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RFP tags for labeling secretory pathway proteins

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ABSTRACT

Red fluorescent proteins (RFPs) are useful tools for live cell and multi-color imaging in biological studies. However, when labeling proteins in secretory pathway, many RFPs are prone to form artificial puncta, which may severely impede their further uses. Here we report a fast and easy method to evaluate RFPs fusion properties by attaching RFPs to an environment sensitive membrane protein Orai1. In addition, we revealed that intracellular artificial puncta are actually colocalized with lysosome, thus besides monomeric properties, p*Ka* value of RFPs is also a key factor for forming intracellular artificial puncta. In summary, our current study provides a useful guide for choosing appropriate RFP for labeling secretory membrane proteins. Among RFPs tested, mOrange2 is highly recommended based on excellent monomeric property, appropriate p*Ka* and high brightness.

1. Introduction

Since the discovery of the first fluorescent protein (FP), the green fluorescent protein (GFP) from jellyfish, FPs have become invaluable imaging tools in the biomedical sciences and in cell biology studies. The appearance and further evolution of red FPs (RFPs) with red-shifted excitation and emission spectra make it possible to label two or more proteins simultaneously in a single cell, as well as extend the Forster resonance energy transfer (FRET) approach to protein–protein interaction studies [1,2]. Despite the growing role of currently developed photoactivatable [3,4] and photoswitchable RFPs [5,6] in advanced diffraction-unlimited imaging approaches, common RFPs is still the choice among FPs for many standard biological applications. Currently, numerous RFPs have been developed for protein labeling. However, they still have many pitfalls compared to the enhanced GFP (EGFP) and its derivatives.

Some RFPs, such as DsRed, are obligate oligomers and are therefore highly unsuitable for protein labeling because oligomerization can lead to mislocalization and inappropriate distribution of the fusion proteins [7,8]. Several "fruit" RFPs were subsequently developed to be monomeric [9]. However, when used for labeling membrane proteins in the secretion pathway, they tend to aggregate into bright punctate structures. In this study, we tried to understand the reason why RFPs tend to form artificial puncta by studying commonly used RFPs: mOrange1, mOrange2, mCherry, mApple, mKate2 and TagRFP, and we developed a membrane protein based assay to assess the fusion properties of RFPs. According to the results, a guideline was provided for choosing right RFPs.

2. Materials and methods

2.1. Plasmids

mCherry, mKate2, mOrange1, mApple, TagRFP and mOrange2 were PCR-amplified and inserted into mEos3.2-pRSETa with BamHI and NotI sites to replace the mEos3.2 and generate mCherry-pRSETa, mKate2-pRSETa, mOrange1-pRSETa, mApple-pRSETa, TagRFP-pRSETa and mOrange2-pRSETa, respectively. To express Orai1-fused fluorescent proteins in mammalian cells, cDNAs of mCherry, TagRFP, mOrange2, mApple, mKate2, were PCR-amplified and inserted into Orai1-EGFP-N1 with BamHI and EcoRI sites to replace the EGFP. Orai1-mGFP-C1 was obtained by inserting PCR-amplified mGFP into Orai1-EGFP-C1 with BamHI and EcoRI to replace the EGFP.

2.2. Protein expression and purification

The proteins of mCherry, mKate2, mOrange1, mApple, TagRFP and mOrange2 were expressed in *Escherichia coli* strain BL21

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(DE3) and purified with Ni-NTA His-Bind resin (Qiagen), followed by a gel-filtration step using a Superdex200 column (GE Healthcare). For the followed analysis, purified proteins were concentrated by ultrafiltration and diluted in PBS.

2.3. Analysis of oligomerization

All proteins are concentrated to 3–4 mg/ml before analysis. The oligomeric states of all proteins were checked by analytical ultracentrifuge assays. Sedimentation equilibrium experiments were performed on a Beckman Optima XL-I analytical ultracentrifuge at 20 °C as previously described [10]. Purified proteins were loaded into 6-channel centrifugation cells and normalized to the corresponding dialysis buffer (PBS, pH 7.4). Samples were centrifuged at 15,000 r.p.m. and 20,000 r.p.m. sequentially. The data were analyzed by nonlinear least-squares analysis using the software package (Origin 6.0) supplied by Beckman coulter. Simultaneously, all purified proteins were diluted to 1.3 mg/ml with PBS and loaded into 2-channel centrifugation cells for sedimentation velocity experiments. Samples were centrifuged at 60,000 r.p.m. and the data were analyzed by SEDFIT software.

2.4. Cell culture and transfection

HEK293 cells were incubated at 37 °C with Dulbecco's Modified Eagle Medium (DMEM) complete medium (Gibco) containing 10% FBS in a humidified incubator containing 5% CO_2. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After transfection, cells were cultured with DMEM complete medium (Gibco). 24 h after transfection, the cells were plated at lower density on clean coverslips (Fisher Scientific) to induce spreading for another 24 h in DMEM (Gibco) complete medium for live cell imaging.

