Experimental protocol PIPE

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Abstract

PIPE is a fluorescence perturbation technique that works by measuring the expansion of a laser induced perturbation of photo convertible fused protein in the cytosol of cells. By measuring the expansion of the Gaussian distribution to find the mean square displacement (MSD) as a function of time , which is a measure of the spatial extent of random motion in the case of diffusion , PIPE calculates the diffusion coefficient of a cytosolic protein in question. The diffusion coefficient can then compared to a standard which could be a similar protein of the same size or even the same protein under different condition to evaluate changes in the protein structure or interactions. Here we describe how Dendra2, a green (ex: 488) to red (ex: 651) photoconvertible protein fused to a cytosolic protein can be used to measure the diffusion coefficient of different proteins.

1 Transfection

We preformed our experiment in COS-7 cells which are easily handled and transfected but other types of cells can also be used, however as the perturbation expands it reaches the cell membrane which changes the Gaussian distribution of the diffusing protein, therefore it is best to choose a cell line with medium to large cell volume.

1. Three days prior to performing the experiment determine the cells confluency. When cells are about 80-90 percent confluent, dilute the cells 1:4 in culture medium and grow them in a 4-well 35mm glass bottom plate until cells reach 50% confluency.

2. When cells reach 50% confluency, which should take about one day, prewarm PEI (Polyethylenimine 1 $\mu g/\mu l$) and the transfer vector DNA to room temperature and prepare a DNA:PEI mix in 1:2.5 ratio in 50 μl of culture medium (without serum). Incubate the mix for 30 minutes at room temperature.

3. Wash the cell with PBS. and add the DNA:PEI mix to 450 μl of culture medium and add the medium with the DNA:PEI mix to the prewashed cells.

4. Incubate the cells for 2-3 hours at a temperature suitable for cell growth.
5. Remove the DNA/PEI mix and wash twice with PBS. Add $500\mu l$ of culture medium.
6. The cells start expressing the protein fused to Dendra2 within a few hours of transfection and should be ready to image within 24-48 hours of transfection

2 Microscopy

PIPE can be performed on any commercial confocal microscope system that has a linear intensity response to fluorescent signal and can perform photo-conversion experiments. We performed our experiments on a NIKON A1r system in resonant mode which is a fast acquisition mode. However, other settings can be used as long as the guidelines below are met:

Important notes:

- Prior to beginning the experiment it is recommended that a sample with an isotropic fluorescence, for example an auto-fluorescent plastic slide, be used to check the spatial evenness of illumination. In other words illuminating a uniform sample could be used to make sure the uniformity of the imaged area. By acquiring a series of 10-100 frames and averaging these frames, data collected could be corrected for non uniform illumination.
- Before starting the experiment the photo-converting laser pulse power and pulse duration should be adjusted such that the amplitude of the photo activated region is maximized without causing damage to the cell.

1. Photo-conversion setup
   (a) Set the 405nm activation laser power to 100% (on our system we use a 50mW laser)
   (b) Set the activating laser time interval. We recommend using 100ms as a starting point and gradually increasing the time interval in case the resulting photo-converted fluorescent intensity is too low. Data analysis and accuracy depend on identifying the photo-activation pulse as it expands. Therefore a stronger pulse will be visible further away from the initial pulse position. However, longer time intervals allow for more time for the initial pulse to expand away from the photo-conversion point, therefore users should check as at least that the initial fluorescence after the pulse is clearly visible to the eye.
   (c) Set the total experiment duration for about 30 seconds and the time interval for acquisition before the photo activation session to about 4 seconds. The frames acquired prior to photo-activation could be used as a reference later on to measure background levels. Note that depending on the rate of diffusion the total acquisition time and frame

rate will need to be adjusted. For slow moving proteins, such as proteins that aggregate or have many binding partners, longer acquisition time to capture the pulse expansion with longer time intervals between individual frames to reduce bleaching. Faster smaller proteins that freely diffuse will require shorter acquisition time with a high frame rate.

(d) Select the photo-activation position to the center of the imaged area.

2. Image acquisition setup

   (a) Set the image scanning mode to bi-directional and the frame rate be 30 frames per second. Some commercial systems that do not allow such high frame rate, can decrease the ROI size in order to gain an increase in frame rate. Also depending on the protein in question, lower frame rates could be used.

   (b) Using a 60x objective pick a few cells (about 5-10 cells should be enough) and adjust the gain and laser power using the 488nm laser, make sure the green image is not saturated. These values might require adjustment during the experiment to allow cells with different protein concentration to be imaged.

   (c) Move the cells in the center of the ROI and zoom in until most of the cell edges are not in the ROI. Make sure that photo-activation pulse is aimed at the desired cellular compartment while making sure to avoid, as much as possible, large organelle that are sequestered from the cytoplasm, such as the nucleus.

   (d) Adjust the the gain to the same setting as the 488 channel the and laser power of the red channel (561nm) to be three times larger than that of the green channel. These values should be adjusted and checked while acquiring data to make sure that:

      i. The initial pulse after photo-activation is not saturated. Detectors or camera have limited capabilities to translate the intensity of an area in the imaged object to a digital signal (aka digital levels). This range known as the dynamic range sets the number of values that span the different fluorescence intensities in the imaged cell. Smaller values or larger values that do fit in this range are assigned the smaller or larger accordingly in the dynamic range resulting in a cutoff. For example, if the initial pulse intensity is too strong the result will be that the center of the Gaussian pulse, which has the higher values, will be cutoff, flattening the peak.

      ii. That the pulse is visible for a maximum number of frames after photo activation (there are no minimum or maximum requirement, rather this note should be regarded as a recommendation). This can achieved by making sure that the initial pulse values

are at the top of the dynamic range while keeping that no pixels are saturated. Another efficient way to do this is to increase the laser in the expanse of the gain which increases the span of values which could be represented by the dynamic range.

