Methods 115 (2017) 42–54

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Quantifying receptor trafficking and colocalization with confocal microscopy

Jeremy A. Pike [a,b,c,*], Iain B. Styles [d], Joshua Z. Rappoport [b,e], John K. Heath [b]

[a] PSIBS Doctoral Training Centre, School of Chemistry, University of Birmingham, Birmingham B15 2TT, UK
[b] School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK
[c] Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge CB2 0RE, UK
[d] School of Computer Science, University of Birmingham, Birmingham B15 2TT, UK
[e] Center for Advanced Microscopy and the Nikon Imaging Center, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form 20 January 2017
Accepted 23 January 2017
Available online 26 January 2017

Keywords:
Colocalization
Trafficking
Endocytosis
Confocal
Receptor
EGFR

ABSTRACT

Confocal microscopy is a powerful tool for the study of cellular receptor trafficking and endocytosis. Unbiased and robust image analysis workflows are required for the identification, and study, of aberrant trafficking. After a brief review of related strategies, identifying both good and bad practice, custom workflows for the analysis of live cell 3D time-lapse data are presented. Strategies for data pre-processing, including denoising and background subtraction are considered. We use a 3D level set protocol to accurately segment cells using only the signal from fluorescently labelled receptor. A protocol for the quantification of changes to subcellular receptor distribution over time is then presented. As an example, ligand stimulated trafficking of epidermal growth factor receptor (EGFR) is shown to be significantly reduced in both AG1478 and Dynasore treated cells. Protocols for the quantitative analysis of colocalization between receptor and endosomes are also introduced, including strategies for signal isolation and statistical testing. By calculating the Manders and Pearson coefficients, both co-occurrence and correlation can be assessed. A statistically significant decrease in the level of ligand induced co-occurrence between EGFR and rab5 positive endosomes is demonstrated for both the AG1478 and Dynasore treated cells relative to a control. Finally, a strategy for the visualisation of co-occurrence is presented, which provides an unbiased alternative to colour overlays.

© 2017 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

The signalling and trafficking of cellular receptors are highly interlinked processes [1–3]. Ligand induced signalling regulates endocytosis and receptor trafficking within the endocytic network, which in turn attenuates receptor signalling. Moreover, the hypothesis of signalling endosomes, for which there is now extensive evidence, implies that the subcellular location of activated receptor triggers distinct signalling responses [3–8]. Homeostatic receptor trafficking is essential for organism development [9,10], and aberrant activity is implicated in numerous diseases [11,12].

Fluorescence microscopy is commonly used to study ligand induced changes to the quantity of receptor located at the plasma membrane [13], and also colocalization with subcellular structures, such as endosomes [14]. Developing a proper understanding of these experiments requires quantitative, unbiased, and reproducible analysis protocols. In this paper, with these requirements in mind, we describe fully automated image analysis workflows for analysing live cell 3D time-lapse data. Confocal microscopy is used exclusively, but all protocols are equally applicable to deconvolved widefield images [15]. A HeLa cell line expressing fluorescent protein tagged constructs for both epidermal growth factor receptor (EGFR) and rab5 is used as a model system [16]. rab5 is an early endosome associated GTPase and key regulator of receptor trafficking [17]. Inhibitors for EGFR kinase (AG1478) [18] and dynamin (Dynasore) [19] are used to perturb the ligand (EGF) induced trafficking response. We demonstrate the effectiveness of the described workflows, and show that both drug treatments perturb EGFR trafficking and colocalization with rab5 positive endosomes.

The rest of the paper is structured as follows. In Section 2 a brief review of related approaches, identifying both good and bad practice, is presented. Section 3 describes, and shows the use of, the proposed protocols. Finally, Sections 4 and 5 provide a discussion and conclusion.

* Corresponding author at: Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge CB2 0RE, UK.

E-mail address: jeremy.pike@cruk.cam.ac.uk (J.A. Pike).

http://dx.doi.org/10.1016/j.ymeth.2017.01.005
1046-2023/© 2017 The Author(s). Published by Elsevier Inc.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

2. Related approaches

2.1. Workflows to quantify the subcellular distribution of receptor

Confocal microscopy data can be acquired in either two, or three, spatial dimensions. In a 3D approach multiple axial slices are acquired at different focal planes through the sample. A 3D approach is inherently superior to 2D acquisition as the entire cellular volume can be sampled [5]. In a 2D approach only a single plane through the cell is acquired, hence key information can be missed [13]. If the axial position is not set automatically, for example at a set distance from the coverslip, or at the widest nuclear plane, then user bias is introduced to the acquisition [20]. Note that for live samples light exposure should be kept low. Therefore for time-lapse imaging there is a practical trade-off between the number of axial slices, and the frame-rate.

Post-acquisition, data can be processed to isolate biologically relevant regions of interest (ROIs) such as the plasma membrane. Subsequently, the fluorescence intensity of a specific marker can be quantified within each ROI. Selection of ROIs can either be manual or automated. Manual selection should be avoided as it is prone to user bias and error, and time-consuming and difficult to implement in 3D. With time-lapse data the change in (normalised) intensity, within each ROI, over time can be calculated. For example Fortian and Sorkin (2014) acquired 3D time-lapse data with spinning disk confocal microscopy, and used an automated 3D edge based segmentation protocol to identify the cellular ROI [5]. The segmentation was eroded by a set number of pixels to identify ROIs for the intra-cellular region and plasma membrane. This was used to calculate the normalised percentage, of both EGF and Grb2, associated with the plasma membrane over time. This is an excellent example of an automated 3D strategy for the quantification of temporal changes to the subcellular distribution of a fluorescent construct. However, the pre-processing and segmentation protocols are not fully defined, only the software package and associated components are cited. As the specific image processing algorithms are not referenced, reproduction of this methodology has not been possible in an alternative software application.

