Local Generation and Imaging of Hydrogen Peroxide in Living Cells

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Described here is a localized H_2O_2 generation-detection system consisting of a yeast D-amino acid oxidase (DAAO) and two spectrally distinct variants of biosensor, HyPer2 and HyPerRed based on circularly permutated yellow and red fluorescent proteins, respectively, which enables spatiotemporal production and examination of the intracellular H_2O_2 dynamics. The protocol describes using this system in a simple cell culture model. We provide detailed instructions on imaging of H_2O_2 generated by the activated DAAO. The system can be easily optimized for various combinations of cell types, conditions and DAAO/sensor subcellular localizations. © 2017 by John Wiley & Sons, Inc.

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INTRODUCTION

Cellular regulatory molecules are the subject of intensive studies and there are particular complications in studying each of them. Reactive oxygen species (ROS) are extremely toxic at high concentrations and are capable of causing damage to almost all biomolecules under pathological conditions called oxidative stress (Sies, Cadenas, Symons, & Scott, 1985; Valko et al., 2007). However, at low concentrations, some of ROS, namely H_2O_2, act specifically on protein thiol groups and participate in fine-tuning of cell signaling cascades (Garcia-Santamarina, Boronat, & Hidalgo, 2014; Kishida & Klann, 2007). Most cell types express NADPH-oxidase (Nox) family enzymes, inducible protein complexes that generate ROS in response to growth factors, cytokines and other stimuli (Bedard & Krause, 2007; Huang, Chen, Hang, Liu, & Zhong, 2015; Kim & Kim, 2015; Leto, Morand, Hurt, & Ueyama, 2009; Liang et al., 2016; Mishina et al., 2011; Rochfort, Collins, Murphy, & Cummins, 2014).

H_2O_2 is chemically stable compared with other ROS. It may even pass through biological membranes (Winterbourn, 2008). But to control its potential harmfulness in cells there are several antioxidant systems that provide a rapid breakdown of all ROS, including H_2O_2 (Antunes & Cadenas, 2000). Due to this fact, H_2O_2 acts locally at the points of its production and it reacts only with local environment (Chen, Kirber, Xiao, Yang, & Keaney, 2008; Mishina et al., 2011; Mishina et al., 2015).

Still, evaluation of biological effects of H_2O_2 requires either an external supply of the oxidant or stimulation of endogenous ROS production. In the first case, externally

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added H_2O_2 would react with all redox-sensitive cysteines nonspecifically, therefore the spatial location of any redox signaling cannot be determined. In the second case, the inevitable activation of numerous upstream signaling and metabolic pathways complicates interpretation of the results. A good example is the activation of Nox enzymes: sometimes it requires simultaneous activation of several upstream pathways such as PI3K, PKC, Rac1 and others. Even in case of resulting ROS production by Nox it is difficult to draw conclusions about the relative input of redox signaling on cellular physiology.

A good approach in this case is the involvement of enzymes capable of ROS production from substrates that do not participate in main metabolic pathways of the system studied. In this article, we describe an application of a fully genetically encoded system for localized H_2O_2 production by yeast catabolic enzyme D-Amino acid oxidase (DAAO) and its detection using biosensors HyPer2 and HyPerRed. DAAO can be localized to various cellular compartments where it generates H_2O_2 when supplied with D-amino acid. In this case no upstream signaling pathways are induced to generate ROS and therefore H_2O_2 production is the first step of the cascade.

STRATEGIC PLANNING

Making Localized Versions of DAAO and HyPer

The original HyPer2 and HyPerRed plasmids are available from Addgene (plasmid #42211 and #48249).

HyPer probes generally can be used in a non-localized version that diffuses between nucleus and cytoplasm (Belousov et al., 2006; Bilan et al., 2013; Ermakova et al., 2014; Markvicheva et al., 2011). This is preferable when no information except temporal H_2O_2 dynamics in the cell is needed. However, the distribution of H_2O_2 in the cell is very inhomogeneous with localized sources and targets (Ermakova et al., 2014; Mishina et al., 2011; Mishina et al., 2015). If particular compartments are of specific interest, we recommend that localized versions of the sensors be used as they provide much higher spatial and temporal resolution. Examples are mitochondrial matrix and intermembrane space, the cytoplasmic sides of endoplasmic reticulum membrane, plasma membrane and endosomes (Belousov et al., 2006; Ermakova et al., 2014; Mishina et al., 2011; Mishina et al., 2015). The same principle can be applied to DAAO as described in the current protocol.

