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Observing the assembly of protein complexes in living eukaryotic cells in super-resolution using refSOFI

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Abstract

Few approaches are currently available that allow the detection of protein-protein interactions (PPIs) in super-resolution, and the observation of the assembly of protein complexes in living cells has been particularly challenging. We developed reconstituted fluorescence-based stochastic optical fluctuation imaging (refSOFI), which is based on bimolecular fluorescence complementation (BiFC) and SOFI and allowed us to detect protein complex assembly 30 minutes after the induction of complex formation. Here we describe how to use refSOFI to map the assembly of two proteins of interest into a complex within living cells at super-resolution.

Keywords

Super-resolution imaging; Stochastic optical fluctuation imaging (SOFI); Protein complexes; Protein-Protein interactions; bimolecular fluorescence complementation (BiFC)

1. Introduction

In the last decade, tremendous progress has been made in the field of super-resolution imaging, allowing us to characterize cellular structures below the diffraction limit, thus having a vast impact on research in several biological fields [1]. Several strategies have been developed to overcome the diffraction limit, which are either based on sophisticated illumination strategies, such as stimulated emission depletion (STED) [2] and saturated structured illumination microscopy (SSIM) [3], the localization of single molecules represented by photoactivated localization microscopy (PALM) [4] and stochastic optical reconstruction microscopy (STORM) [5], or higher order correlation analyses of fluorescence intensity fluctuations in the case of stochastic optical fluctuation imaging (SOFI) [6, 7]. While some of these methods permitted a resolution of up to 20 nm in living cells under optimal conditions, the number of methods that could directly visualize the interaction of proteins beyond simple co-localization is rather limited. Recently, several methods that combine the bimolecular fluorescence complementation (BiFC) of fluorescent proteins [8] with a super-resolution strategy have been introduced [9–12]. The approach we developed is using SOFI with BiFC-compatible fragments of Venus [13] or DronpaMVF

(DMVF), a variant of the photoswitchable fluorescent protein Dronpa [14] that we specifically optimized for this task. We termed this strategy reconstituted fluorescence-based SOFI (refSOFI) [12] and demonstrated that it is specifically advantageous to map protein interactions in living cells and to observe the assembly of protein complexes within 30 minutes when using Venus. We could show that in the case of subunits of the pore forming Ca^{2+} release-activated Ca^{2+} (CRAC) channel ORAI1 and stromal interaction molecule 1 (STIM1) at endoplasmic reticulum (ER)-plasma membrane (PM) junctions, super-resolution information is critical to assess the characteristics of clusters of STIM1/ORAI1 complexes. Here we describe how to apply refSOFI to detect protein complexes and to observe complex formation in living cells in a general way. In brief, first the proteins of interest are fused to BiFC-compatible fragments of Venus or DMVF and transiently expressed in the cells of interest. Subsequently, the cells can be stimulated if desired, image series of the fluorescence intensity fluctuations are recorded, and the data is analyzed with the software package Localizer [15], yielding super-resolution images of the complexes formed by the proteins of interest.

2. Materials

2.1 Plasmids & Molecular Cloning

1. Templates for molecular cloning: pcDNA3 [Lyn-FRB-DMVF-181-N], pcDNA3 [FKBP-DMVF-181C], pcDNA3 [Lyn-FRB-Venus-173-N], and pcDNA3 [FKBP-Venus-173-C] from our study[12]
2. Plasmids with cDNA of the proteins of interest
3. Restriction enzymes *NheI*, *HindIII*, *BamHI*, *KpnI*, *SpeI* (FastDigest, Thermo Fisher Scientific)
4. TAE Buffer: 40 mM Tris, 20 mM Acetate, 1 mM EDTA
5. Agarose
6. Agarose gel DNA extraction kit
7. Phusion High-Fidelity DNA Polymerase (NEB: M0530)
8. T4-Ligase
9. LB media: 1 % (w/v) bacto-tryptone, 0,5 % (w/v) yeast extract, 1% (w/v) NaCl, autoclaved
10. Ampicillin
11. SOC media: 2 % (w/v) bacto-tryptone, 0,5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, MgCl_2 10 mM, MgSO_4 10 mM, autoclaved, 20 mM Glucose has to be added after autoclaving using a sterile filtered stock solution
12. Aliquots of chemically competent E. Coli bacteria (DH5 α strain)
13. LB agar plates with Ampicillin (100 $\mu\text{g/ml}$): LB with 1.5 % (w/v) agar, autoclaved, Ampicillin added after cooling down to 60 °C from stock solution, poured in sterile plates

