Chapter 3

Live-Cell STED Imaging with the HyPer2 Biosensor

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Abstract

Stimulated emission depletion (STED) microscopy is a popular super resolution imaging technique. Not only synthetic dyes and fluorescent proteins can be utilized as STED fluorophores, but also genetically encoded biosensors. Fusing the biosensor with proteins of interest allows subdiffraction imaging of intracellular macromolecular architecture with simultaneous extraction of functional information about cellular activities. Here, we describe a protocol for live-cell STED microscopy of the HyPer2 biosensor fused to cytoskeletal filaments.

Key words Super-resolution microscopy, STED, Live-cell imaging, Biosensors, HyPer2, Hydrogen peroxide

1 Introduction

HyPer2 is a genetically encoded biosensor for the detection of H_2O_2 in living cells [1]. It is an improved variant of biosensor HyPer [2] with twice expanded dynamic range. The biosensor consists of fluorescent protein cpYFP and a bacterial H_2O_2-sensitive domain OxyR. Previously published protocols [3, 4] cover the HyPer usage in live-cell conventional fluorescent microscopy, all the recommendations on immobilizing biosensor within cell, choosing cell lines and stimuli. Most of these protocols can be straightforwardly applied to HyPer2. HyPer2 was neither specifically designed nor optimized for STED microscopy; nevertheless, biosensor had good performance in the STED super-resolution microscopy, allowing for simultaneous H_2O_2 detection and a resolution improvement [5].

Here, we present a detailed protocol for live-cell STED imaging of intracellular H_2O_2 production including detailed step-by-step sample preparation instructions, experimental setup, and image analysis guidelines. This protocol may be used as a starting point to adapt live-cell imaging of HyPer2 and similar biosensors for the requirements of STED microscopy.

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2 Materials

2.1 Reagents

1. Vector pHyPer2-C (Addgene, cat. no. 42211) for HyPer2 fusion protein construction by means of molecular cloning.
2. DMEM.
3. Minimum Essential Medium Eagle (EMEM).
4. OPTI-MEM.
5. Hanks Balanced Salt Solution (HBSS).
6. Dulbecco-PBS.
7. Fetal calf serum, FCS.
8. Penicillin/streptomycin.
9. L-Glutamine.
10. Trypsin/EDTA solution.
11. HEPES.
12. Bovine Serum Albumin (BSA), cell culture grade.
13. Paraformaldehyde.
14. Mowiol.
15. FuGene6 transfection reagent or similar.
16. Mammalian cells. This protocol describes the use of HeLa-Kyoto and NIH-3T3 cell lines. However, the overall technique is applicable to any transfectable or transducible cell culture.
17. Sodium bicarbonate.
18. H_2O_2. Caution. It may cause severe skin burns and eye damage. Follow the safety guide.
19. PDGF.

2.2 Equipment

1. Glass-bottom dishes.
2. Cover slips #1.5 (optimal: 0.170 ± 0.01 mm thick).
3. Microscopy slides.
4. Standard equipment for eukaryotic cell culturing.
5. Custom-built STED microscope or a commercial STED microscope (e.g., Leica Microsystems GmbH, Mannheim, Germany) equipped with a thermostating box.
6. Image processing software: ImageJ or FIJI (National Institutes of Health).
7. Data quantification and analysis software: Excel (Microsoft) or Origin (OriginLab Corporation).

2.3 Reagent Setup

1. Complete growth medium: We use DMEM supplemented with penicillin/streptomycin, L-glutamine, and 10% FCS.
2. Cell starvation and imaging medium: EMEM supplemented with either 2.2 g/l sodium bicarbonate and 20 mM HEPES (if CO_2 supply for thermostating box available) or just 20 mM HEPES (use bicarbonate free media in case no CO_2 supply for thermostating box is available in order to prevent alkalinization of the imaging media), penicillin/streptomycin, L-glutamine, and 0.5% BSA.
3. Preparation of growth factors for experiment: Growth factor suppliers recommend the optimal protocol for storage. Just before the experiment, dilute a small aliquot of the growth factor in EMEM to prepare a 1000 stock solution (10 mg/ml PDGF). Keep on blue ice.

2.4 Equipment Setup

We used Leica TCS SP8 microscope with a time-gated stimulated emission depletion (STED) module (Leica Microsystems GmbH, Mannheim, Germany), equipped with a white light laser (WLL), STED 592-nm laser, HCX PL APO 100×/1.40 oil objective, and a hybrid detection system (Leica HyD).

