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Challenges in imaging cell surface receptor clusters

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ABSTRACT

Super-resolution microscopy offers unique tools for visualizing and resolving cellular structures at the molecular level. STED microscopy is a purely optical method where neither complex sample preparation nor mathematical post-processing is required. Here we present the use of STED microscopy for imaging receptor cluster composition. We use two-color STED to further determine the distribution of two different receptor subunits of the family of receptor serine/threonine kinases in the presence or absence of their ligands. The implications of receptor clustering on the downstream signaling are discussed, and future challenges are also presented.

1. Introduction

In solid tissues, like bone and connective tissue, the interaction of a cell with its microenvironment, namely the extracellular matrix (ECM), is mediated by cell surface receptors. Adherent cells use their surface receptors for sensing and responding to physical and chemical cues presented in the ECM, which in turn regulate many different processes, like adhesion and migration, as well as proliferation and differentiation [1]. At the ventral side, multi-molecular assemblies, recruited upon clustering of cell surface receptors, build up discrete adhesion structures at the interface with a surface, i.e. a 2D substrate [2]. For cells embedded in a 3D ECM meshwork, these types of structures are rather diffuse and mostly located in protruding parts or throughout the whole cell body [3]. These adhesion structures, called focal adhesions (FAs), are highly dynamic and act as signaling hubs thereby transducing information between the ECM and the cell cytoskeleton. Mature FAs are elongated structures (ca. 3–5 μm in length and 0.5–1 μm wide) consisting, as for today, of over 100 different proteins [4], which, in response to different stimuli, associate and dissociate within the complex.

Integrins are among the most important cell surface receptors involved in FAs assembly. These heterodimeric transmembrane receptors directly interact with domains presented in various ECM proteins, such as fibronectin, vitronectin and collagen. The adhesive interactions between cells and the ECM are not just mediated by integrins: a plethora of transmembrane proteins, such as growth factor receptors, have been identified as co-receptors, being not just distributed homogeneously in the plasma membrane, but rather confined to discrete regions, namely FAs. Vinculin is a focal adhesion protein which links integrins to the actin cytoskeleton and its recruitment at adhesion sites is dependent of force generation [2]. The bone morphogenetic protein (BMP) receptors (BMPRs) are transmembrane proteins and belong to the family of serine/threonine kinases that comprises BMPR type I and II. The confinement in lateral mobility and accumulation of signaling receptors in microdomains at the cell membrane is crucial not only for binding to the ligand, but also for activation of signaling cascades [5,6]. Additionally, the different modes of receptor oligomerization dictate the downstream signaling pathways and the resulting transcriptional responses [7,8].

The BMPRs recognize BMPs, which are important members of the TGF-β family and exert pleiotropic action on several tissues, regulating normal and transformed cell growth. While an emerging role of BMP-mediated signaling in regulating cell cytoskeleton dynamics has been reported [9,10], the type of interaction still remains unclear, in terms of receptor localization, oligomerization and clustering, between different types of BMPRs and integrins at FAs. Using standard confocal microscopy and protein complex

Abbreviations: BMP, bone/body morphogenetic protein; BMPR, bone/body morphogenetic protein receptor; ECM, extracellular matrix; FA, focal adhesion; STED, stimulated emission depletion

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immunoprecipitation, Lai et al. [11] proposed that integrins regulate BMP-2 activity by close association of αv and β1 integrins with BMPRs, which results in the activation of differentiation signaling. However, analyzing the BMPR subunit distribution and the possible interactions with other proteins is a challenging task to achieve with conventional microscopy approaches, being labeling methods and imaging processing the limiting factors.

Light microscopy techniques are advantageous for the investigation of protein localization, especially when two or more different proteins, and their possible interaction, are of particular interest. The use of fluorophores conjugated with antibodies directed against the protein of interest enhances the contrast between the structure of interest and the background in comparison to conventional bright field imaging where the contrast is caused by absorbance of the transmitted light by the specimen. Additionally, the combination of different fluorophores with sufficiently separated emission spectra easily allows for multicolor images. Protein–protein interactions can be investigated either biochemically, with methods such as immunoprecipitation, or optically, for example by specific labeling of the proteins of interest. Nowadays laser scanning confocal microscopy is a widely used technique for imaging cells. With a lateral resolution of about 250 nm and an axial resolution of about 500 nm larger protein assemblies such as FAs can be easily imaged. However, to resolve the spatial organization of molecules within these structures the resolution is not sufficient, resulting in a blurred image. Resolving the fine structure of these complexes requires super-resolution techniques such as electron microscopy, STimulated Emission Depletion (STED) [12] or localization microscopy techniques such as Photoactivated Localization Microscopy (PALM) [13], STochastic Optical Reconstruction Microscopy (STORM) [14] or Ground State Depletion microscopy followed by Individual Molecule return (GSDIM) [15]. All these techniques besides electron microscopy rely on the principle of switching, where super-resolution is gained by a state transition of the fluorophore [16,17]. Whereas STED is a purely optical method without the need of mathematical post-processing, PALM, dSTORM or GSDIM images are obtained by numerical image processing.

