Methods 66 (2014) 139–152

Contents lists available at ScienceDirect

Methods

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

Automated selection of regions of interest for intensity-based FRET analysis of transferrin endocytic trafficking in normal vs. cancer cells

Ronak Talati [a], Andrew Vanderpoel [b], Amina Eladdadi [b], Kate Anderson [a], Ken Abe [a], Margarida Barroso [a,*]

[a] Center for Cardiovascular Sciences, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA
[b] Department of Mathematics, The College of Saint Rose, 432 Western Avenue, Albany, NY 12203, USA

ARTICLE INFO

Article history:
Available online 28 August 2013

Keywords:
Förster resonance energy transfer (FRET)
Transferrin (Tfn)
Transferrin receptor (TFR)
Image processing
Region of interest (ROI)
Strictly standardized mean differences (SSMD)

ABSTRACT

The overexpression of certain membrane-bound receptors is a hallmark of cancer progression and it has been suggested to affect the organization, activation, recycling and down-regulation of receptor–ligand complexes in human cancer cells. Thus, comparing receptor trafficking pathways in normal vs. cancer cells requires the ability to image cells expressing dramatically different receptor expression levels. Here, we have presented a significant technical advance to the analysis and processing of images collected using intensity based Förster resonance energy transfer (FRET) confocal microscopy. An automated Image J macro was developed to select region of interests (ROI) based on intensity and statistical-based thresholds within cellular images with reduced FRET signal. Furthermore, SSMD (strictly standardized mean differences), a statistical signal-to-noise ratio (SNR) evaluation parameter, was used to validate the quality of FRET analysis, in particular of ROI database selection. The Image J ROI selection macro together with SSMD as an evaluation parameter of SNR levels, were used to investigate the endocytic recycling of Tfn–TFR complexes at nanometer range resolution in human normal vs. breast cancer cells expressing significantly different levels of endogenous TFR. Here, the FRET-based assay demonstrates that Tfn–TFR complexes in normal epithelial vs. breast cancer cells show a significantly different *E*% behavior during their endocytic recycling pathway. Since *E*% is a relative measure of distance, we propose that these changes in *E*% levels represent conformational changes in Tfn–TFR complexes during endocytic pathway. Thus, our results indicate that Tfn–TFR complexes undergo different conformational changes in normal vs. cancer cells, indicating that the organization of Tfn–TFR complexes at the nanometer range is significantly altered during the endocytic recycling pathway in cancer cells. In summary, improvements in the automated selection of FRET ROI datasets allowed us to detect significant changes in *E*% with potential biological significance in human normal vs. cancer cells.

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1. Introduction

The endocytic trafficking of membrane bound receptors has been shown to be defective in a wide range of human cancers [1–3]. Altered expression and/or activity levels of proteins involved in the regulation of membrane trafficking can affect the endocytosis, degradation and/or recycling of membrane bound receptors, leading to increased cell proliferation and motility as well as metastatic behavior [4,5]. Key endocytic proteins such as Rabaptin5, a Rab5 GAP involved in regulating endosome fusion [6] and Numb, a clathrin coat adaptor, have been found to be disrupted or significantly reduced in human cancers [7,8]. Moreover, the overexpression of certain membrane-bound receptors, which is a common occurrence during cancer progression [9–11], has been suggested to affect the organization, activation, recycling and down-regulation of receptor–ligand complexes in human cancer cells [2]. Receptor overexpression may provide an alternative manner to disrupt endocytic trafficking by saturating the limited availability of endocytic proteins involved in vesicle formation, sorting and fusion [1,12]. Therefore, it is crucial that we develop a better understanding of how the endocytic trafficking pathways of receptor–ligand complexes are regulated in normal cells and how these processes are disrupted in cancer [2]. However,

