2013

Tracking Protein Dynamics With Photo-Convertible Dendra2 on Spinning Disk Confocal Systems

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Introduction

Eukaryotic cells regulate protein function by controlling their access to sub-cellular compartments. Knowledge of the dynamic associations of proteins with sub-cellular compartments is therefore a key determinant in understanding their function. In this context, we present two complementary techniques intended for characterising protein trafficking pathways in living cells. Firstly, we introduce the FRAP-PA unit (Andor) combined with the Nikon Eclipse Ti E Spinning Disk Confocal Microscope (SDCM) as a new & robust platform to exploit the photo-convertible properties of the fluorescent protein Dendra2 (Evrogen). A major advantage of the SDCM is the rapid acquisition speed, enabling high-temporal resolution of cellular processes. Furthermore, photo-conversion and imaging are less invasive on the SDCM as the cell exposure to illumination power is reduced, thereby minimising photo-bleaching and increasing cell viability. Using two fast trafficking proteins (UBC9, Fibrillarin) as proof of principle, we describe step by step procedures, with emphasis on image acquisition & processing parameters, to successfully characterise Dendra2-fused proteins trafficking pathways in live cells & in real-time. Subsequently, we present novel analytical software comprised of a simple user interface which allows the user to track the fluorescence of selected point over time, and we describe in depth the steps required to process the acquired data & analyse the resultant images. The image processing stage includes red/green image identification & separation, noise filtering, background extraction, contrast stretching & temporal smoothing. Image analysis includes the construction of mean & standard deviation images, classification of cell regions & photo-conversion point approximation.

Acquisition

Spectral properties of Dendra2. [Image]

Dendra2c and Dendra2Fibrillarin vectors (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia) were kindly provided by Prof. Elena Woods, Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia.

Conway Institute, UCD, for his assistance with image acquisition and photo-conversion on the Spinning Disk Confocal Microscope (funded by Science Foundation Ireland). We thank the authors who acknowledge Dr. Konstantin Labunsky for his extremely generous gifts of the Dendra2 and Dendra2Fibrillarin vectors (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia). This work is funded by the National Virus Reference Lab (NVRL), University College Dublin.

Table 1

<table>
<thead>
<tr>
<th>Channel</th>
<th>Laser</th>
<th>Linear Intensity</th>
<th>Pixel dwell</th>
<th>Confocal Modality</th>
<th>Expansion time(s)</th>
<th>Emission filter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>488</td>
<td>488</td>
<td>2000</td>
<td>Acquire</td>
<td>0.5</td>
<td>C2821 (50:50)</td>
</tr>
<tr>
<td>Red</td>
<td>561</td>
<td>561</td>
<td>2000</td>
<td>Acquire</td>
<td>0.5</td>
<td>C2821 (50:50)</td>
</tr>
</tbody>
</table>

Data Acquisition: 34 hours post-transfection, HeLa cells expressing Dendra2 (Green) were imaged in the Green channel (Table 1 acquisition parameters) on a Nikon Eclipse Ti E Spinning Disk Microscope. Pre-conversion events are monitored by recording a preliminary image in the Red channel. Next, a sub-cellular region is targeted with the FRAP-PA unit and photo-converted using a 40Watt diode laser at 25μW laser power for 300ms/µm². In parallel, an image is recorded in the Green channel to confirm the target accuracy of the photo-conversion laser. Subsequently, Dendra2 (Red) trafficking events are monitored through acquisition of a Time series in the Red channel using an 8.87 MHz acquisition software.

Analysis

Data Analysis: The data is exported as a Multi-FL file and analysed using an algorithm developed in MATLAB. The image sequence is first processed to remove noise, enhance contrast and generally improve image quality across the sequence. Dendra2 trafficking is characterised by analysing the variation in red mean fluorescence intensity within cellular Regions of Interest (ROIs) over the time series. Selection and demarcation of the ROIs depend on the protein of interest trafficking characteristics and is a user input to the system. A mean image of the entire sequence is generated to aid the user in their selection. Three points of interest have been selected, the algorithm automatically selects out connected points in the same area using a seeded region growing technique. The mean fluorescence of these regions is then tracked through the image sequence allowing graphs to be automatically produced of mean fluorescence over time. Finally a “migration map” is generated by classifying the signal variation (5%) of individual pixels over the time series, providing an overview of Dendra2 trafficking during the time series with respect to the point of photo-conversion. The migration map is coded to represent different trafficking behaviours. Within Accumulation within a region. Dark Grey: Migration away from the region of interest. Blue: No photo-conversion grey: Migration into a region. Black: Unclassified.

Applications

1. Tracking the inter-nuclearal shuttling of nuclear proteins

![Image]

Dendra2Fibrillarin traffics between nucleus via the nucleoplasm

A. Dendra2Fibrillarin displays a nuclear distribution in HeLa cells. B. Snapshots of Dendra2Fibrillarin sub-nuclear trafficking. Dendra2Fibrillarin migration was tracked by acquiring a time series in the red channel (1 frame per 500 ms) for 120 frames. Green = Red image series merged with the Green channel. Dendra2Fibrillarin migration was confirmed by monitoring the time of conversion. Bars, 10 μm. C. Migration Map of Dendra2Fibrillarin sub-nuclear trafficking. Dendra2Fibrillarin trafficking between two Nuclei (ROI1 and ROI2), via the nucleoplasm (FC, ROI3).

2. Tracking protein nuclei-cytoplasmic & sub-nuclear trafficking

![Image]

Dendra2UBC9 traffics between nuclear sub-compartments

A. Dendra2UBC9 is distributed between the nucleus and cytoplasm in HeLa cells. B. Snapshots of Dendra2UBC9 sub-nuclear trafficking. Dendra2UBC9 migration was tracked by acquiring a time series in the red channel (1 frame per 40 μs) for 120 frames. Green = Red image series merged with the Green channel. Dendra2UBC9 migration was confirmed by monitoring the time of conversion. Bars, 10 μm. C. Migration Map of Dendra2UBC9 sub-nuclear trafficking. Dendra2UBC9 trafficking to PML bodies (ROI1 and ROI2) and cytoplasm (ROI3).

3. Delineating protein associations with sub-nuclear bodies

![Image]

Dendra2UBC9 is dynamically associated with PML Nuclear Bodies.

A. Dendra2UBC9 accumulates in sub-nuclear PML Nuclear bodies in HeLa cells. B. Snapshots of Dendra2UBC9 migration between PML bodies. Dendra2UBC9 migration was tracked by acquiring a time series in the red channel (1 frame per 40 μs) for 120 frames. Green = Red image series merged with the Green channel. Dendra2UBC9 accumulation was confirmed by monitoring the time of conversion. Bars, 10 μm. C. Migration Map of Dendra2UBC9 trafficking between PML bodies. D. Analysis of Dendra2UBC9 shedding between PML bodies (FC, ROI2) via the nucleoplasm (FC, ROI1) and cytoplasmin (ROI4).

Acknowledgements:

The authors wish to acknowledge the access to the use of the UCD Conway Imaging Core Technologies, Conway Institute for Biomolecular and Biomedical Research, University College Dublin. In particular, we wish to acknowledge Prof. Dimitri Scholtz director of Biological Imaging, Conway Institute, UCD, for his assistance with image acquisition and photo-conversion on the Spinning Disk Confocal Microscope (funded by Science Foundation Ireland). We thank the authors who acknowledge Dr. Konstantin Labunsky for his extremely generous gifts of the Dendra2 and Dendra2Fibrillarin vectors (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia). This work is funded by the National Virus Reference Lab (NVRL), University College Dublin.