Two-color STED microscopy in living cells

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Abstract: Diffraction-unlimited resolution provided by Stimulated Emission Depletion (STED) microscopy allows for imaging cellular processes in living cells that are not visible by conventional microscopy. However, it has so far not been possible to study dynamic nanoscale interactions because multicolor live cell STED microscopy has yet to be demonstrated and suitable labeling technologies and protocols are lacking. Here we report the first realization of two-color STED imaging in living cells. Using improved SNAP₂ and CLIP₂ technologies to label epidermal growth factor (EGF) and EGF receptor (EGFR), we report resolutions of 78 nm and 82 nm for 22 sequential two-color scans in living cells.

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1. Introduction

Fluorescence microscopy allows for real-time imaging in living cells and has greatly expanded our knowledge of protein function and cellular processes. However, conventional fluorescence microscopy methods are limited by the diffraction barrier and are unable to resolve structures smaller than ~200 nm. Only recently have super-resolution microscopy methods overcome this limitation and are gaining widespread use in cell biology [1]. Of these methods, STED microscopy [2] has been one of the most successful methods regarding live cell applications [3,4].

Compared to techniques relying on single molecule localization which require relatively long image acquisition times and post imaging data processing [5–7], the purely physical resolution improvement provided by STED is advantageous for live cell applications. In STED microscopy, excited fluorophores at the periphery of the focal volume are quenched by overlaying the excitation beam with a doughnut-shaped depletion beam. The intensity of this depletion beam which is red-shifted relative to the peak of the fluorescent emission spectrum is chosen high enough to saturate the stimulated emission transition. This targeted switching typically results in a 3 to 10-fold resolution improvement and is fundamentally diffraction-unlimited [1,8].

Single color STED has been used to image a variety of biological structures in fixed cells. However, to better understand how proteins function in cellular processes, the relative spatial distributions of two or more proteins are required at sub-diffraction resolution. Towards this goal, two-color STED microscopy has been demonstrated in fixed cells [9,10]. While multicolor imaging in fixed cells provides useful biological information, it does not allow for studying protein dynamics. Cellular processes are inherently dynamic and observing protein movement in living cells as they happen is vital to fully understanding biological functions. Although live cell STED microscopy has been successfully applied to the imaging of gap junctions [4], vimentin [4], actin [11], and synaptic vesicles [3], these experiments were restricted to a single color only.

Therefore, the major breakthrough, live-cell multicolor STED imaging, has yet to be achieved. To accomplish this goal, several hurdles must be overcome. Firstly, fluorophores suitable for live cell two-color STED must be identified. While fluorescent proteins provide the advantage of being genetically encodable, they suffer from poor photostability and fitting STED pairs have not been identified yet. Organic dyes provide an appealing alternative but often lack live-cell compatible targeting methods. Thus, a convenient live cell labeling strategy poses a second hurdle. Finally, an optimal imaging scheme must be realized for two-
Color live cell STED imaging since previous approaches of two-color STED have utilized sequential imaging of two channels [9,10].

To overcome these obstacles, we first used a new SNAP-tag based labeling technology that has faster binding kinetics than commercially available versions. This methodology is both genetically encodable and compatible with organic fluorophores and therefore optimal for live-cell imaging applications. We then screened for optimal dye pairs compatible with our STED setup. Combining these approaches with an imaging scheme that alternates line by line between different excitation beams to acquire two STED images quasi-simultaneously, we report the first example of two-color STED microscopy in living cells.

2. Materials and Methods

2.1 Expression Constructs

pSNAP<sub>f</sub>-tag(T7) and pCLIP<sub>f</sub>-tag(T7) were constructed by replacing the SNAP-26m coding region of pSNAP-tag(T7)-2 (New England Biolabs) using the unique EcoRI and SbfI sites with the coding regions of SNAP<sub>f</sub> and CLIP<sub>f</sub>, respectively. The mouse EGF coding sequence was fused in-frame to the 5′ end of CLIP<sub>f</sub>, and a 6-histidine tag fused to the 3′ end of CLIP<sub>f</sub> in pCLIP<sub>f</sub>-tag(T7). The resulting plasmid, pEGF-CLIP<sub>f</sub>-6xHis, was used for expression of EGF-CLIP<sub>f</sub> fusion protein in E. coli and subsequent affinity purification by Ni-NTA agarose (Qiagen). A linker encoding the signal sequence of EGFR was inserted into 5′ MCS of pSNAP<sub>f</sub> vector (New England Biolabs) using the unique NheI and EcoRI sites. Subsequently, the coding sequence of mature EGFR (GeneCopoeia) was amplified by PCR and subcloned into the plasmid pSNAP<sub>f</sub>-EGFR.