Abbreviations: Acc, acceptor; A:D, internalized acceptor:donor ratios; $A{:}D_{calc}$, calculated A:D ratios; AF488, Alexa Fluor 488; AF555, Alexa Fluor 555; AF488-Tfn, Alexa Fluor 488-Transferrin; AF555-Tfn, Alexa Fluor 555-Transferrin; D, donor; *E*%, energy transfer efficiency; ED, edge detection; FRET, Förster resonance energy transfer; HME, human mammary epithelial cells; ID, integrated density; qD, quenched donor; MDCK-PTR, Madin-Darby canine kidney epithelial cells stably expressing human TFR; %*F*, area fraction; PBS, phosphate buffered saline solution; PFRET, precision FRET; uFRET, unquenched FRET; ROI, region of interest; SBT, spectral bleed-through; SNR, signal to noise ratio; SSMD, strictly standardized mean differences; STD, standard deviation; Tfn, transferrin; TFR, transferrin receptor.

* Corresponding author.
E-mail address: barrosm@mail.amc.edu (M. Barroso).

1046-2023/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymeth.2013.08.017

comparing receptor trafficking pathways in normal vs. cancer cells requires the ability to image cells expressing dramatically different receptor expression levels, which can raise significantly technical issues when using standard fluorescence based imaging approaches.

Therefore, the development of novel quantitative imaging techniques is crucial to increase our understanding of the regulation of the intracellular endocytic trafficking of membrane receptors in both normal and cancer cells. Recently, Förster resonance energy transfer (FRET)-based imaging approaches have been developed to assay receptor dimerization/oligomerization as well as receptor–ligand interactions during endocytic trafficking pathways [13–16]. In particular, a quantitative FRET assay has been used to follow the endocytic/recycling trafficking of transferrin receptor (TFR) and its respective ligand, iron-bound transferrin (Tfn), at nanometer range resolution [17–22]. TFR is a well-known membrane-bound receptor which is responsible for cellular iron uptake upon the binding of iron-loaded Tfn [23]. Increased iron uptake is necessary to sustain the increased cell proliferation typical of cancer cells. Thus, elevated levels of TFR expression have been found in various malignancies (e.g., 74% breast carcinomas, 76% lung adenocarcinomas, and 93% lung squamous cell carcinomas) [24–31]. Thus, Tfn has been used as a target for molecular imaging techniques in breast tumors [32–35] and it has been widely used as carrier for anti-cancer drug/siRNA delivery systems [31,36,37]. We have capitalized on the homodimeric nature of the TFR [23,31] and employed FRET to quantitatively analyze the endocytic trafficking of Tfn–TFR complexes. As part of this assay, Tfn molecules conjugated with FRET donor or acceptor fluorophores are brought to nanometer-range proximity upon binding to the homodimeric TFR at the plasma membrane [17–22,38]. These Tfn–TFR complexes are internalized via clathrin-coated vesicles into endosomes, where the iron is released from Tfn upon endosomal acidification via ATPase proton pumps [23,39]. Then, the Tfn–TFR complexes are recycled back to the plasma membrane, where the apo-Tfn is released into the extracellular space [23,39]. FRET imaging was performed to test whether the TFR–Tfn receptor–ligand complexes undergo conformational changes throughout the endocytic recycling pathway as they are exposed to different endosomal luminal environments. Those conformational changes may lead to slight changes in the distance between Tfn molecules bound to TFR, which would result in variations in FRET levels since FRET is a highly sensitive proximity assay. Thus, changes in FRET levels may be due to the ability of TFR–Tfn complexes to undergo conformational changes that are mediated by alterations in the endocytic environment.