The sample preparation for both optical and electron microscopy is crucial; however, unlike the rather complex sample preparation for electron microscopy methods, sample preparation for super-resolution light microscopy techniques do not require particular equipment and treatment. The samples in fact, can be prepared as for conventional fluorescence microscopy.

A current challenge in imaging is still represented by discriminating different receptors clustered in discrete regions of the cell. Here we present images of cell membrane receptors where we show the valuable contribution of super-resolution microscopy in determining receptor cluster composition.

Two-color STED imaging has been done with two general approaches: either the super-resolved images are created by two STED point spread functions (PSFs), requiring two STED lasers and two well-aligned optical beam paths [18], or the two-channel super-resolved image is created with as single STED PSF using two appropriate dyes with different emission colors and sufficient STED efficiency at the used STED wavelength, thus eliminating the donut alignment issue [19]. Both approaches have their advantages: using two different STED lasers and PSF gives the chance to select suitable dyes with optimal STED efficiency and guaranties best resolution, while at the same time the lateral alignment of two STED PSF might compromise the co-localization results. The alignment can be checked with addition of e.g. beads labeled with both dyes in the same sample, however the complexity of sample preparation is increased. The approach with a single STED PSFs eliminates the need for optical alignment, but the achievable resolution for both colors can be different because the STED efficiency can be differing for the dye/STED wavelength combination, especially when the selected dyes are far apart in there emission spectrum for best possible cross-talk reduction. The resolution reduction can be very well compensated by applying the gated detection technology [20].

The data shown in this paper are recorded with two different newly available STED wavelengths in red and far-red regime, allowing the use of commonly available dyes either from green (500–540 nm) to orange (580–640 nm) emission or orange (580–640 nm) to red (660–740 nm) emission range. Two-color experiments designed with single STED wavelength (either 660 nm for green/orange dye combination; or 775 nm for orange/red dye combination) and phase mask combination guaranty precise co-localization results and low cross talk between channels. We applied gated detection technology to compensate for resolution reduction for the second color.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from Promocell were cultured in endothelial cell basal medium supplemented with a mix of growth factors (all from Promocell), at 37 °C and 5% CO_2. According to the supplier's recommendation cells were plated at a density of 5000–10,000/cm^2. Cells were seeded on fibronectin-coated (5 μg/cm^2 human cellular fibronectin (Sigma)) glass coverslips (diameter of 10 mm; Menzel) 24 h prior to immunofluorescence staining.

2.2. Indirect immunofluorescence

Cells were fixed with 4% (w/v) paraformaldehyde (PFA, Riedel-de Haen) for 20 min followed by permeabilization with 0.1% (v/v) Triton-X-100 in PBS at RT for 5 min. Then the samples were blocked with 1% (w/v) bovine serum albumin (BSA, Carl Roth) in PBS at RT for 10 min. All primary antibodies were used at a final concentration of 1–10 μg/ml diluted in 1% BSA in PBS and incubated at RT for 1 h. The following primary antibodies were used: anti-BMPR II rabbit IgG (Cell Signaling), anti-BMPR IB mouse IgG (R&D Systems), anti-BMPR I rabbit IgG (detects BMPR IA and BMPR IB, Santa Cruz) and anti-vinculin mouse IgG (Sigma-Aldrich, St. Louis, USA). Following several washing steps with 1% BSA in PBS, the respective secondary antibodies (confocal: Alexa532 goat anti-mouse IgG, Alexa488 goat anti-rabbit IgG; 1-color STED: Alexa488 goat anti-mouse IgG; two-color STED: TMR sheep anti-mouse IgG, OregonGreen514 goat anti-rabbit IgG) were used at a final concentration of 10–20 μg/ml diluted in 1% BSA in PBS and likewise incubated at RT for 1 h. After several washing steps with PBS to remove unbound secondary antibodies, the samples were mounted in Mowiol. Before imaging the samples were left overnight at RT to allow polymerization of the mounting medium.

2.3. Confocal and STED imaging

Two-color confocal images were acquired with a Leica TCS SP8 X equipped with a white light laser source (Leica Microsystems CMS, Mannheim, Germany). With a scanning speed of 400 Hz and line average 4, the two channels were acquired sequentially using HyDs (Hybrid GaAsP detectors).

Single- and two-color STED imaging was performed on Leica TCS SP8 STED 3X equipped with a white light laser and gated STED technology (Leica Microsystems CMS, Mannheim, Germany). Two-color STED images were acquired sequentially (line by line) at a