2.2 Chemical Synthesis of SNAP and CLIP-Tag STED Probes

SNAP-tag and CLIP-tag fluorescent probes were synthesized from BG-NH<sub>2</sub> [12,13] or BC-NH<sub>2</sub> [14] (New England Biolabs, Inc.) and commercially available N-hydroxysuccinimide (NHS) activated fluorescent dyes using standard procedures [15]. DY-480XL NHS and DY-521XL NHS were purchased from Dyomics GmbH (Jena, Germany). Chromeo494 NHS was purchased from Active Motif Chromeon GmbH (Tegernheim, Germany). ATTO620 NHS, ATTO633 NHS, ATTORhod14 NHS, ATTO647N, and ATTO655 NHS were purchased from ATTO-TEC GmbH (Siegen, Germany). KK114 NHS [16,17] was provided by Prof. Dr. Stefan W. Hell, Max Planck Institute for Biophysical Chemistry (Göttingen, Germany).

2.3 Mammalian Cell Culture

Human embryonic kidney (HEK 293) cells stably expressing pSNAP<sub>f</sub>-EGFR were selected with 400 µg/ml G418 in phenol red free DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C, 5% CO<sub>2</sub>, and maintained in growth medium with 200 µg/ml G418.

2.4 EGF-CLIP<sub>f</sub> Isolation and Labeling

Expression of recombinant EGF-CLIP<sub>f</sub>-6xHis was performed in Shuffler® T7 E. coli (New England Biolabs). EGF-CLIP<sub>f</sub>-6xHis was purified from E. coli cell lysate using Ni-NTA Agarose (Qiagen). Analysis of protein expression and purification was done with Coomassie Blue stained SDS-PAGE. Labeling of EGF-CLIP<sub>f</sub>-6xHis was carried out with 5 µM EGF-CLIP<sub>f</sub>-6xHis, 10 µM BC-ATTO647N (or BC-Chromo494), 1 mM DTT and 1x PBS on ice for 5 hours. 15% Glycerol was added at the end of the reaction.

2.5 Live Cell Labeling

Human embryonic kidney (HEK 293) cells stably expressing pSNAP<sub>f</sub>-EGFR were plated on #1.5 cover glass dishes and allowed to adhere overnight in phenol red free DMEM with 0.1% FBS. Prior to imaging, cells were incubated with freshly prepared solutions of 10µM BG-Chromo494 in phenol red free DMEM with 0.1% FBS for 10 min at 37°C. Cells were washed 3 times with fresh media and placed on the microscope stage for STED imaging.
After focusing, 100 ng/mL of EGF-CLIP-ATTO647N was added directly to the imaging media and cells were imaged by two-color STED microscopy.

Fig. 1. Schematic of experimental labeling procedure using SNAP<sub>f</sub> or CLIP<sub>f</sub> reactions to specifically label EGFR and EGF. (A) In living cells, SNAP<sub>f</sub> recognizes its substrate, BG, and undergoes a Sn2 type reaction that displaces BG, resulting in a permanent covalent bond between the EGFR and Chromeo494 (green star). (B) EGF-CLIP<sub>f</sub> is recombinantly expressed and purified, labeled with BC-ATTO647N (BC-647N) producing exogenous EGF-CLIP<sub>f</sub>-ATTO647N. (C) Live cells expressing EGFR-SNAP<sub>f</sub> are labeled with Chromeo494, followed by incubation with EGF-CLIP<sub>f</sub>-ATTO647N. This results in the double labeling of the EGFR-EGR complex for two-color STED imaging. EGFR is known to form a homodimer upon ligand binding, but is shown as a single receptor in this schematic for clarity. Cyto = cytoplasm, PM = plasma membrane and ECM = extracellular matrix.

2.6 STED Microscopy

Two-color STED images were obtained using a commercial Leica TCS STED microscope. This setup features pulsed diode lasers (PDL 800-B, PicoQuant) emitting at 640 nm (~8-65 µW) and 532 nm (~150 µW) for excitation and a tunable, modelocked Ti:Sapphire laser (Mai Tai, Spectra Physics) for depletion (~130 mW). Laser powers were measured at the objective back aperture and corrected for objective transmission efficiency to represent the power at the sample. A 100x/1.4 NA oil immersion objective lens (HCX PL APO STED, Leica Microsystems) was used for imaging. Fluorescence signal was collected by the objective lens, passed through a 0.5 Airy unit pinhole, split by a dichroic mirror (650 nm long-pass), bandpass filtered (FF01-685/40 for 640 nm excitation or FF01-582/75 for 532 nm excitation, both from Semrock), and detected by avalanche photodiodes. For resolution comparison, confocal and STED images were taken sequentially line by line with a scan speed of 1000 lines per second, a pixel size of 25.3 nm (1024 x 1024 pixels), and 32 line averages. Two-color STED time series were also obtained sequentially line by line by alternating the excitation lasers and the STED laser tuned to 760 nm for both ATTO647N and Chromeo494.
Crosstalk between channels was below the noise limit (<5%) as determined by imaging singly labeled samples. Frames (1024 x 1024 pixels) were acquired at a scan speed of 1400 lines per second with a pixel size of 30.3 nm. Each frame consisted of 16 line averages and required ~11.9 seconds recording time. Images were smoothed with a 2.5 pixel full width half-maximum Gaussian filter using Imspector (written by Dr. Andreas Schönle, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, available via Max-Planck-Innovation GmbH, Munich, Germany) and the color map rescaled.

