Live-cell monochromatic dual-label sub-diffraction microscopy by mt-pcSOFI

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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We expand photochromic super-resolution optical fluctuation imaging (pcSOFI) to monochromatic dual-channel sub-diffraction microscopy. Multi-tau (mt-)pcSOFI unmixes spectrally identical reversibly switchable fluorescent proteins (RSFPs) based on their blinking kinetics. We show that mt-pcSOFI can be used to simultaneously image two structures in living cells with existing RSFPs and the newly developed ffDronpa-F.

Questions in biological and biomedical research generally revolve around the spatiotemporal presence, activity and interactions of specific molecules, and are often studied with fluorescence microscopy because of its high specificity, selectivity and sensitivity. The inherently complex nature of cells and organisms necessitates the use of multicolour microscopy to provide adequate context.

An important restraint of conventional fluorescence microscopy is the limited spatial resolution, which impedes the visualization of many biologically relevant processes at or close to their true scale. This issue has been circumvented in recent years by the development of methods that circumvent this diffraction limit.¹ Many live-cell sub-diffraction microscopy methods make use of controllable or “smart” fluorescent proteins (FPs), because these do not require specific buffer conditions.^{2–4} Examples include Photo-Activated Localization Microscopy (PALM),^{5,6} Reversible Saturable Optical Linear Fluorescence Transitions (RESOLFT),⁷ Patterned Activation Non-Linear Structured Illumination Microscopy (PA NL-SIM)⁸ and pcSOFI.⁹

The currently available super-resolution methods are finding their way into non-expert labs where they are being used to help solve acute questions. However, in order to become rooted as the go-to method, the availability of reliable and easy-to-

perform live-cell multicolour sub-diffraction imaging is required.

Many sub-diffraction imaging methods have reported multicolour or multi-channel results, relying on spectrally distinct fluorophores that are imaged sequentially or on multiple detectors or detector regions. Unfortunately, in addition to the need for fiducial markers and the chromatic aberrations inherently present,^{10,11} the number of spectrally distinct, adequate “smart” FPs is limited and the most-used phototransformable FPs occupy the green, the red or both spectral regions.^{4,12–14} Alternative methods to acquire multicolour sub-diffraction information make use of photoswitching and fluorescence lifetime information,^{15,16} or spectral unmixing or resolving.^{17–20} Most of these methods provide additional hardware requirements in order to perform the experiments.^{21,22}

Within this work we present multi-tau (mt-)pcSOFI, a novel monochromatic, multi-channel sub-diffraction imaging method based on pcSOFI; that does not require additional hardware or imaging steps, limits chromatic aberrations and temporal offsets, and makes optimal use of the large range of suitable green reversibly switchable (RS) FPs.

pcSOFI extracts sub-diffraction information from fluctuations in the emission of photochromic fluorophores, such as RSFPs, through correlation analysis, and works well even under challenging conditions,^{23–26} making it suitable for super-resolution biochemical activity imaging.^{27,28} At present, pcSOFI achieves a spatial resolution below 100 nm and a temporal resolution down to a few seconds or less.^{29,30} Because pcSOFI images are calculated from a series of fluorescence images, they contain information on the fluorescence dynamics of the emitters present in the sample.³¹ The blinking relaxation time (τ), which reports on the decrease of the SOFI signal for images calculated with increasing time lags, is different for distinct blinking kinetics. (Supplementary Note) Fast blinking fluorophores, whose signals decorrelate rapidly in time, have a short blinking relaxation time, and SOFI images calculated with high time lag values therefore contain little information. Slow

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

blinkers decorrelate more slowly and thus retain more information in SOFI images calculated with high time lag values. We harnessed this additional blinking information to unmix spectroscopically similar RSFPs. (Supplementary Note)

Figure 1 shows the analysis of simulated SOFI data using mt-pcSOFI. A dataset containing the emission of fast and slowly blinking emitters (fig 1a) was used to calculate multiple pcSOFI images with increasing time lag values using the Localizer package (fig 1b).³² These images were subsequently used to compute unmixed images of the fast and slowly blinking components (fig 1c), after which the unmixed images were used to generate a merged dual-colour representation (fig 1d). Comparing the SOFI images calculated with time lag zero and one clearly illustrates the large reduction in signal for the fast blinking emitters due to the rapid decorrelation. Initially, unmixing of fast and slowly blinking fluorophores was implemented using solely the images calculated with times lags zero and one. However, in order to achieve a more reliable unmixing, we additionally incorporated the SOFI signal calculated with a time lag equal to two. (Supplementary Note and Supplementary Figure 1)

In pursuance of a practical and reliable analysis method, we calculated lookup tables containing the weights needed for unmixing different combinations of fast and slow blinking relaxation times (τ_{fast} and τ_{slow}). (Supplementary Note) As the results in figure 1 show, this method allowed us to reliably separate fast and slowly blinking emitters, when present in separated structures (fig. 1d fast and slow), and in areas with mixed presence (fig. 1d mixed).