Localization of specific proteins can be accomplished in two ways. First, the encoding DNA sequence can be modified by the addition of special localization signals in-frame with the protein open reading frame (ORF). Most of the sequences for organelles' localization signals are well known and are easy to find in the literature or in a public plasmid repository. Some of the subcellular localization tags are added to N-terminus (for example, mitochondrial localization signal), some are C-terminal (examples are nuclear localization and peroxisomal targeting signals). Alternatively, it is possible to use proteins with strict cellular compartmentalization as an anchor. In this case, two proteins must be fused with a flexible or rigid linker. Examples are fusions of HyPer with EGF receptor bringing the probe to the plasma membrane and cytoplasmic surface of the endosomes (Mishina et al., 2011) or with microtubule-binding protein EB3 anchoring the sensor on the microtubules (Mishina et al., 2015). A high-resolution crystal or NMR structure can serve as a useful guide for choosing linker characteristics as well as previously described similar constructs (usually fusions with conventional fluorescent proteins). Correct localization of the fused proteins has to be confirmed by co-localization studies with fluorescent proteins with well-established proper compartmentalization or organelle-specific synthetic dyes, or using -cytochemical staining with antibodies.

Choosing Transfection Method

Liposome transfection is the simplest way to obtain transient transfection of cells like HeLa or HEK293 or other easy-to-transfect lines. We routinely use FuGeneHD transfection reagent, another good option is Lipofectamine LTX with Plus Reagent. For hard-to-transfect cell types (primarily cell lines, some immortalized cell lines) lentiviral transduction or nucleofection are preferred.

The transfection protocol must be optimized for successful co-transfection of several DNA constructs. Different amounts of DNA, the DNA/transfection reagent ratio and the ratio of transfected plasmids can affect greatly transfection efficiency and cell viability. Manufacturers' protocols and other sources (Asgharian, Banan, & Najmabadi, 2014; Jacobsen, Calvin, Colvin, & Wright, 2004; Jordan & Wurm, 2004) contain many tips on transfection optimization.

Choosing Excitation and Emission Wavelengths

Ratiometric time series acquisition is simple with green-emitting versions of HyPer (HyPer, HyPer2, HyPer3). They have two excitation peaks. The 420-nm peak is excited on a wide-field microscope using a filter set transmissive for the 400 to 430 nm range. The 500-nm peak can be excited with a wide-field filter set enabling excitation in the 470 to 500 nm range. In both cases, the emission is detected in the 510 to 550 nm range. Sequential frame-by-frame switching between ex420/em520 and ex500/em520 results in two series of images. Ratiometric series are produced using frame-by-frame division of ex500/em520 images by ex420/em520 images.

HyPerRed has an excitation peak at 575 nm and an emission peak at 605 nm. It requires a filter set enabling excitation in the 550 to 590 nm range and detection of emission at 600 to 660 nm.

We performed all imaging procedures on a Leica DMI6000 microscope equipped with the Leica CFP/YFP FRET filter system (set 11522073). This system consists of CFP excitation (CFPex, BP 427/10) and emission filters (CFPem, BP 472/30) and the same components for YFP (BP 504/12 and BP 542/27 for YFPex and YFPem respectively). The high-speed HyPer2 imaging can be carried out with this system, acquisition settings for the 420-nm peak—CFPex/YFPem, for 500-nm peak—YFPex/YFPem. The "FRET" filter cube with a dichroic mirror only (440/520 nm) was used for HyPer2 acquisition, HyPerRed was imaged with filter cube TX2 (excitation: BP 560/40, dichroic mirror 595, emission: BP 645/75). However, for both HyPer2 and HyPerRed other suitable filter sets can be used.