14. Plasmid DNA miniprep kit

2.2 Cell cultivation

1. Growth media for the specific cell line of interest
2. Lipofectamine 2000 (Thermo Fisher Scientific)
3. Opti-MEM® I reduced serum medium (Thermo Fisher Scientific)
4. Hank's balanced salt solution (HBSS)
5. Imaging dishes (35 mm) with glass bottom

2.3 SOFI-Imaging

1. Download the software package Localizer [15], which also requires either the software IGOR Pro (Wavemetrics) or Matlab (MathWorks). Follow the included instructions to integrate the software.
2. Analysis computer with sufficient CPU power and memory to handle imaging files up to 1 GB
3. Microscope with TIRF capability and appropriate filters to excite and detect Dronpa (excitation peak at 503 nm and emission peak at 518 nm) and Venus (excitation peak at 515 nm and emission peak at 528 nm), optical components that result in a camera image exhibiting a pixel size close to or below the diffraction limit, and a 488 nm laser (for DMVF or Venus) and/or a 514 nm laser (for Venus) with at least 25 mW output (see Note 1). Furthermore, the hardware and software should allow recording image sequences with an exposure time of 35 ms or less.
4. Imaging buffer: HBSS with 2.0 g/l D-glucose, adjust pH to 7.4 with NaOH, filter-sterilized using 0.22 µm filters

3. Methods

3.1 Generating the constructs

The protocol below describes a typical approach to obtain expression plasmids containing c-terminal fusion constructs of the proteins of interest with the BiFC fragments of DMVF or Venus based on the constructs we generated. Accordingly, several alternative molecular cloning strategies can be used to combine the fragments with the proteins of interest on a genetic level more individually, which are not covered here.

1. The original plasmids are derived from pcDNA3 and follow a certain cloning scheme, therefore the cDNA of the proteins of interest has to be furnished with the appropriate restriction sites in order to insert them. The plasmids contain the

¹The required laser power is affected by a variety of microscope- and sample-dependent factors, such as camera sensitivity, optical efficiency, and protein expression. In order to examine the appropriate excitation power required for a given system, cells expressing membrane targeted Dronpa or Venus (full fluorescent proteins) can be used. To avoid excessive photobleaching, follow the instruction in 3.3.9 and compare the decrease in signal between two consecutive SOFI images.

BiFC-suitable fragments of Venus or DMVF (see Note 2) but are constructed similarly otherwise. The plasmids possess restriction sites for NheI, HindIII, BamHI, SpeI, KpnI, EcoRI and XbaI (5' to 3') and contain other designed features, illustrated in Fig. 1.

2. Design PCR primers for the cDNAs of your proteins of interest and add the suitable restriction sites so that it can be inserted into the original constructs (most likely HindIII and KpnI). Make sure that these restriction sites are not present in the original cDNA (see Note 3). Furthermore, insert at least 3 extra bases to ensure efficient restriction enzyme digestion and make sure that the cDNA is in frame. Also, add a Kozak sequence including a start codon when using HindIII. The SpeI site is unique in the construct, but not in the plasmid, so it can be utilized using alternative cloning strategies (see Note 4).
3. Prepare the samples for PCR reactions, for example by mixing 33 µl water, 10 µl 5X Phusion HF Buffer, 1 µl 10 mM dNTPs, 2.5 µl of the forward and reverse primer, 0.5 µl template DNA, and then adding 0.5 µl Phusion DNA Polymerase at the end. Run the PCR reactions in a thermocycler. The following is a sample protocol: preheat the block to 98 °C; 98 °C for 30 s; repeat for 25 times: 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s per kb; 72 °C for 10 minutes; hold temperature at 4 °C
4. Add 6 µl 10x restriction enzyme buffer and 2 µl of the enzymes corresponding to the primers to each PCR product and incubate at 37 °C for 30 minutes.
5. Mix 1 µg of the original plasmids containing the fragments of the fluorescent proteins, 2 µl 10x restriction enzyme buffer, 1 µl of each enzyme corresponding to the PCRs, and water for a total volume of 20 µl. Incubate the reaction at 37 °C for 30 minutes.
6. Prepare a 1 % agarose gel with TAE buffer, load the gel with the digested PCR products and original plasmids and run a agarosegelelectrophoresis at 110 V for 30 minutes.
7. Cut the DNA bands of the backbones of the original plasmids and the PCR products out of the gel and use an agarose gel DNA extraction kit to obtain the DNA.
8. Mix 2 µl of the backbone DNA solution, 13 µl of the PCR product, 4 µl of the 5x ligase buffer and 1 µl T4 DNA ligase and incubate at room temperature for 15 minutes.