Intensity-based FRET using confocal microscopy is the most widely used imaging technique to perform nanometer scale measurements in live cells [15,17]. Although, the use of filter-based intensity FRET together with organic dye FRET pairs, such as Alexa Fluor 488 (AF488; donor) and 555 (acceptor; AF555) can be applied to most optical microscopy systems, confocal microscopy still provides the best approach to measure FRET signals in time-lapse and/or pulse-chase experiments using live-cell intensity-based FRET imaging. Confocal images are processed to remove background noise as well as spectral-bleedthrough (SBT) by the custom generated processed FRET (PFRET) algorithm Image J plugin software, as developed by Dr. Periasamy at the Keck Center for Cellular Imaging, University of Virginia [19,21,40–45] [17,19–21,40,42–46]. The PFRET algorithm removes SBT from uncorrected FRET images, taking into consideration intracellular variability of fluorophore expression levels. The PFRET algorithm allows for the calculation of apparent energy transfer efficiency (E%) as a percentage of the unquenched donor (D), providing an expression for the actual energy transferred from donor to acceptor molecules [19–21,41–44]. However, apparent E% measurements do not allow for the separation between donors that participate in FRET events (FRET donors) and those that do not (non-FRET donors). To calculate precise energy transfer efficiencies (E) and distances between donor and acceptor fluorophores, one must determine an imaging factor (λ) that contains information about the donor and acceptor quantum efficiencies and the device detection efficiency [15,40,41,47,48]. Recently, the use of a standard donor–acceptor tandem construct with known energy transfer efficiency as measured by fluorescence lifetime has been used to determine the value of imaging factor and to normalize the relative FRET efficiencies [15,48]. However, generating such a construct is technically challenging, when using a system that involves the cellular uptake of fluorescently labeled ligand molecules. Furthermore, most biological experiments are by nature relative, since they require the comparison of a control vs. a test experiment in which cell samples undergo changes under specific conditions, such as time, temperature, pH, pharmacological treatments or genetic-based approaches. Given that the γ factor does not affect the relative comparison of E% measurements used in filter-based intensity FRET analysis, for simplicity, in our FRET analysis, we used $\gamma = 1$, as described previously [17,19–22,43–45]. However, filter-based intensity FRET assays carried out with different imaging parameters will by definition have distinct γ factors. Importantly, previously, the TFR–Tfn model system has been characterized using both intensity- and lifetime-based imaging analysis in MDCK-PTR epithelial cells stably expressing TFR [17–22,49]. Similar E% values (15–20%) were detected demonstrating the strength of our intensity based FRET analysis [49].

There are two main issues that prevent the application of filter-based intensity FRET to an even wider variety of biological questions. The first issue is the application of filter-based intensity FRET to low signal FRET images such as those generated by the binding of AF488-Tfn and AF555-Tfn to TFR endogenously expressed in HME cells. The second is the ability to develop automated FRET image analysis that can reliably and consistently evaluate numerous images generated by time-lapse live cell imaging or high content high-throughput imaging. Previously, semi-automated quantitative FRET processing algorithms, such as the PFRET Image J plugin, FRET stoichiometry calculator [48], the FRET and colocalization analyzer [50], the PixFRET [51] and the RiFRET custom-based software [52], have been developed to implement SBT correction and to calculate precise E values. To analyze a large number of low FRET signal images processed by the PFRET algorithm, we developed a novel algorithm that implements standardized background correction procedures as well as the automated selection of defined x,y coordinates (non-grid) and non-overlapping ROIs based on different intensity and statistical based thresholding parameters [53]. Moreover, we have established a statistical signal-to-noise ratio (SNR) parameter, i.e., the strictly standardized mean difference (SSMD) [54–56], to validate changes to selected FRET ROI datasets as they affect the reliability and consistency of average E% values. Here, we have compared TFR–Tfn endocytic FRET in three well-known epithelial cell lines: human mammary epithelial (HME) cells, human breast cancer T47D cells and Madin-Darby canine kidney epithelial MDCK-PTR cells, a well-known epithelial cell model system [57]. These cell lines express TFR at various levels; whereas HME cells endogenously express low levels of TFR, T47D cells show significantly higher endogenous TFR expression levels [58] and MDCK-PTR cells, stably express human TFR at intermediate levels [59]. Our results suggest that the organization of Tfn–TFR complexes at nanometer range resolution is significantly altered during the endocytic recycling pathway in cancer cells. These results are particularly important since Tfn–TFR system is widely used to target anti-cancer drugs to tumors in vivo, making it crucial to analyze in detail the Tfn–TFR endocytic system in normal vs. cancer cells.

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