H_2O_2 GENERATION IN THE NUCLEUS OR CYTOPLASM AND REAL-TIME IMAGING AT BOTH COMPARTMENTS

BASIC PROTOCOL

D-amino acid oxidase (DAAO) from yeast Rhodotorula gracilis catalyzes oxidative deamination of D-amino acids producing hydrogen peroxide (Fig. 1) (Matlashov, Belousov, & Enikolopov, 2014; Pollegioni et al., 1993). Mammalian cells use mostly L-amino acids and the concentration of D-amino acids in the cells is negligible. Therefore, it is possible to regulate DAAO activity (and thus production of H_2O_2) by substrate availability. Moreover, DAAO can be targeted to different cellular compartments using special localization signals.

A genetically encoded ratiometric fluorescent sensor for H_2O_2 HyPer consists of two parts: bacterial OxyR regulatory domain and circularly permutated YFP (cpYFP) (Fig. 1) (Belousov et al., 2006). OxyR part is capable of selective oxidation by a low concentration of H_2O_2. This causes structural changes in the domain and consequently in cpYFP.

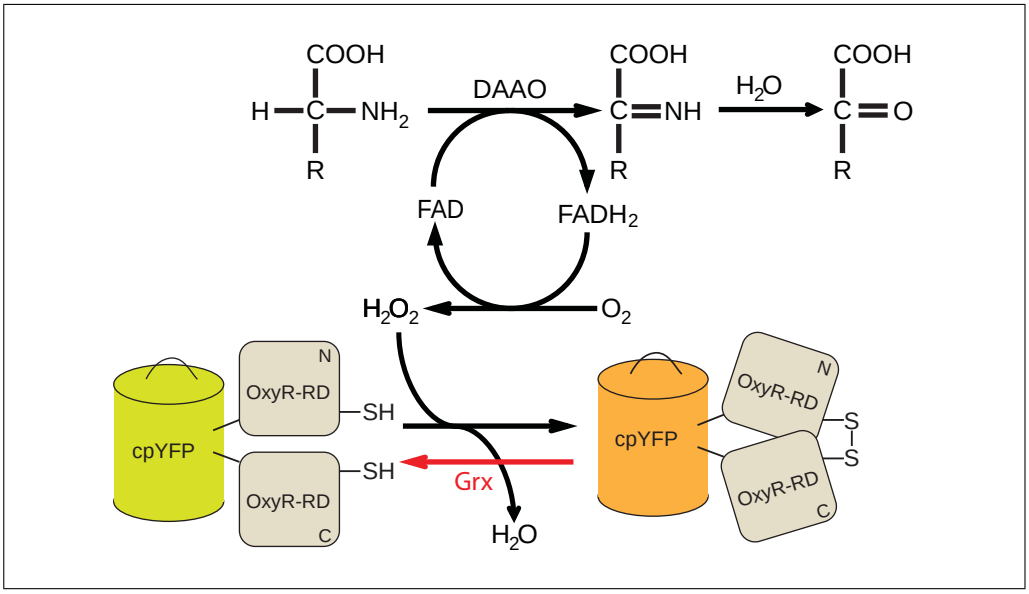

Figure 1 Scheme of the reaction catalyzed by DAAO with H_2O_2 detection by HyPer. Oxidative deamination of D-amino acid produces H_2O_2 that can be detected by the HyPer family probes. Reduction of HyPer by glutaredoxin (Grx, red arrow) makes the sensor signal reversible.

Therefore, in the presence of H_2O_2 the fluorescence excitation spectrum of HyPer changes in a ratiometric manner: decrease in 420 nm peak and increase in 500 nm peak. Because of the protein nature of the sensor it can be localized at almost any cellular compartment with the addition of a special targeting tag to its amino acid sequence (Belousov et al., 2006; Markvicheva et al., 2011). Second and third versions of HyPer were developed, both having expanded dynamic range compared to the original sensor (Bilan et al., 2013; Markvicheva et al., 2011). In addition, HyPer2 has greater brightness compared to HyPer and HyPer3 when expressed in eukaryotic cells, and HyPer3 has faster oxidation and reduction. A later red fluorescent version of the biosensor, HyperRed, was developed to enable multiparameter imaging in different compartments simultaneously (Ermakova et al., 2014).

