probable position of each photoactivatable molecule. This information is used to create the super resolution image of the cell (**Figure 7 and Figure 8**).

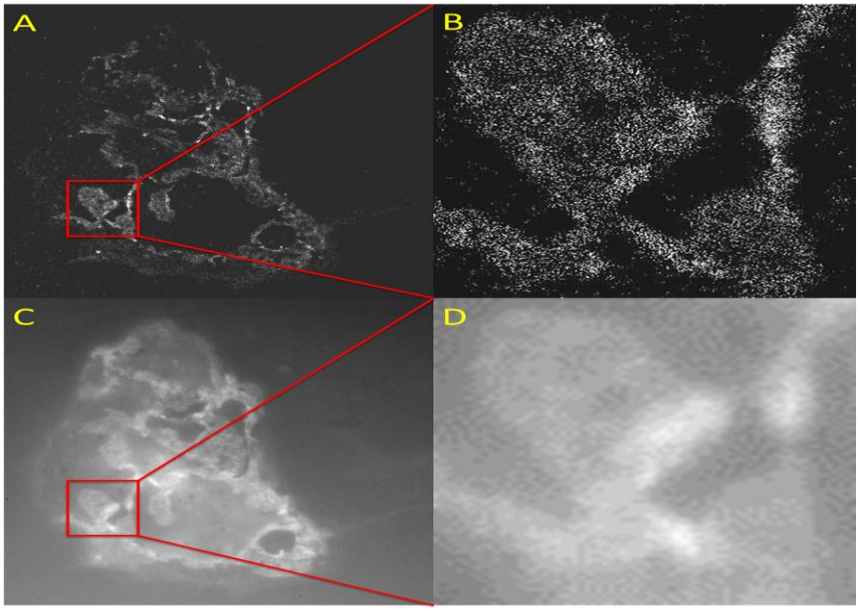

Figure 7 Reconstructed PALM images of FcR-paGFP

A and **B** are reconstructed images using the ImageJ plugin, "QuickPalm." **C** and **D** are the summed images of all of the raw data with no reconstruction, which is the TIRF equivalent image of the Cos-7 cell. The cell is expressing the FcR receptor fused to paGFP. Over 150,000 molecules were localized to acquire the reconstructed PALM image (**A**). **B** shows a zoomed in view of the PALM image compared to **D** which is the same section of the cell but the TIRF equivalent. The better resolution afforded by the PALM reconstruction is evident when comparing the magnified images.

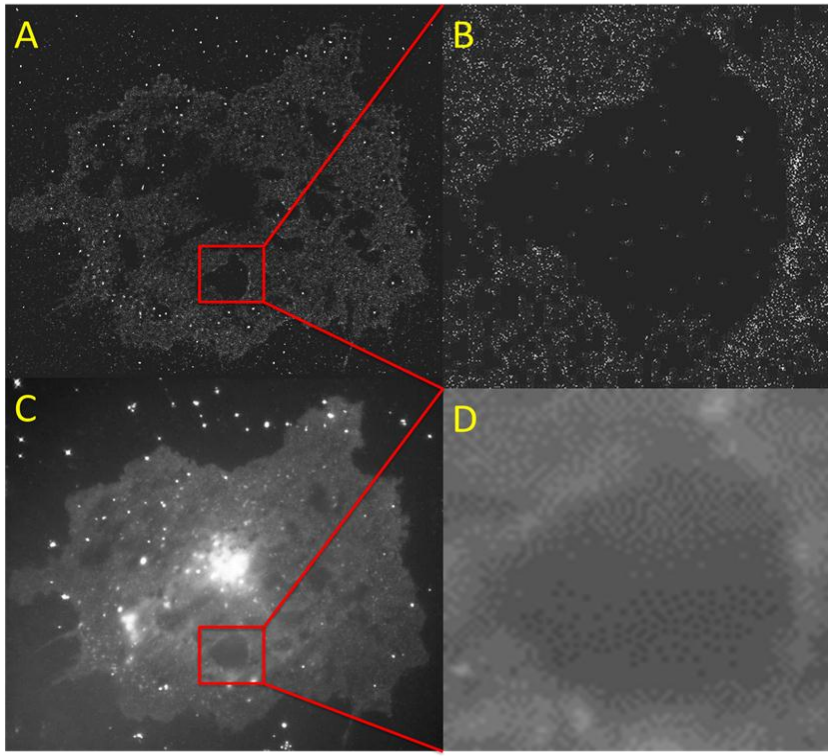

Figure 8 Reconstructed PALM Image, transferrin pamCherry

A and **B** are reconstructed PALM images of a Cos-7 cell expressing the Transferrin receptor fused to pamCherry. Over 300,000 molecules were localized to obtain these images. **C** and **D** are the summed images of the raw data, which are equivalent to a TIRF image. **B** is a magnified section of **A** that can be compared to the magnified section **D** of the TIRF equivalent image. It is apparent that the resolution of the PALM image exceeds that of the TIRF-equivalent image.

The better resolution afforded by PALM is apparent when comparing the magnified images seen in **Figure 7** and **Figure 8.** A problem associated with imaging the pamCherry molecule was the rapidity of activation associated with using the 405 nm laser, on the timescale of 5 to 10 ms. Multiple molecules in a diffraction limited area (or point spread

function) caused problems in the process of image reconstruction. The goal is to only activate one molecule per diffraction limited area in order to most efficiently localize as many molecules per image as possible. Better localization precision yields greater final resolution. Significant gains were made in developing this method for imaging structures using PALM. From trial to trial, the resolution improved. More molecules were imaged and resolved using the technique and it continues to develop into a useful method for visualization.

DISCUSSION (AND/OR CONCLUSIONS)

The groundwork has been laid for acquiring and analyzing PALM data in the lab. Some microscope settings still need adjustments but the outlook is good. We will need to investigate a well-characterized cellular structure, such as a clathrin coated pits in order to determine how much information can be obtained from sub-diffraction limit PALM imaging. Future goals include expanding this technique, utilizing the exponentially decaying evanescent field of total internal reflection fluorescence microscopy. With this characteristic excitation, we are hoping to utilize intensity measurements of single molecules to reconstruct three-dimensional super-resolution images. We are also hoping to establish two-color PALM to determine the distribution of signaling receptors relative to key subresolution regulators of signaling. It will be a challenge to find a balance in order to image both the paGFP molecules and the pamCherry molecules at the same time for two color PALM because of the differences in brightness and sensitivity for photoactivation between the two molecules.

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