### 3. Results and Discussion

Two-color STED imaging can be realized by using either different STED wavelengths for the two fluorophores or the same wavelength for both. In the first case, fluorophores are distinguished by imaging different color channels [9]. However, the blue-shifted STED laser beam can usually excite the red-shifted fluorophore so efficiently that the probe will rapidly bleach [18]. While acceptable for fixed samples where the red-shifted label can be imaged first, it poses an insurmountable problem for acquiring two-color time-series required in live cell applications. We therefore chose to follow the scheme using only one STED laser which is red-shifted relative to both fluorophores. To separate the two labels in imaging, one fluorophore features a large Stokes-shift so that probes can be excited by different laser lines and distinguished this way [10]. By alternating the excitation laser line by line, the two channels can be recorded quasi-simultaneously, minimizing temporal shift between the images.

#### Table 1. Fluorophores Evaluated for Two-Color Live Cell STED Microscopy

| Fluorophore  | $\lambda_{ex}$ (nm) | $\lambda_{STED}$ (nm) | Non-specific binding |
|--------------|---------------------|-----------------------|---------------------|
| DY-480XL     | 500                 | 730                   | -                   |
| DY-521XL     | 523                 | 750                   | -                   |
| Chromeo494   | 494                 | 760                   | -                   |
| ATTO620      | 619                 | 760                   | -                   |
| ATTO633      | 629                 | 760                   | mitochondria        |
| ATTORho14    | 625                 | 770                   | mitochondria        |
| ATTO647N     | 644                 | 760                   | mitochondria        |
| ATTO655      | 663                 | 780                   | -                   |
| KK114        | 650                 | 760                   | -                   |

As a biological application, we fused EGFR with the SNAP-tag and its ligand, EGF, with the CLIP-tag (both New England Biolabs, Ipswitch, MA). SNAP and CLIP-tags are human DNA repair enzymes that have been engineered to specifically recognize benzylguanine (BG) and benzylcytosine (BC) derivatives, respectively [14]. BG and BC substrates can be conjugated to any fluorophore, and form a covalent bond upon reacting with the SNAP or CLIP-tags (dashed box in Fig. 1(A)). In this study, a new pair of fast labeling SNAP and CLIP-tag variants, termed SNAPf and CLIPf, were utilized as fusion partners. SNAPf and CLIPf are described in detail in [19]. These variants have increased labeling rates compared to the commercially available versions and are advantageous for monitoring fast molecular events in living cells. These technologies are highly specific for their substrates and provide a convenient way to functionalize different proteins with organic fluorophores. This approach provides a significant advantage over fluorescent proteins for 2-color live cell STED imaging applications because it allows for facile introduction of STED compatible probes that have increased photostability and brightness.
Fig. 2. Resolution measurements for ATTO647N and Chromeo494 in living HEK293 cells. HEK293 cells were treated with 100 ng/mL EGF-CLIP$_f$-ATTO647N and imaged with (A) STED and (B) confocal microscopy. The area depicted by the white box in (A) is shown in (C) STED and (D) confocal. The boxes in (C) and (D) were used to generate the line profiles for ATTO647N in (E) and (F). HEK293 cells stably expressing EGFR-SNAP$_f$ were treated with 10 μM BG-Chromeo494 for 10 min at 37°C and imaged by (G) STED and (H) confocal microscopy. The area depicted by the white box in (G) for Chromeo494 is shown in (I) STED and (J) confocal. The boxes in (I) and (J) were used to generate the line profiles for Chromeo494 in (K) STED and (L) confocal. Solid red lines indicate the Lorentzian (STED) and Gaussian (confocal) least squares fits used to determine the full width at half-maximum (FWHM) values.

