White Paper

Correlative Protein Localization in Yeast

High-Resolution Localization of Fluorescent Proteins Using Shuttle & Find for Superresolution and Scanning Electron Microscopy.

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Correlative Protein Localization in Yeast

High-Resolution Localization of Fluorescent Proteins Using Shuttle & Find for Superresolution and Scanning Electron Microscopy.

Authors: Dr. Jeffrey Caplan, Dr. Carissa Young,
Jean Ross, Samuel Jacobs, Shannon Modla
University of Delaware Newark, DE USA

Dr. Kirk Czymmek
Carl Zeiss Microscopy, LLC.

Dr. Alexandra F. Elli
Carl Zeiss Microscopy GmbH

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Specific recognition signals that govern quality control within the secretory pathway provide a basic framework for determining trafficking mechanisms of recombinantly expressed G-Protein coupled receptors (GPCRs). Our long term goal is to improve and verify functional production of GPCRs, and to achieve this we used rational engineered chimeras to assess protein structure/function.

Introducing

To this end, we have developed high-resolution microscopy approaches for the yeast model organism, *Saccharomyces cerevisiae* (Young et al. 2012, Wei et al., 2012), to better understand processes that regulate the heterologous expression of a family of human adenosine receptors such as hA1aR, hA2aR and hA3aR. By implementing DNA recombination strategies combined with complementary high-resolution imaging techniques, we optimized a correlative microscopy method using both super-resolution microscopy (ZEISS ELYRA.PS1) and field emission scanning electron microscopy (ZEISS AURIGA 60) to determine the intracellular localization of the heterologous GPCR, hA1aR. In order to significantly enhance the efficiency of this correlative work, we took advantage of the benefits of Shuttle & Find to allow easy and rapid relocation of multiple sites of interest between the two microscopes. Specifically, we investigated discrete subpopulations of the tagged protein hA1aR-Cerulean using the super-resolution techniques, dSTORM (Alexa Fluor® 647) and SIM (Calcofluor White) followed by field emission scanning electron microscopy. We describe an optimized protocol using cryo-fixation of high-pressure frozen freeze-substituted yeast cells followed by Lowicryl® resin infiltration regimen. One important criterion of this work was to obtain fluorescence signal for super-resolution imaging steps as well as maintain a high quality morphological preservation for electron microscopy when using rapid relocation with Shuttle & Find. With this approach, we were able to facilitate the identification of sub-cellular organelles and associated compartments where the targeted GPCR fusion protein was localized. In general, we expect variations on this procedure will be broadly applicable for correlative SEM combined with fluorescence immunolocalization and/or super-resolution microscopy on resin sections.

Sample Preparation

Yeast were prepared by high pressure freezing and freeze substitution based on the protocol of Giddings (2003). Yeast pastes were high pressure frozen and freeze substituted with 0.1% uranyl acetate and 0.2% glutaraldehyde in 100% acetone for 95 h at -80°C. Samples were warmed to -20°C over a period of 20 h, washed 3 x 15 min with 100% acetone at -20°C, and then infiltrated with MonoStep Lowicryl® HM-20 embedding resin at -20°C. Briefly, samples were infiltrated in 30% and 50% resin in 100% acetone for 4 h each and then overnight in 70% resin.
Yeast samples were infiltrated twice in 100% resin for 2 h each followed by an overnight incubation in 100% resin. The samples were exchanged with fresh 100% resin for 2 h at -20°C, embedded in gelatin capsules, and then UV polymerized for 24 h at -45°C. Samples were sectioned on a Reichert-Jung Ultracut E ultramicrotome, and ultrathin sections (80 nm) were dried onto 22 x 22 mm correlative ITO coated coverslips (Indium Tin Oxide) with chrome fiducial markers.

Immunofluorescence labeling

The sections on ITO coverslips were treated for 30 min with a freshly prepared 0.1% solution of sodium borohydride in 1X PBS to quench autofluorescence, rinsed and further treated with 0.05 M glycine in 1X PBS for 15 min to inactivate residual aldehyde groups. The coverslips were transferred to a humidity chamber for all further steps. Protein blocking with a solution of 5% BSA and 5% normal goat serum in 1X PBS was carried out for 1 h. The primary antibody, Abcam rabbit anti-GFP polyclonal (ab6556), was diluted 1:120 in blocking solution and the coverslips were incubated for 1 h at room temperature (RT).
The coverslips were thoroughly washed in several changes of the blocking solution and then incubated with a 1:300 dilution of Alexa Fluor® 647 goat anti-rabbit antibody in blocking solution for 1 hour at room temperature in the dark. After washing with several changes of 1X PBS, the coverslips were stored overnight at 4°C in a 1:100 dilution of stock Calcofluor White to label the cell walls in 1X PBS. The coverslips were rinsed 3 times with 200 mM Tris buffer, incubated in a 1:5 dilution of 100 nm gold beads (used for acquisition autofocus image alignment) for ten min and washed three times with 200 mM Tris buffer prior to imaging.