ROI intensities, and colocalization measures, can be calculated using either the raw or pre-processed data. Raw data refers to the unprocessed data as acquired by the microscope. There is extensive literature on both image denoising and deconvolution [15,21,22]. These techniques respectively aim to remove corruption and out of focus contributions within image data. Although these approaches can be inaccessible for biological researchers, due to either lack of knowledge or user-friendly tools, working with raw data cannot be considered best practice. However, the use of unjustified or poorly specified methods is worse as results cannot be reproduced. Following the initial pre-processing steps, data can be further processed to enhance, or isolate, biologically meaningful components. Note there is no generalised workflow for image pre-processing and care should be taken to match the approaches used to both the data and the biological context. For example, Dunn et al. (2011) suggest that background subtraction, as calculated with a median filter, is appropriate for the quantification of signal within endosomes [14]. When the width of the filter is at least twice as large as the endosomal structures a reliable estimation of local background is produced.

2.2. Colocalization analysis

Colocalization analysis is typically used to determine if labelled proteins colocalize, or cluster, to the same subcellular structures. High quality analysis relies on high quality data, and particular care must be taken to avoid detector saturation and cross-talk between channels [23]. The spatial sensitivity of colocalization analysis is limited by the resolution limit of the microscope, which is determined by the point spread function (PSF) [24]. According to the Nyquist criterion the pixel size, and the axial spacing, should be less than approximately half this limit to accurately represent the sample at this resolution [25]. However, sampling at this optimal rate may be practically infeasible for live experiments, or large scale screens. When using larger pixels, or axial spacing, artefacts can be introduced and it is the pixel size, not the resolution of the microscope, which limits the spatial sensitivity of the colocalization analysis. For example, consider the imaging of endosomes using a pixel size of 0.25 μm, and axial spacing of 0.5 μm. When using standard imaging wavelengths, and an objective with a numerical aperture of 1.4, this is larger than the size defined by the Nyquist criterion. Two endosomes, can only be distinguished if they are separated by more than approximately 0.5 μm laterally, or 1 μm axially. Therefore colocalization analysis, even using super-resolution techniques, is poorly suited to the identification of direct protein-protein interaction [26]. Techniques such as Förster Resonance Energy Transfer (FRET) are more appropriate for this purpose [27]. Conversely, when there is no direct interaction between the proteins but association within subcellular structures, such as endosomes, FRET cannot be used.

In studies of receptor trafficking, quantitative analysis is often neglected, and colour merges are used to provide qualitative evidence for colocalization [6,28–30]. This can leave interpretation and presentation of results open to user bias, either through the image display settings, or the choice of *representative* images. Visualisation of correlation is better performed using joint-histograms, not colour merges [14]. There are two distinct strategies for colocalization analysis. The first is based on the overlap, or correlation, between pixels [14,23,25]. The second detects objects within the data and uses the centre of mass for each object to determine clustering statistics such as Ripley's K-function [25,26,31]. Object based methods have shown promising results for localization and TIRF microscopy, where the data is well modelled by point, or spot like, objects [26]. However, for the application of receptor trafficking using confocal microscopy, the receptor is typically localised to either the plasma membrane or endosomal structures, the former of which is not well represented by a point distribution. Therefore we will focus on pixel based measures which can be split into two categories; co-occurrence and correlation [32]. Co-occurrence measures quantify how often, or how much, signal from each channel overlaps with the other channel based only on the presence, or absence, of signal. For example, 50% of channel 1 signal overlaps with channel 2 signal. Correlation measures assess the extent of a relationship between the signals from each fluorophore. For example, if there is high positive linear correlation a pixel with high intensity in channel 1 would typically also have high intensity in channel 2. For high negative linear correlation a pixel with high intensity in channel 1 would typically have low intensity in channel 2.

The Manders Coefficients (MCs) (M1 and M2) are well-established co-occurrence measures which simply calculate the percentage of total signal from one channel which overlaps with signal from the other, such that [33],

$$M1 = \sum_i \frac{C1_{i,coloc}}{C1_i} \qquad M2 = \sum_i \frac{C2_{i,coloc}}{C2_i} \tag{2.2.1}$$

where $C1_i$ and $C2_i$ represent the intensities of individual pixels for channels 1 and 2 respectively. $C1_{i,coloc}$ and $C2_{i,coloc}$ represent the colocalizing pixels such that $C1_{i,coloc} = C1_i$ when $C2_i > 0$ and $C1_{i,coloc} = 0$ otherwise. Similarly $C2_{i,coloc} = C2_i$ when $C1_i > 0$ and $C2_{i,coloc} = 0$ otherwise. The Pearson coefficient (PC), R is a well-established measure of linear correlation, defined such that [34],

Download English Version:

https://daneshyari.com/en/article/5513513

Download Persian Version:

https://daneshyari.com/article/5513513

Daneshyari.com