activated fluorophores be relatively sparse in the field of view. Yet, both isolated and moderately crowded fluorophores provide valuable information about the image that is only partially degraded by overlap. Analyzing the complete fluorescence data set could accelerate super-resolution image estimation for dynamic biological samples. Whereas recent work has improved methods for labeling and imaging biological structures, less has been done to optimize the localization computation at the heart of these techniques to extract as much image information as possible from the data. We propose a novel computational analysis procedure for fluorescence imaging data based on iterative image deconvolution. Our algorithm exploits the sparse structure of data arising from dilute fluorophores, but does not require that molecules' images are non-overlapping. We use the expected statistical properties of fluorescence imaging data, including the image sparseness and temporal correlations induced by intermittent activation of emitters, to constrain a deconvolution procedure that iteratively improves the estimated super-resolution image. We validated our approach and compared it with single- and multi-emitter fitting procedures using simulated data. We also applied our algorithm to stochastic optical reconstruction microscopy (STORM) data from immunohistochemically labeled microtubules. Our algorithm achieves accurate super-resolution image reconstruction even in moderately crowded conditions in which analysis by localization fails. This improved analysis allows a ~5-fold speedup in imaging by permitting the use of a higher density of active emitters.

3671-Pos Board B532
Multiphoton and STED Imaging Nanoscopy
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The coupling of multi photon excitation microscopy with optical nanoscopy allows envisaging developments towards bioimaging of thick samples at nanoscale resolution. Fluorescence depletion applied to multi photon excitation allows overcoming one of the main drawbacks related to multi photon imaging, i.e. poorer resolution with respect to confocal microscopy. In order to have a potentially better control of distortions when imaging thick highly scattering specimens we focused on using the very same wavelength for excitation and depletion. To this end, we developed a new class of 2-photon excitation - stimulated emission depletion microscope (2PE- STED) using the very same wavelength for excitation and depletion. We show the opportunity to perform super-resolved fluorescence imaging, exciting and stimulating the emission of a fluorophore by exploiting the very same laser source properly split. The broadening of the cross section in the 2PE regime allows the excitation of several dyes in the visible window without changing wavelength. We show that a red-emitting fluorophore widely used for STED applications, ATTO647n, could be 2-photon excited at a wavelength that sits on its emission spectrum. This fact opens the possibility to perform super-resolved imaging at a resolution 5-6 times better than conventional 2PE microscopy.

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GraspJ - An Open Source, Real-Time Analysis Package for Super-Resolution Imaging
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In the past decade, a number of techniques have been developed that overcome the diffraction limit in fluorescence microscopy. Among these techniques, the ones that are based on single molecule photoactivation and localization, such as STORM, PALM and GSDIM, heavily depend on image analysis for achieving high resolution. With most current analysis software, the post-processing takes orders of magnitude longer than data acquisition. In particular, recent application of these techniques in live-cells came with tremendous advancements in temporal resolution, further increasing the gap between acquisition and analysis speed.
To fill this gap we developed an open source, full featured, real-time analysis package (GraspJ - **G**PU-**R**un **A**nalysis of **S**TORM and **P**ALM data for Image**J**) that keeps up with the highest possible acquisition speeds of modern EMCCDs. By taking advantage of the parallel processing power of the Graphics Processing Unit (GPU), we were able to achieve more than 1,000,000 molecule fits/second, using a maximum likelihood estimator. Therefore, with GraspJ, analysis can be performed in real-time in parallel to data acquisition, using almost any acquisition software. During real-time analysis, complex post processing (e.g. intrinsic drift correction, trailing of molecule peaks over several frames, high resolution rendering and dynamic zooming) can be easily done without reducing the analysis time significantly. For example, processing times of less than 1ms per frame were achieved using typical imaging conditions (256x256 pixels, ~50-100 molecules per frame). In addition, GraspJ can be run as an ImageJ plugin, leveraging thousands of features and plugins readily available for ImageJ and therefore providing a convenient and familiar environment.
Overall, we believe that the ability to see the drift corrected, high quality super-resolution image building up during acquisition using GraspJ will be of great benefit to the experimenter.

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Investigating the Impact of Photo-Blinking on Photo Activated Localization Microscopy: From Single Molecules to Cell Membrane Receptors
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Fluorescent proteins are known to display in certain cases an on-off blinking and switching behavior. We explore the consequences of this behavior in super-resolution fluorescence microscopy techniques based on the sequential photo-switching and bleaching of individual fluorophores, such as Photo Activated Localization Microscopy (PALM).
We show that in a typical in-vitro experiment a significant fraction of isolated single molecule time traces display multiple reactivations after a relatively long lived dark state. Starting from the complex photophysical features of one of the most recent and promising photoconvertible fluorescent proteins for PALM studies, mEos2, we have systematically investigated the effect of molecular photo-blinking and fluorescence dark times on a typical PALM experiment.
The multiple counting of even a small number of molecules may clearly have an impact in the imaging of biological systems such as proteins expressed on the plasma membrane, where phenomena such as oligomerization and clustering can be properly identified only if the same molecule is not counted multiple times. Comparing simulations and experiments we have identified a parameter, the dark time td, depending on which it is possible to move from an overcounting to an undercounting condition. We therefore outline different regimes to conduct a PALM measurement and propose an approach to more accurately quantify the number of fluorophores activated in the sample.
We compare a negative and a positive clustering control by using plasma membrane-bound proteins to determine how photo-blinking or reactivation induced artifacts may be erroneously interpreted as biological clusters. Screening for concomitant spatial and temporal clusters proves an effective tool to identify potential artifacts originated by photo-blinking in super-resolution images of small G protein-coupled receptor aggregates.

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Probes for Two-Photon-Activation FPALM
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Biplane fluorescence photoactivation localization microscopy (FPALM) [1], a localization based super-resolution technique, uses photo-activatable fluorescent proteins (PAFPs) to image samples at resolutions beyond the diffraction limit. These PAFPs undergo a controlled change in their emission spectrum upon absorption of a photon typically of the UV range. By repeatedly activating and localizing PAFPs in the sample a three-dimensional map of molecule positions is generated that has a far better resolution than typical light microscopy techniques can achieve.
For improved performance, especially when imaging thicker samples, the 405 nm activation light can be replaced by a focused mode-locked laser beam of 700-1000 nm wavelength. This type of setup then uses two-photon-activation to confine the activation axially to the focal plane [2-5], increasing the fraction of usable labels in the sample because molecules out of focus will not be activated and bleached. For the same reason background will be reduced which opens up the way to imaging thicker samples that exhibit too high background levels in conventional FPALM.
In order to choose the best probe, it is important to know how each one performs using two-photon-activation. Here we present a method that allows determining the two-photon-activation cross section of any photo-activatable fluorescent probe. We show results for a number of commonly used established and new PAFPs as well as images recorded using two photon activation.
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