²The fragments of the two fluorescent proteins DMVF and Venus that we utilized have different advantages. The fragments of DMVF have been optimized for BiFC assays with the reconstituted DMVF retaining the photophysical properties of Dronpa. It can be used to image complex assemblies that are formed by either constitutive or inducible but long-lasting interactions between proteins. The Venus fragments allow to detect complex formation within 30 minutes, therefore it is most suitable to observe acute signal-induced protein assembly.

³If the important restriction sites are also present in the cDNA of the protein of interest, restriction enzymes with compatible cohesive ends can be used to extend the PCR product. For example, BglII can be used to ligate to BamHI, and XbaI to ligate to NheI.

⁴If an alternative cloning strategy is used, ensure a flexible linker (e. g. GGGSGGGS) is used between the fragments and the proteins of interest.

9. Transform 5 µl of the ligation reactions into chemically competent DH5α bacteria using a common heat-shock protocol, spread the bacteria on LB agar plates with ampicillin, and incubate the plate over night at 37 °C.
10. Pick several single colonies from the plate to inoculate bacterial cultures (2 ml LB media with ampicillin). Incubate the bacterial cultures at 37 °C overnight.
11. Use a plasmid DNA miniprep kit to obtain the plasmids from the bacterial cultures. Verify that the plasmids contain the fusion construct of the cDNA of the proteins of interest and the fluorescent protein fragments, either by sequencing or by performing a test digest with the appropriate restriction enzymes.

3.2 Transfection of cells

1. Plate cells of the cell line of interest in imaging dishes one day prior to the transfection. Select the number of cells to seed so that the confluency reaches 75 % to 90 % on the following day.
2. For each imaging dish, mix 50 µl Opti-MEM® with 0.5 µg of each construct and 50 µl Opti-MEM® with 1.5 µl Lipofectamine 2000. Add the DNA solution dropwise to the Lipofectamine mix and incubate for 15 minutes at room temperature (see Note 5). It can be useful to add an additional construct with a different-colored FP serving as a marker for co-imaging and to facilitate imaging in case the interaction is constitutively weak.
3. Add the mix dropwise to the imaging dish and keep it in an incubator under the standard conditions for the employed cell line for at least 24 hours.

3.3 Imaging

1. Remove the growth media from the imaging dish and wash the cells twice with HBSS before adding the imaging buffer (HBSS with glucose).
2. Perform the imaging of the dish at your microscopy setup and at the appropriate temperature. Fix the dish to the microscope stage to prevent any movement.
3. Set the microscope up for imaging DMVF or Venus, and any other appropriate markers (see Note 6).
4. Switch the imaging software to the 'live'-mode to directly observe the fluorescence intensity as it would be recorded in subsequent experiments. Focus and adjust the TIRF angle appropriately (see Note 7) to ensure a high intensity of the excitation light exclusively close to the surface of the imaging dish. Select an exposure time of 35 ms or less and adjust the laser power and EM gain so that the fluorescence intensity values make proper use of the camera dynamic range.

⁵The transfection protocol was optimized for the systems we have described. Different inserts may require optimization by testing several DNA/Lipofectamine ratios. Read the manufacturer's protocol for the most updated information.

⁶The FKBP/FRB model system can be readily utilized to establish a refSOFI protocol on new equipment. The constructs we described can be transfected into HeLa cells; the interaction can be induced using 100 nM rapamycin.

⁷While TIR condition is not necessary for refSOFI, the observation of single-molecule fluctuation benefits from substantial z-axis rejection, which lends directly to high signal-to-noise ratio in refSOFI and high image contrast. It is worthwhile to optimize this factor for better results.

5. For reconstituted DMVF: Increase the laser power until you observe clearly visible fluorescence intensity fluctuations. Higher laser power improves the fluctuations behavior but increases photobleaching (permanent photodamage), therefore the laser power has to be carefully chosen to allow imaging over 10 s to 30 s without significant signal reduction due to photobleaching.
6. For reconstituted Venus: In our experience, the population of fluorescent Venus often has to be decreased (via photobleaching) in order to obtain suitable single-molecule fluorescence intensity fluctuations in protein complexes. If the cellular fluorescent image is bright, saturated, and intensity fluctuations could not be observed, increase the 514nm laser power to photobleach a fraction of the Venus molecules. This may only take several seconds, and single-molecule fluctuations should come into view. After the average fluorescence intensity of the image decreased by approximately half, reduce the laser power and check for visible single-molecule fluorescence intensity fluctuations again. Repeat if necessary.
7. Acquire an image series. To obtain a 2nd order refSOFI image (up to double resolution), record at least 200 images. For a 3rd order refSOFI image (up to triple resolution), record at least 800 images.
8. Use the imaging software to save your image sequence as a multipage-TIFF file (a single TIFF file containing all of the images in the sequence).
9. If the interaction of the proteins of interest can be induced, record an image series before induction and multiple image series after induction. For Venus, the formation of clusters of protein complexes can be monitored 30 minutes after they occur, while for DMVF, the cells should be incubated for at least 3 hours to allow reconstitution and maturation.
10. If an additional marker is used, also take images of it shortly before or after the image sequence for refSOFI is recorded. A marker that contains a photoswitchable FP such as rsTagRFP can also be used for SOFI, so that a super-resolution image of the protein complexes and the marker can be obtained from the same cell (example in Fig. 2).