Signaling H_2O_2 acts locally, near the sites of its generation (Mishina et al., 2011). Therefore, both instruments, DAAO and HyPer family probes, comprise a unique set of tools for local generation and monitoring of the oxidant. In this protocol, we use versions of DAAO, HyPer2 and HyPerRed with nuclear export (NES) and nuclear localization (NLS) signals. Nuclear transport systems interact with short signal peptides added on the C-termini of the proteins and actively transport them inside or outside the nucleus. These two compartments are easily recognizable under the microscope and can be confirmed with DAPI staining. Therefore, H_2O_2 will be generated either within the nucleus or cytoplasm and detected simultaneously in both compartments.

Materials

Mammalian cells
Appropriate cell culture medium (e.g., complete growth medium; see recipe)
Plasmids: HyPer2-NES (version with nuclear export signal, NES), HyPerRed-NLS (version with nuclear localization signal, NLS), and one of the DAAO variants: DAAO-NES or DAAO-NLS
OPTI-MEM (ThermoFisher Scientific, cat. no. 31985-047)
FuGeneHD transfection reagent (Promega, cat. no. E2311)
Tyrode's Salts (Sigma-Aldrich, cat. no. T2145)
HEPES (Promega, cat. no. H5302)
Imaging medium (see recipe)

H_2O_2 (Sigma–Aldrich, cat. no. 516813)
Alanine stock solution (see recipe)

Glass-bottom 35-mm dishes (MatTek, cat. no. P35G-1.5-10-C)
37°C, 5% CO_2 incubator
Wide-field fluorescent microscope equipped with a thermostatic box
Mercury or xenon arc lamp
ImageJ or Fiji open software

Transfect cells

1. Plate cells on glass-bottom dishes in the culture medium (e.g., complete growth medium) recommended for the particular cell line. Wait for at least 12 hr prior to the transfection procedure.
2. Transfect the cells with three plasmids encoding HyPer2-NES (version with nuclear export signal, NES), HyPerRed-NLS (version with nuclear localization signal, NLS), and one of the DAAO variants: DAAO-NES or DAAO-NLS. Use FuGeneHD transfection reagent mixed with the plasmid DNA in OPTI-MEM medium or other appropriate transfection methods.

   Intensive mixing of the plasmids is absolutely necessary before and after addition of transfection reagent for uniform transfection.

3. Culture the cells for 24 to 48 hr at 37°C, 5% CO_2.

   HyPer maturation proceeds quickly under these conditions. However, depending on the cell line used appropriate intracellular localization of the proteins achievement of a reliable signal brightness level may take some time. We do not recommend imaging cells earlier than 24 hr post-transfection.

Prepare transfected cells for the experiment

4. Begin to warm the thermostatic box to 37°C 2 hr before placing the dish onto the microscope stage.
5. Replace complete medium with 1.5 ml preheated (37°C) Tyrode's salt solution supplemented with 20 mM HEPES and additional 20 mM glucose or another imaging medium of choice.

   The use of media containing phenol red and/or high riboflavin causes unwanted background fluorescence.

   Avoid using HEPES concentration higher than 25 mM in order to avoid cellular toxicity.

Perform primary visualization

6. Use a mercury or xenon arc lamp with GFP or FITC filter sets for HyPer2-NES and TX2 or TRITC filter sets for HyPerRed-NLS to carry out transfection control and primarily visualization of the probes.

   At this step, pseudobleaching of HyPer2-NES can be seen. The deprotonated form of YFP chromophore undergoes photoconversion to the dark state upon irradiation with near-500 nm light. It can be easily reversed by brief (1 to 2 sec) illumination with near-420 nm light exciting the protonated form of the chromophore. For visualization of cellular compartments, we recommend using 40 or 63 Plan Apochromat objectives with high numerical aperture or other high magnification objectives.