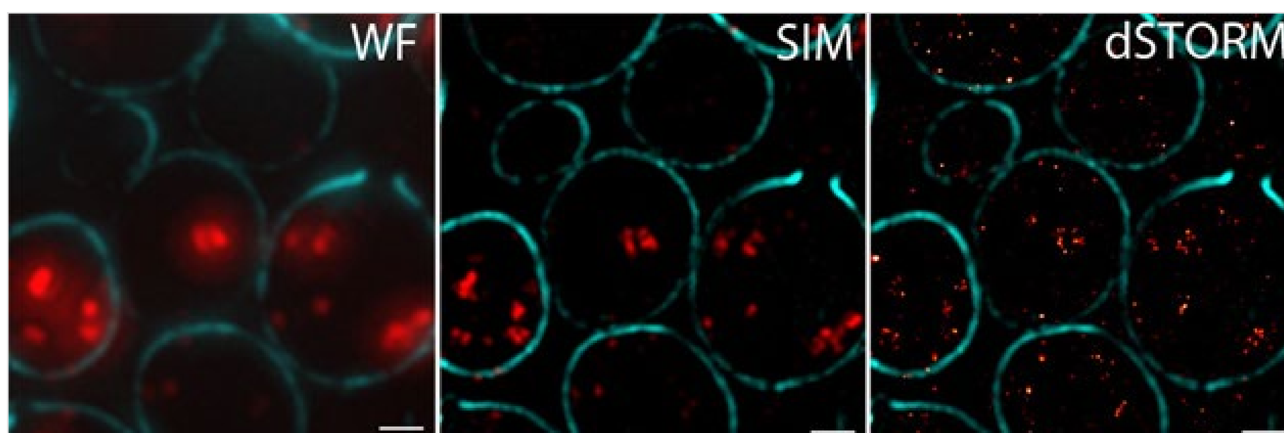

Figure 1. *A comparison of widefield and super-resolution imaging modes with ultrathin sections of baker's yeast Saccharomyces cerevisiae. Calcofluor White labeled yeast cell walls (blue) and Alexa Fluor® 647 labeled GFP- hA1aR (red) with widefield microscopy (WF), Structured Illumination Microscopy (SIM) and direct Stochastic Optical Reconstruction Microscopy (dSTORM) showed resolution differences between approaches. Scale bar = 1µm.*

Imaging

Fiducial coverslips were first calibrated on ZEISS ELYRA PS.1 and all subsequent images were acquired using an alpha Plan-Apochromat 100x/1.46 oil objective. Initially, regions of interest with cells were identified and acquired in laser widefield mode to store image coordinates at all selected locations. A 5 slice z-stack SIM dataset was acquired and processed using an auto noise filter, baseline cut processing with an experimental point spread function. Following SIM acquisition, the sample was prepared for dSTORM imaging using a buffer containing 43.8 µM glucose oxidase (Sigma-Aldrich) and 20 mM cysteamine (Sigma-Aldrich) and applied to the holder with sections. dSTORM of Alexa Fluor® 647 was acquired with the 642 nm laser in TIRF mode (approximately 30,000 frames) and autofocused and aligned using gold fiducials (Figure 1 dSTORM). After super-resolution imaging, the residual oil on the coverslip was carefully removed with a cotton tip swab and lens cleaning solution before further processing. Subsequently, the sections on the fiducial coverslips were post stained with saturated uranyl acetate in methanol and Reynolds lead citrate. Following the calibration of the fiducial coverslips on ZEISS AURIGA 60 Crossbeam Workstation, the same position was recovered using Shuttle & Find on the widefield images and BSE images using the Energy Selective Backscattered (EsB) detector were acquired (Figure 2).