During the development and application of the rsGreens,³³ we noticed they displayed a significantly faster blinking behaviour than (ff)Dronpa,^{34,35} which seemed to be in agreement with the large difference in photoswitching speed between the RSFPs. We therefore hypothesized that RSFPs with large differences in photoswitching speed would display sufficiently distinct

Figure 1: The mt-pcSOFI principle illustrated with simulated data. (a) Example frames of a simulated SOFI dataset containing fast and slowly blinking fluorophores in separate zones and mixed. Simulated blinking rates were comparable with those measured for rsGreen1 (fast)³³ and Dronpa (slow).³⁴ Arrows at the bottom indicate the frames used in the cumulant analysis (time lags). (b) Second order SOFI images calculated using time lag values of zero and one. (c) Unmixed images calculated from the distinct contributions of the different fluorophores in the lag 0, lag 1 and lag 2 images. (d) Pseudo-coloured merged overlay of the unmixed images. Large arrows indicate the mt-pcSOFI analysis workflow.

blinking for unmixing in mt-pcSOFI. Proof-of-concept experiments were conducted on single-labelled cells expressing rsGreen1 and Dronpa or ffDronpa in various fusion constructs. Figure 2 summarizes the performance of the different RSFPs. As expected, the large difference in photoswitching speed between the different FPs (fig 2a-b) resulted in markedly different blinking relaxation times (fig 2c). This lead to significantly different contributions to time lag one pcSOFI images (fig 2d-g). The data acquired with rsGreen1 labelled structures retains barely any information in the time lag one images in contrast to Dronpa and ffDronpa time lag one images. To identify additional candidates for the mt-pcSOFI approach, we performed an initial photoswitching screening experiment on liquid *E.coli* cultures expressing different green RSFPs. (Supplementary Figure 2) While several RSFP variants display similar or faster photoswitching compared to (ff)Dronpa under identical illumination conditions, the only slower switching

Figure 2: (a - b) Photoswitching curves of rsGreen1 and Dronpa. Switching was induced with 488 nm irradiation (0.48 W/cm², cyan bars) and 405 nm irradiation (0.034 W/cm², purple bars) until completion. (c) Blinking relaxation time (τ) curves of the displayed images. (d - g) Comparison between averaged widefield, time lag zero second order pcSOFI (SOFI² lag 0) and time lag one second order pcSOFI (SOFI² lag 1) images of rsGreen1 fused to microtubule-associated protein 4 (MAP4) (d) and Keratin 19 (Krt19) (e), ffDronpa fused to MAP4 (f) and Dronpa fused to Krt19 (g). Time lag zero and time lag one are displayed with identical colourscaling, revealing that time lag one pcSOFI images of rsGreen1 yield very little information, in contrast to Dronpa and ffDronpa images. All pcSOFI images were calculated from 1000 frames acquired at 100 Hz. Scalebars = 5 μ m. (e - f) (g)

RSFPs we could identify were known dimerizing and tetramerizing RSFPs such as 22G, PDM1-4 and mGeos2-F.^{34,36,37} The faster switching RSFPs and specifically faster switching Dronpa variants such as Dronpa-2 and -3, rsFastLime and Dronpa-M159T suffer from an undesirable decrease in fluorescence brightness.^{38,39} We therefore initiated a search for a (ff)Dronpa mutant that retained the high brightness, but displayed faster off-switching to expand the range of useful fluorophores for mt-pcSOFI

Through screening of rational, semi-rational and random mutagenesis libraries we obtained a ffDronpa mutant with two mutations (K45I and F173V) in addition to the V60A mutation previously identified for optimized maturation. The most crucial role is played by the F173V mutation, which increases the colony fluorescence two- to threefold as well as the off-switching speed by approximately a factor of ten. (Table 1 and Supplementary Figure 3) We dubbed this mutant ffDronpa-F, with “F” referring to the faster photoswitching as well as the most essential mutated residue, Phe173. A similar effect on the fluorescence and photoswitching properties of Dronpa was previously identified during the development of cpDMVF.²⁷ The K45I mutation provided an additional slight improvement to the colony fluorescence. While the photoswitching and colony fluorescence of ffDronpa-F were altered in respect to Dronpa and ffDronpa, the quantum yield of fluorescence and extinction coefficient remained largely unaffected (Table 1 and Supplementary Figure 3). Although the photoswitching speed is not as high as that of the rsGreens,³³ the high molecular and colony brightness of ffDronpa-F coupled to a decent off-switching speed led us to suspect it would make a worthy candidate as a partner in mt-pcSOFI, and a potential starting point for additional mutagenesis efforts.