Adjust imaging settings

7. Set up the microscope for HyPer time-lapse imaging. Use three channels for imaging: two for the 420 nm and 500 nm excitation peaks of HyPer2-NES and one for HyPerRed-NLS. Activate camera binning, the same for all channels, if needed. This minimizes light exposure and avoids phototoxicity.

8. Use filter cube "FRET"equipped with a dichroic mirror (440/520 nm).
9. Activate excitation filter CFP (BP 427/10) for the 420-nm peak excitation.
10. Activate excitation filter YFP (BP 504/12) for the 500-nm peak excitation.
11. Activate emission filter "YFP" (BP 542/27) for both channels.
12. Use filter cube "TX2" equipped with a dichroic mirror (BP 560/40 nm) for HyPerRed-NLS.
13. Set up 420-nm peak excitation: light intensity 2 to 5, exposure time 20 to 200 msec.
14. Set up 500 nm peak excitation: light intensity 2–5, exposure time 20–200 msec.
15. Set up RFP excitation: light intensity 2 to 5, exposure time 20 to 200 msec.

    Light can cause phototoxic effects in imaged cells. Try to minimize light exposure.

16. Add transmitted light channel if cell morphology visualization is needed.
17. Adjust rate of recording. Usually, one frame per 5 to 10 sec is enough to visualize time course of H_2O_2 production.

Image cells

18. Place the dish onto the microscope stage with lid open. Find the appropriate focus.
19. Change system to live mode.
20. Find a group of cells characterized by normal morphology, bright sensors and lack of fluorescent protein aggregates.

    In a case of low transfection efficiency, two or three groups of cells clearly originated from different transfected precursor cells increase the chance to find cells expressing cryptic non-fluorescent DAAO. In addition, multiposition imaging can be a good option if available on the microscope.

21. Proceed with several single scans. At this step set final focus, control light intensity and exposure time. Fluorescence intensities at all channels should be similar within intracellular ROI at different compartments.

    HyPer2 signal excited at 500 nm at the maximum oxidation point will be up to 5-fold higher than in the reduced state. HyPerRed demonstrates ~2-fold maximal signal change. Avoid excessively bright images of the cells at the baseline to prevent further saturation of the signal upon oxidation of the probes. Hi-Lo display of scans may be convenient for the adjustment.

22. Start time-lapse imaging recording. The microscope focus should be stable during baseline recording time. Re-start imaging after proper dish fixation on the microscope stage in case of out of focus frames. Whenever autofocus can be engaged, activate this option.
23. Stimulate cells with D-amino acid: After 2 min of imaging add 200 to 500 µl of the prewarmed D-alanine (alanine stock solution) diluted from stock solution in Tyrode to final concentrations 0.2 to 8 mM. Changes in HyPer2 and HyPerRed fluorescent intensities start within minutes after stimulation.

    The addition should be performed with great care, drop-by-drop over the entire surface of the media, without touching the microscope stage or dish. The L isoform of the amino acid can serve as a negative control. HyPer is sensitive to pH. For pH control use C199S mutant form of sensor, SypHer.

24. At the end of imaging series, make an addition of H_2O_2 diluted in 100 µl of Tyrode to final concentrations 50-200 µM for maximal oxidation of HyPer2 and HyPerRed.

    CAUTION: *H_2O_2 may cause severe skin burns and eye damage. Follow the safety guide.*

Analyze data

25. Import the time series into ImageJ (*https://imagej.nih.gov/ij/*) or Fiji (*http://fiji.sc*) open software. LOCI plugin allows importing various bioimage formats. Activate the "split channels" option in the LOCI pop-up menu. For each channel choose background ROI, measure the mean intensity and subtract it from all frames in the stack. Convert HyPer2 stacks to 32 bit. Use threshold function for the 420-nm stack to eliminate pixel values in the background. Divide the 500-nm stack by 420-nm stack frame by frame using **Image Calculator** to obtain the ratio stack. HyPerRed stack requires only BG subtraction. Calculate HyPer2 ratio or HyPerRed fluorescence for ROI chosen inside the cytoplasm or the nucleus within the ratio stack or HyPerRed stack, respectively.