2.5. Confocal microscopy and cell imaging

Confocal microscopy images of mCherry, mKate2, mApple, TagRFP, mOrange2 and mGFP were obtained by using FV1000 laser-scanning confocal microscope (Olympus). All the pictures were obtained using a 60×, 1.4 numerical aperture (NA) oil-immersion objective. For lysosome alkalization experiment, 75 mM NH_4Cl was added to cell before image acquisition. Images were quantified and analyzed using FluoView software (Olympus) and ImageJ (US National Institutes of Health).

2.6. TIRFM imaging

We used an Olympus IX71 inverted microscope equipped with a 100×, 1.45 NA oil objective (Olympus PLAN APO). The fluorescence signals were acquired using an electron-multiplying charge coupled device (EMCCD) camera (Andor iXon DV-897 BV). The maximum power near the rear pupil of the objective was 2.54 mW for the 561 nm laser (Cobolt Jive). Data analysis was performed by ImageJ (US National Institutes of Health) and Igor Pro 6 (Wave Metrics).

3. Results and discussion

3.1. The monomeric properties of commonly used RFPs

One main reason of RFPs oligomerization is that they are not true monomers as previously reported. When expressed at high levels as fusion tags in living cells, they tend to form obligate oligomers, and aggregation may follow oligomerization. Many RFPs are identified as monomers using an *in vitro* gel-filtration method, which is largely dependent on the concentration of the loaded purified RFP proteins. While the exact dissociation constant (K_d) values for these RFPs are not complete, in the current study, we provide the K_d values for commonly used RFPs using an accurate ultracentrifuge analysis [6,11]. The results show that mKate2 and TagRFP are weak dimers with K_d of 49.2 and 38.4 μM, respectively, and are not monomeric as previously reported [9,12]. In contrast, mCherry, mApple, mOrange1 and mOrange2 are true monomers with K_d beyond the detection limit of our instruments (Table 1, Supplementary Fig. 1).

Table 1
The monomeric properties of RFPs.

RFP	M_1 (15,000 rpm)	M_2 (20,000 rpm)	K_d	Oligomerization
mCherry	21,699	21,844	/	Monomer
mApple	25,046	20,444	/	Monomer
mKate2	41,672	43,752	49.6 μM	Dimer
mOrange2	28,402	21,261	/	Monomer
mOrange1	27,642	21,602	/	Monomer
TagRFP	51,512	44,463	38.4 μM	Dimer

/ represents a very high K_d that is beyond the limit of our instrument. The concentration of RFPs is 3–4 mg/ml.

3.2. Distribution of the artificial puncta

Besides the true monomeric properties of RFPs, another reason for the artificial punctate localization is that some proteins, such as membrane proteins, has confined or constrained microenvironments, which make the proteins very sensitive to the oligomeric state of the FP tag. The local concentration of the RFP-labeled membrane proteins might be higher than that used for an *in vitro* gel-filtration assay. Currently, two visual assays in living cells have been developed to report on the tendency of fluorescent proteins to oligomerize using intracellular fusion proteins as probes [13,14]. However, a plasma membrane protein-based assay has not been developed. Here we show that the calcium channel protein Orai1, which is important for multiple biological processes, is very sensitive to RFP-tag aggregation. Under confocal microscopy and TIRFM, mKate2 and TagRFP-tagged Orai1 showed apparent punctate distribution on the plasma membrane, which was absent when Orai1 was labeled with mCherry, mApple, and mOrange2 (Fig. 1 and Supplementary Fig. 2). These results are consistent with the K_d data of the RFPs, suggesting that the distribution of Orai1-FP on the plasma membrane can be used as an assay to report on the oligomerization tendency of fluorescent proteins in living cells. In contrast, in intracellular perinuclear regions, mOrange2 and mApple showed no or little punctate localization, while other RFPs showed apparent puncta distribution (Figs. 1 and 3).

3.3. The intracellular puncta are located in lysosome

We next identified the exact cellular localization of these puncta. We found that the intracellular puncta of Orai1-mCherry and Orai1-TagRFP colocalized with the lysosomal marker (Fig. 2), suggesting that some of the RFP-tagged proteins are destined for degradation in the lysosome. It is reported that all fluorescent proteins have pH dependent absorption and emission spectrum, which can be described by p*K*a, a key parameter represents the pH sensitivity of the FP. The p*K*a value equals to the pH at which the absorbance or the emission fluorescence of the FP reaches the half maximum [15,16]. Thus considering the very acidic environment of lysosomes and the different pK_a of RFPs [9], we proposed that the diminished puncta localization of mOrange2 and mApple was largely due to their relatively high pK_a values. To prove that, we expressed mOrange2-labeled Orai1 in HEK293 cells