Imaging was performed on a commercially available STED microscope (TCS STED, Leica Microsystems) featuring a tunable mode locked Ti:Sapphire laser for depletion and two synchronized pulsed lasers at 532 nm and 640 nm for fluorophore excitation. To determine the best combinations of fluorophores for two-color live cell experiments, we systematically tested dyes compatible with our STED microscope and evaluated their usability for live cell imaging by assessing the amount of non-specific staining in cells expressing EGFR-SNAP$_f$ (Table 1). The fluorophores were conjugated to BG and all were efficiently recognized by SNAP$_f$. Of the three long Stokes-shift dyes tested, Chromeo494 was the brightest and showed minimal non-specific staining. For the red-shifted dyes, ATTO647N, ATTO655 and KK114 were the brightest, while ATTO620 and ATTO633 bleached relatively quickly. ATTO633, ATTO647N and ATTORh14 showed non-specific staining of mitochondria. To reduce non-specific binding of ATTO647N, purified recombinant EGF-CLIP$_f$ was labeled with BC-ATTO647N (Fig. 1(B)) and then used to label EGFR. Non-specific staining was reduced in comparison to the direct labeling of EGFR-SNAP$_f$ with BG-ATTO647N. Therefore we chose to label EGFR-SNAP$_f$ with Chromeo494 and EGF-CLIP$_f$ with ATTO647N (Fig. 1(C)).

Next, we compared the achievable resolution in living cells using STED microscopy with Chromeo494 and ATTO647N (Fig. 2). Following a protocol that labeled the plasma membrane of clustered HEK293 cells with either dye as outlined in section 2.5, we could determine the resolution by measuring the profile through membranes that were primarily
oriented perpendicular to the focal plane. The STED resolution obtained for Chromeo494 and ATTO647N was 82 and 78 nm, respectively. These values are close to the performance limit of our microscope in fixed bead samples (data not shown), and represent a 3 to 4-fold increase in resolution over confocal microscopy.

To investigate the dynamics of EGFR and EGF in living cells, we took a two-color STED time series of EGFR-SNAP$_F$ labeled with BG-Chromeo494 and EGF-CLIP$_F$-ATTO647N at 11.9 sec/frame. While bleaching certainly is observable, we were able to image 22 and more sequential two-color STED images at acceptable signal levels (Fig. 3). The time series shows that internalization of EGF occurs rapidly after EGF stimulation and that EGFR-SNAP$_F$-Chromeo494 and EGF-CLIP$_F$-ATTO647N co-localize in endosome-like structures (arrowhead, Fig. 3).

![Fig. 3. Live cell two-color STED time series of HEK293 cells labeled with EGF-CLIP$_F$-ATTO647N (magenta) and EGFR-SNAP$_F$-Chromeo494 (green). Data has been normalized to correct for bleaching. The shown images have been cropped from the original raw data. Scale bar = 1 μm.](image)

Shortly after EGF stimulation an endosome-like structure that is EGF-CLIP$_F$-ATTO647N positive, but EGFR-SNAP$_F$-Chromeo494 negative, began to form (arrows, Fig. 3). The post internalization trafficking of the EGFR-EGF complex is not completely understood. It is hypothesized that the complex remains intact before being degraded in lysosomal compartments [20] followed by subsequent down regulation of the receptor [21]. Our results showing co-localization of labeled EGFR and EGF are in agreement with the hypothesis that EGFR-EGF complexes remain intact early in the endosomal pathway. The formation of an only EGF-CLIP$_F$-ATTO647N positive structure is indicative of internalization of a pool of EGFR that was newly synthesized or in the process of being recycled to the plasma membrane. Labeling of internal pools of EGFR in combination with two-color STED microscopy will allow for an in depth analysis of the recycling and trafficking of EGFR. This new approach enables further live cell co-localization and pulse-chase studies to help fully deduce EGFR-EGF trafficking.

### 4. Conclusions

Our presented approach overcomes the limitations of specifically targeting STED fluorophores to the proteins of interest and provides a convenient imaging scheme that allows for 2-color live cell STED imaging for the first time. We believe this method is a powerful new way to address biological questions in living cells. SNAP$_F$ and CLIP$_F$ technology can readily be applied to other proteins and the versatile labeling methodology allows for unique combinations of localization, pulse-chase and pull-down type experiments. These
technologies can also be combined with far-red fluorescent proteins compatible with STED, such as TagRFP657 [22] and E2-Crimson [23].

The diffraction limit associated with conventional confocal microscopy impairs accurate evaluation of protein distributions, and the sub-diffraction resolution provided by STED microscopy can help to obtain more meaningful co-localization data. We expect that utilizing novel approaches such as distinguishing labels by their lifetime [24] and improving axial resolution using two opposing objectives [25] or total internal reflection [26] will expand live cell STED microscopy to three or more colors, 3D imaging and improved optical sectioning.

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