3 Methods

3.1 Plasmid Construction

Clone the protein of interest in-frame with HyPer2 using the appropriate cloning vector (*see* **Note 1**).

3.2 Transfection

1. Plate the cells on glass-bottom dishes for live-cell imaging or grow them directly on cover slips for subsequent cell fixation.
2. Transfect the cells with the HyPer2 fusion encoding vector by any appropriate method. We used FuGene6 transfection reagent, after 4-h incubation with the liposome:DNA complex, we recommend rinsing the cells with HBSS and changing the medium to fresh prewarmed liposome-free complete culture medium.
3. Culture the cells for 24–48 h at 37 °C, 5% CO_2.

3.3 Preparation of Cell Samples for an Experiment

We suggest three types of samples: choose cell fixation (**step 1**) for image settings adjustment and verifying HyPer2-fusion construct localization. Choose live-cell H_2O_2 addition (**step 2**) for HyPer2-fusion sensor performance tests and finally prepare for live-cell experiment in physiological conditions (**step 3**).

1. Cell fixation. Rinse the cover slip in PBS. Fix the cells by incubating in 4% paraformaldehyde in PBS for 15 min at room temperature. Wash the cells twice with PBS. Mount cover slip with the sample on a microscope slide with a small

drop (~15 μl) of Mowiol [6]. Leave slide for several hours in the dark to allow the Mowiol to harden.

2. Live-cell experiment with H_2O_2 addition. Replace the complete medium with 1.5 ml preheated (37 °C) HBSS or PBS.
3. Cell stimulation with growth factor. Replace the complete medium with 1.5 ml preheated (37 °C) MEM. Incubate for 2 h (for HeLa-Kyoto) or 4–6 h (for NIH-3T3) at 37 °C. Keep the cells out of the CO_2 incubator if using bicarbonate-free MEM.

3.4 Imaging Settings (General Considerations for Leica TCS SP8 STED Users)

Use fixed cells while setting up the microscope. HyPer2 can be excited with the 488 nm or 496 nm laser lines, emission can be detected in the 500–550 nm range (*see* **Note 2** and Table 1). As the high power of the lasers could induce phototoxicity, minimize white light laser (WLL) and STED laser power and reuse the line average number in live-cell experiments with growth factors.

1. Activate WLL, set the laser power to 70%. Choose 488 or 496 nm line.
2. Activate STED 592 nm laser, set the laser power to 100%.
3. Align STED and imaging lasers at the beginning of imaging session, then once again after 20 min and every 3–4 h thereafter.
4. Set the scanning resolution to 1456 × 1456 pixels.
5. Set the number of line averages equal to 8.
6. Set pinhole size to 1 Airy units and the pixel size to 20 nm.
7. For confocal imaging set the intensity of the WLL to 5%.
8. For STED imaging set the intensity of the WLL to 25% and the intensity of STED 592 nm laser to 100%.
9. Choose HyD as active detectors.
10. Use gating, starting with 0.7–1 ns.
11. Balance WLL power and gating.

3.5 Time Series Acquisition

1. At least 2 h before imaging, heat up the thermal incubator box to 37 °C.
2. Place the dish with cells onto the microscope stage. Set up focus.
3. Switch the system to a scanning mode.
4. Perform a single scan in confocal mode. Zoom selected cell.
5. Perform several single scans in confocal and STED modes to set the final focus, adjust laser power and gating. Adjust the delay between frames (*see* **Note 3**).

Table 1
Example of image acquisition settings for short and long experiments

Parameter	Short experiment (H_2O_2 addition), STED	Short experiment (H_2O_2 addition) confocal	Long experiment (stimulation with PDGF), STED	Long experiment (stimulation with PDGF), confocal
Scan speed, Hz	1000	1000	400	400
Line average	8	8	5	5
Filter - notchFW2	NF 594	–	NF 594	NF 594
Filter - polarization FW	NF 488	NF 488	–	–
STED (592 nm), output power %	100	0	91.2	0
STED (592 nm), intensity %	100	off	36.2	off
Intensity (488 nm), %	15	5	0.01	–
Intensity (496 nm), %	–	–	23.8	9
Detector	HPD (500–550 nm)	HPD (500–550 nm)	HPD (562–616 nm)	HPD (562–616 nm)
Gain	380	380	421	421
Gate start, ns	0.75	0.75	0.61	0.61
Gate end, ns	12	12	4.39	4.39
Time delay between frames	12 s	–	2 min	–