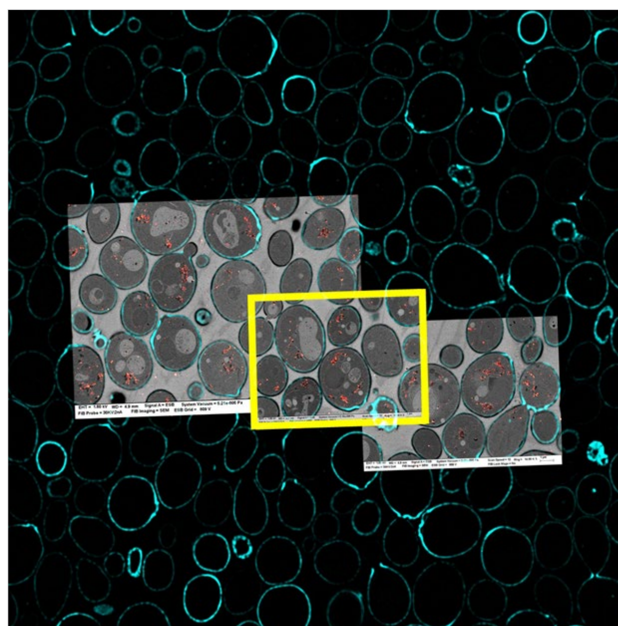

Figure 2. *Overview correlative super-resolution microscopy of yeast ultrathin sections. Cell wall (blue, SIM) with Alexa Fluor® 647 labeled hA1aR-Cerulean (red, dSTORM) overlaid with three nearby FE-SEM BSE images.*
Locations imaged by super-resolution microscopy were automatically recovered in the SEM using Shuttle & Find. Yellow Box denotes single FE-SEM image shown in more detail in ***Figure 3.***

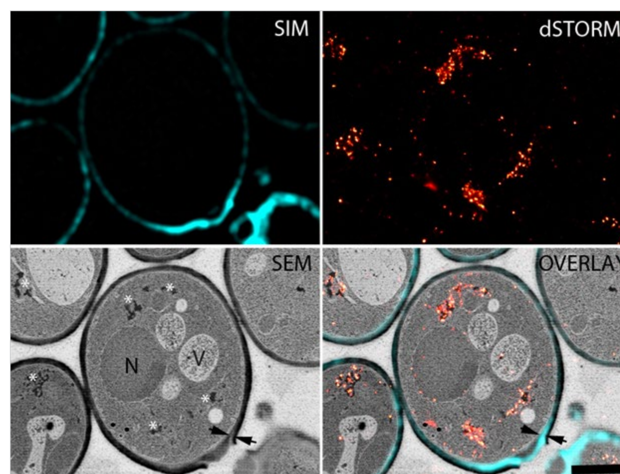

Figure 3. *High Magnification Correlative Super-Resolution/FE-SEM Microscopy of yeast cell walls (SIM), dSTORM Alexa Fluor® 647 (dSTORM) targeted Cerulean-hA1aR and overlaid (OVERLAY) with FE-SEM BSE image (SEM) after automated recovery using Shuttle & Find. Electron dense cisternal compartments (Golgi equivalents) (white asterisks) in SEM image were directly identified as the primary structure correlated to localization using dSTORM super-resolution microscopy. Also note that Calcofluor White labeled a thin electron translucent layer on the yeast inner cell wall (between black arrows-SEM & OVERLAY). Same region as yellow box in Figure 2. Nucleus (N), Vacuole (V). Scale Bar = 1 µm.*

Results

Overlaid super-resolution and SEM images revealed that following ER stress, the hA1aR localized to electron dense cisternal compartments (Golgi equivalents) and low levels associated with the ER and plasma membrane of yeast cells. Localization patterns are consistent with immunogold localization patterns (data not shown). While freeze-substitution fixation is known to result in low contrast for some membranes, highly convoluted three-dimensional nature of the cisternal surface, and resolution limits of the approach make definitive conclusions of localization to this membrane complex problematic. However, low level plasma membrane localization would be consistent with GPCR function and the cisternae pattern observed. Use of correlative SIM to localize Calcofluor White served as a useful internal alignment aid and also demonstrated that the resolution gains with this approach allowed localization of a thin electron translucent layer within the inner yeast cell wall and at bud scars, presumably representing chitin.

Conclusion and Outlook

In conclusion, our final protocol showed the possibility to obtain high-quality correlative SEM in combination with super-resolution microscopy with Shuttle & Find. We chose a sequential approach: super-resolution microscopy -> heavy metal staining -> Shuttle & Find FE-SEM. In the future, we believe a similar approach will be fully compatible with most conventional immunofluorescence protocols on thin sections as well as expanding our work to multi-color dSTORM with Alexa Fluor® 488 labeling (i.e., specific subcellular targets) to confirm the localization of hA1aR. With Shuttle & Find, it is currently possible to use either ITO-coverslips with or without fiducial markers, each providing the same rapid relocation of the selected sample position. We confirmed here that coverslips with fiducial markers allowed highly flexible strategies to remove the coverslip from the holder for additional processing (i.e., heavy metal stains) and enabled the relocation of the sample position with very high precision. A similar approach could be combined with other labeling/contrasting protocols and readily adapted to existing sample specific methods.

Acknowledgements

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Carl Zeiss Microscopy GmbH
07745 Jena, Germany
BioSciences
microscopy@zeiss.com
www.zeiss.com/microscopy

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