3.4 Image data analysis

Here we describe how we analyze the image data using Localizer and the graphical user interface (GUI) using the software IGOR pro. Localizer is also available as a subroutine for Matlab and can be used analogously, though the GUI is specific to the Igor Pro version. See Fig. 3 for an illustration of the principles of refSOFI.

1. Open 'Igor Pro' and select the menu 'Localizer\Read CCD data\Read CCD data from disk...'. Use the dialog to select a TIFF file containing an image sequence.
2. Click on the button '>>' to open the localizer GUI. Select the tab 'SOFI'
3. Choose the desired cumulant Order (2nd or 3rd) and the number of pixel combinations. Subsequently calculate the combination weights by clicking on the button 'Calc weights'. The weights are then stored in an Igor Pro data

structure (wave) and can be used for the same order and to analyze image sequences that were recorded under the same conditions. Select the wave and the combination weights.

4. Choose a range of frames within the image sequence that will be used to calculate a SOFI image. It is important to exclude frames at the beginning that exhibit strong photobleaching and frames at the end that barely show a signal. For that purpose, the average intensity trace of the image sequence can be calculated to aid with estimating an appropriate range. Select the tab 'Other', pick 'Average Intensity Trace' from the drop-down list in the 'Basis analysis' area, and click the button 'Do it' in order to obtain the trace showing the average intensity with respect to the frame number. Furthermore, the image sequence has to be inspected for sections in which cells that are visibly moving by scrolling through it, and these sections have to be excluded as well (see Note 8).
5. Calculate the SOFI image by clicking the button 'Do it'.
6. The GUI also offers to apply the built-in 'Richardson-Lucy Deconvolution' to the SOFI image. In order to do that, select the standard deviation of the point spread function (PSF) in pixel, choose the number of iterations, and click on the button 'Do it' in the right bottom corner.
7. To save the results, the corresponding waves (Igor datasets) M_SOFI (SOFI image), M_DeconvolvedSOFI (deconvolved SOFI image), and M_SOFI_avg (average image of the image sequence) can be saved as waves using the menu 'Data\Save Waves\Save Igor Binary...' or as a tiff file using 'Data\Save Waves\Save Image...'. A window opens, and the particular waves can be found in the folder 'Packages\Localizer\LocalizerViewerX' where X represents the a counter that increments for every opened image stack. The command can then be executed directly ('Do It') or copied to the command line ('To Cmd Line'). Alternatively, one can make use of the ImageSave command (see appropriate documentation in Igor).

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⁸For live-cell imaging, the effect of probe diffusion could be of concern. However, the accuracy of SOFI imaging under these conditions has been examined, and it has been suggested that diffusion has an overall positive effect on the imaging [16].

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Fig 1.

Cloning scheme of the constructs in the template plasmids. All internal restriction sites except for BamHI are located in frame. The cDNA of FRB and FKBP and eventually the Lyn targeting sequence or the His-tag have to be replaced with the cDNAs of the proteins of interest.

Fig 2.

Using refSOFI to investigate the assembly of STIM1 and ORAI1. (A) representative refSOFI image of an unstimulated HeLa cell expressing STIM1-VC, VN-ORAI1, and ER-localized rsTagRFP (not shown). (B) cell shown in (A) 30 minutes after treatment with 1 μM thapsigargin (TG). (C) Multicolor image showing the STIM1/ORAI1 complexes (green) and the ER-localized rsTagRFP (red) after the treatment.

Fig 3.

Schematic representation of the principles important for refSOFI. (A) Two proteins of interest that are fused to fragments of DMVF or Venus are expressed in cells and the proteins are not interacting. Accordingly, there is no fluorescence when excited with 488 nm (DMVF) or 514 nm (Venus). If interactions take place and the complementary fragments get into nanometer proximity, the fluorescent protein is reconstituted and provides fluorescence intensity fluctuations when excited. (B) An image sequence is acquired, and the software package Localizer is used to carry out cross-cumulant analysis, generating the refSOFI image with an increased resolution.