Table 1: Properties of the newly developed ffDronpa-F compared to Dronpa.

	λ_{ex}	λ_{em}	λ_{act}	ϵ	QY	molec. bright.	col. bright.	switch. time
Dronpa	503	518	390	83	66	55	1	625
ffDronpa-F	504	518	390	77	69	53	2.4-3.0	57

λ (nm): peak wavelength for excitation, emission and activation. ϵ ($\text{mM}^{-1}\text{cm}^{-1}$): extinction coefficient of the anionic chromophore. QY_{fluor} (%): quantum yield of fluorescence. molec. bright.: molecular brightness defined as (extinction coefficient x quantum yield) / 100. col. bright.: average fluorescence brightness of bacterial colonies, scaled to Dronpa. switch time (sec): off-switching half-time defined as the time needed to decay to half the initial value under identical irradiation.

To test the suitability of ffDronpa-F for mt-pcSOFI in combination with Dronpa or ffDronpa, we analysed image sequences of live dual-labelled HeLa cells. The mt-pcSOFI results are shown in Supplementary Figure 4. Comparison with the averaged widefield images illustrates the characteristic increased contrast of pcSOFI in addition to the unmixing of both distinctly blinking RSFPs.

Although we were able to perform mt-pcSOFI using ffDronpa-F, we investigated whether the use of faster photoswitching RSFPs, such as rsGreen1 and rsGreenF, would increase the performance. The obtained results are shown in Figure 3 and Supplementary Figures 5 and 6. mt-pcSOFI again provided

Figure 3: Comparison of the average widefield images and mt-pcSOFI merged dual-colour images of HeLa cells transfected with (a) MAP4-Dronpa & Krt19-rsGreenF, (b) Krt19-Dronpa & MAP4-rsGreen1. Boxed areas are expanded on the right. Images were calculated from 1000 frames acquired at (a) 100 Hz or (b) 33 Hz (b). Main images: scalebar = 5 μm , expanded images: scalebar = 2 μm .

additional contrast and allowed unmixing of the differently labelled structures into two channels. Comparison with the averaged widefield images further illustrated the additional resolving power, revealing details indistinguishable in the diffraction limited image, similar to the second order pcSOFI outcome. (Supplementary Figure 6) Using faster photoswitching RSFPs in combination with (ff)Dronpa for mt-pcSOFI proved to be beneficial indeed, as less input frames were required to calculate the unmixed images, i.e. 4000 frames for ffDronpa-F versus 1000 frames for rsGreen1 and rsGreenF. The larger difference in photoswitching speed, and therefore more distinct τ -values thus allow for a clearer unmixing. The continued development of fast photoswitching RSFPs with high brightness will therefore benefit future mt-pcSOFI experiments.

Because of the unmixing process, some signal is inevitably lost in mt-pcSOFI, slightly reducing the image quality compared to regular pcSOFI. However, we feel that the gained information in the dual-label measurements greatly outweighs this. Especially considering that mt-pcSOFI can be performed without any additional hardware, and does not introduce chromatic aberrations or temporal offsets between the two channels. The monochromatic nature of mt-pcSOFI also frees up a large part of the spectrum, making multiplexing with biosensors or other super-resolution methods in different colours feasible. Furthermore, the continued development and improvement of RSFPs can result in the inclusion of a third RSFP with a sufficiently distinct blinking relaxation time, or can lead to a combined approach of multicolour and mt-pcSOFI using

spectroscopically distinct RSFPs.^{40–43} Since mt-pcSOFI fits well within the original framework of SOFI analysis, it is inherently compatible with all previous developments, including 3D-SOFI, bSOFI and fSOFI.^{29,31,44} Lastly, the mt-pcSOFI concept is not limited to RSFPs. Any change in the fluorescence dynamics of a single fluorophore could be exploited as contrast mechanism for mt-pcSOFI, therefore turning the environmental sensitivity of any dye into a potential ratiometric sensor.^{45,46}

To conclude, we presented the general concept and proof-of-principle of mt-pcSOFI, a live-cell monochromatic multi-label sub-diffraction microscopy technique. The method, which relies on differences in the blinking kinetics of the used fluorophores, can yield dual-colour sub-diffraction images without additional hardware requirements. To demonstrate the method, we used the newly developed ffDronpa-F, a faster switching (ff)Dronpa mutant that retained the high brightness, and rsGreen variants in combination with (ff)Dronpa to image separate structures in live HeLa cells.

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