(e) Usually fluorescence perturbation techniques are very sensitive to bleaching and require that experiments be performed with low laser power. However, PIPE was shown to be less sensitive to bleaching, therefore the red channel laser power can be increased in the expense of reducing the gain. Although increasing the gain increases the image intensity is also increases noise by the same ratio, therefore it is better to increase the laser power as this improves the signal to noise ratio. **Important note**: high laser power can also be damaging to cells, therefore increasing the laser power should be done moderately.

(f) Always make sure to check the shape of the raw data in the graphical reports of the computational analysis (see the separate user manual for that part). If you see that the initial peak is saturated, execute the measurement again with decreased laser power or gain. If the signal vanishes quickly, increase the laser power or gain, or choose a brighter cell.

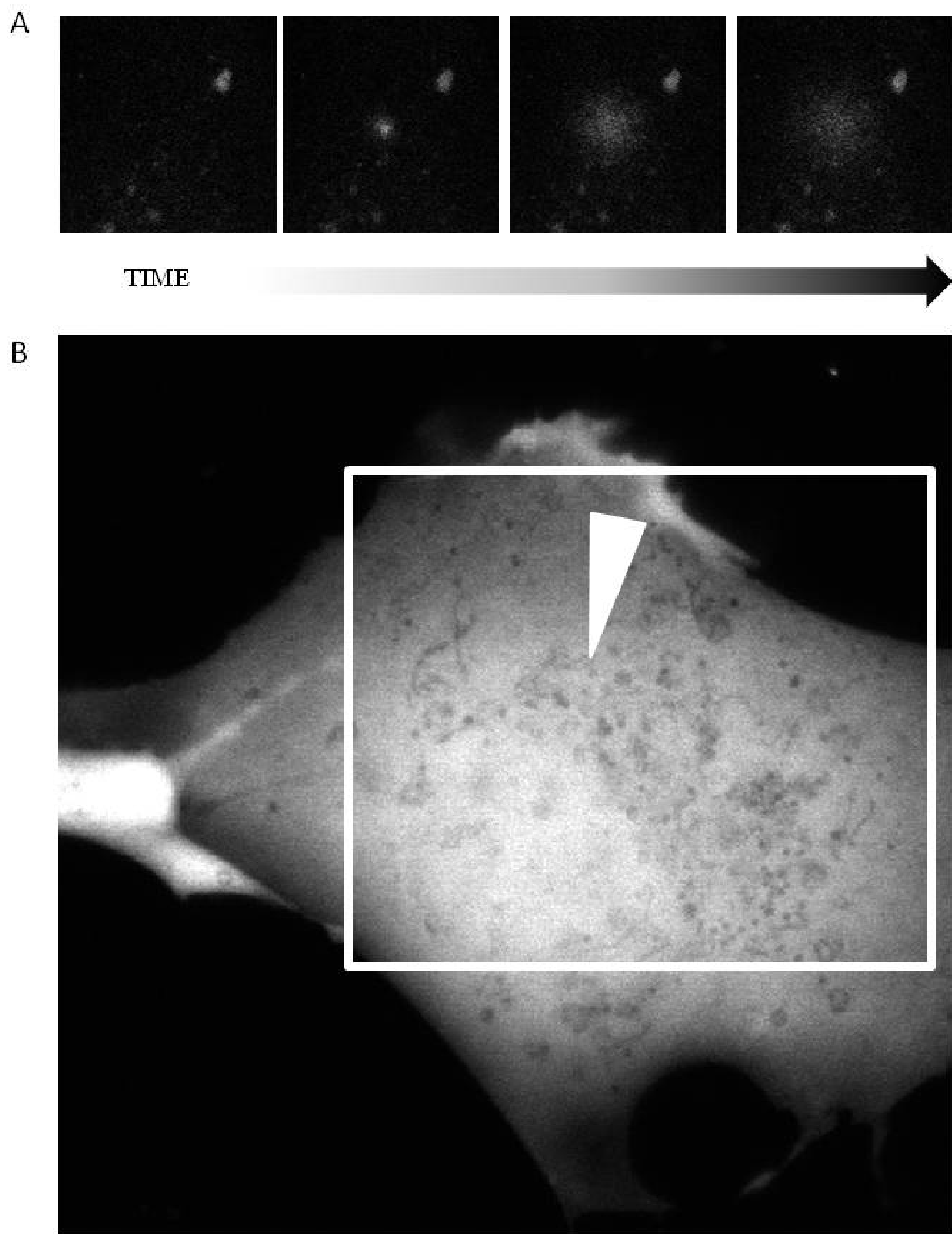

Figure 1: (A) A time series showing the expansion of a photo-converted Dendra 2 in the cytosol of a COS-7 cell following a 100ms pulse. Image size is $36\mu m$ x $36\mu m$. (B) Image of cell expressing cytosolic Dendra2 demonstrating ROI selection (white square) as well as the photo-activation point (white arrow).