6. Start time series acquisition in STED mode. Pay special attention for the microscope focus stability during the entire experiment. The use of an automatic focus adjustment accessories is highly advisable.
7. H_2O_2 addition: After 1 min of imaging, add H_2O_2 diluted in 100 µl of pre-warmed HBSS or PBS dropwise. Changes in the signal occur immediately after H_2O_2 addition. Continue data acquisition for 2–3 min.
8. Growth factor stimulation: After 1–2 min of imaging, add the growth factor dropwise (for NIH 3T3 we add 10 ng/ml PDGF) diluted in 100 µl of prewarmed MEM or 100 µl of serum-free MEM as negative control. Continue data acquisition for 15–30 min (*see* **Note 4**).

3.6 Image Analysis

One of the great advantages of STED over the most of super-resolution techniques is that image processing is not needed to obtain super-resolution images. Similarly, HyPer2 intensity change can be monitored during imaging. Nevertheless, we recommend performing image postprocessing described below for the representation improvement and more precise image analysis if needed.

Analyze acquired data offline in ImageJ software (http://imagej.nih.gov/ij/) (*see* **Note 5**).

1. To aid the visualization convolve the image with Gaussian kernel (**Process/Filters/Gaussian blur**). Use kernel size of 1 or 2 pixels.
2. If sample drift is observed, apply "**StackReg**" plug-in (http://bigwww.epfl.ch/thevenaz/stackreg/) or any other appropriate plug-in for image stack registration.
3. Depict the resulting stack in pseudocolors using a "**Ratio**" lookup table for better visualization of intensity changes.
4. Choose a ROI for the time profiles quantification. The resultant table can be saved and used in .xls format for Excel or Origin.

3.7 Results

Using the method described above, we recorded STED images of vimentin filaments with a resolution of 70–80 nm [5]. Typical results are presented in Figs. 1, 2, and 3. Figure 1 shows a live HeLa-Kyoto cell expressing vimentin-HyPer2 imaged in confocal and STED mode. A line profile plot (Fig. 1c) shows the resolution improvement. Figure 2 demonstrates that upon the addition of H_2O_2 HyPer2 increases brightness. Figure 3 demonstrates STED images of microtubules labeled with EB3−HyPer2 in NIH 3T3 fibroblast. Stimulation of the fibroblast with PDGF resulted in cellular H_2O_2 production reflected by the increase of EB3−HyPer2 brightness.

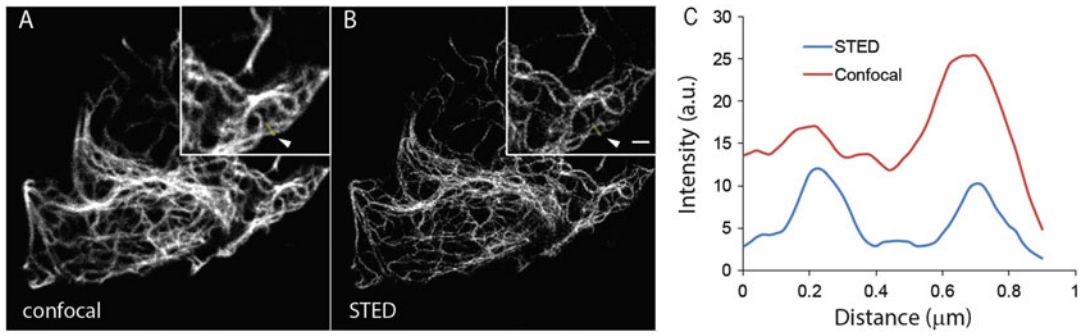

Fig. 1 A representative confocal (**a**) and STED (**b**) images and (**c**) intensity profiles along the linear ROI (see the *arrow*) of the HeLa-Kyoto cell expressing vimentin-HyPer2. Scale bar is 1 μm

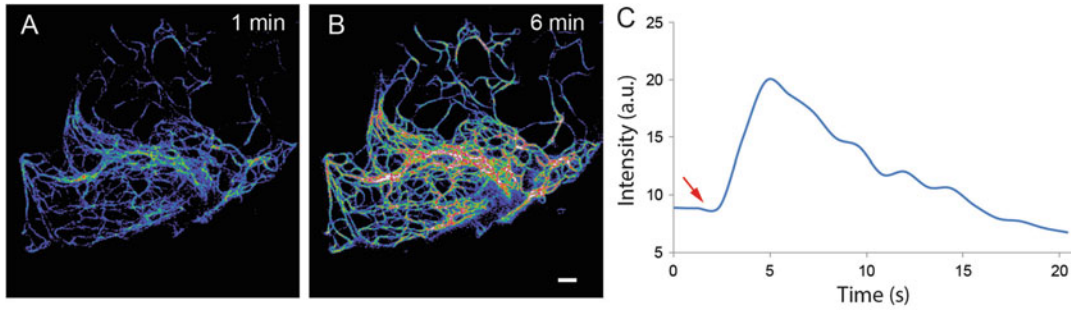

Fig. 2 Pseudocolored STED images of the HeLa-Kyoto cell expressing vimentin-HyPer2 in the course of H_2O_2 addition. Increase in fluorescence is clearly visible shortly after hydrogen peroxide addition (**a**, **b**). Scale bar is 5 μm. (**c**) Time course of HyPer2 intensity change. The *arrow* indicates H_2O_2 addition

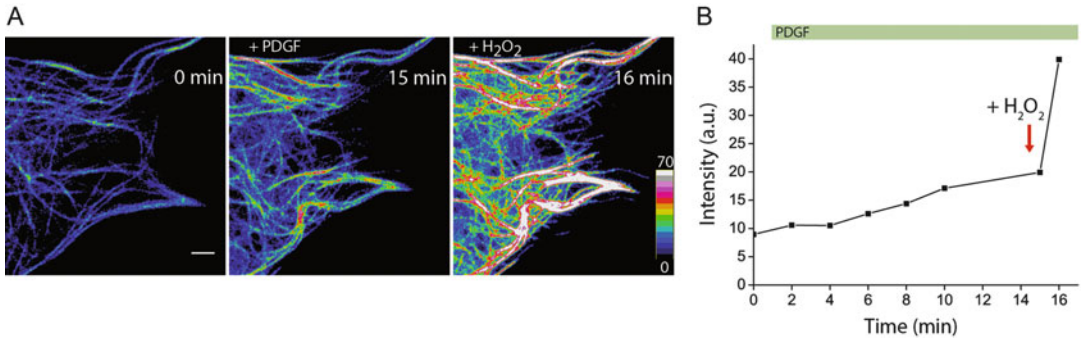

Fig. 3 Imaging of EB3-HyPer2 in live NIH 3T3 cells. (**a**) STED images of EB3-Hyper2 fluorescence intensity change in NIH 3T3 cell at indicated time points after stimulation with 10 ng/ml PDGF and subsequent addition of 200 μM H_2O_2. Scale bar: 2 μm. (**b**) Timing of fluorescence intensity change in cell shown on panel (**a**). Adapted from [5]. Copyright (2015) American Chemical Society

4 Notes

1. In our experiments fusion constructs of vimentin-HyPer2 (labeling of intermediate filaments) and EB3-HyPer2 (labeling of microtubules) worked well for STED microscopy. General considerations for creating HyPer2 fusion constructs can be found here ([3]).
2. The fluorescent core of HyPer2 biosensor is based on cpYFP, thus STED imaging settings for YFP (eYFP, citrine) should be applicable for HyPer2 too. It is important to note that HyPer2 is relatively dim probe; therefore, excitation laser intensity should be set to the level 3–5 times higher than for citrine. When setting the intensity of excitation laser, keep in mind that long exposure at high intensity is phototoxic for living cells and also causes rapid photobleaching of fluorescent probe. Thus,

the balance is needed between laser power, gating, and time delay between frames.

3. For short experiments (H_2O_2 addition) choose "no delay" between frames during time series acquisition. For long experiments (stimulation with growth factor) delay should be about 1–3 min.
4. At the end of the session you can add an H_2O_2 aliquot to the final concentration of 200 μM, in order to completely saturate HyPer2. This step can serve as a positive control for the biosensor performance, and will also help to evaluate the results of the experiment.
5. To compare STED and corresponding confocal images all the postprocessing steps of these images should be identical.

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