REAGENTS AND SOLUTIONS

Use the deionized, distilled water in all steps and recipe, unless indicated otherwise.

Alanine stock solution

Dissolve D- (Sigma–Aldrich, cat. no. A7377) and L-Alanine (Sigma–Aldrich, cat. no. A7627) in Milli-Q purified water or equivalent to a concentration of 1 M. Divide into 0.2-ml aliquots. Store aliquots up to 6 months at −20°C.

Complete growth medium

Dulbecco's modified Eagle's medium (ThermoFisher Scientific, cat. no. 41965-039)
100 U/ml Penicillin-Streptomycin (ThermoFisher Scientific, cat. no. 15070-063)
10% (v/v) fetal bovine serum (ThermoFisher Scientific, cat. no. 16000-044)
Store up to 1 month at 4°C

Imaging medium

Tyrode's Salts solution (Sigma-Aldrich, cat. no. T2145)
20 mM HEPES (Promega, cat. no. H5302)
20 mM D-Glucose
Adjust pH to 7.4 using NaOH, if necessary
Store up to 1 month at 4°C

COMMENTARY

Background Information

D-amino acid oxidase is an enzyme that uses FAD as a cofactor for the oxidative deamination of D-amino acids (Pollegioni et al., 2002). During a catalytic cycle, deamination of D-amino acid occurs with the formation of imino acid and reduction of FAD. Molecular oxygen rapidly re-oxidizes flavin with the generation of H_2O_2, the imino acid undergoes hydrolysis to α-keto acid and ammonia after dissociation from the enzyme. With localized expression of DAAO, production of H_2O_2 at various compartments can be controlled by addition of different amounts of D-amino acid in the cellular medium since typical eukaryotic amino acid transporters have a low specificity for L- or D-isoforms.

Dynamic real-time imaging of H_2O_2 is another challenge in understanding of ROS biological functions. To date, several methodologies for H_2O_2 detection have been developed, and most of them are based on the oxidation of reduced dyes accompanied by changes in fluorescent properties (Gomes, Fernandes, & Lima, 2005). Some of them have distinct localization in mitochondria or nucleus, some are ratiometric, but all of them undergo irreversible oxidation (Woolley, Stanicka, & Cotter, 2013). These flaws can be overcome through the use of genetically encoded biosensors.

GFP and its mutated variants are proteins with very rigid structures. Subtle changes in chromophore conformation have a great impact on the spectral characteristics. A circular permutation method for fusing natural N- and C-termini of fluorescent protein with a peptide linker and inserting a new terminus near

Table 1 Troubleshooting

Step	Problem	Possible cause	Solution
4	Low level of transfection or cotransfection	Suboptimal transfection protocol	Optimize transfection conditions
5	Low brightness level	Low expression level of HyPer	Incubate cells for another 24 hr to obtain more expressed protein
6	Rapid decrease in the fluorescence intensity at yellow-green channel	Photoconversion of the chromophore into the non-fluorescent state	Wait for several minutes or illuminate the sample at cyan channel
7	No H_2O_2 generation after D-amino acid addition	Imaging medium pH is not 7.4	Change imaging medium
		Low concentration of D-alanine	Use greater D-alanine concentrations
	H_2O_2 signal is not localized	Too high D-alanine concentration	Use lower D-alanine concentrations

the chromophore allowed creation of a number of biosensors. As mentioned above, the biosensor HyPer contains circularly permuted YFP integrated into the regulatory domain of transcriptional factor OxyR from *E. coli*. Two amino acids of OxyR are redox-sensitive cysteine residues. In the presence of H_2O_2 one of the residues undergoes oxidation to sulfenic acid, leaves the hydrophobic pocket and interacts with the second cysteine residue. The resulting disulfide bridge changes the conformation of the whole biosensor and the intensity of its fluorescence simultaneously. In case of HyPerRed, circularly permuted version of RFP was integrated into OxyR.

Critical Parameters

Choice of D-amino acid

DAAO is capable of catalyzing the deamination reaction of neutral and polar amino acids. D-Alanine is a preferred substrate for DAAO because of the easily metabolized final product, pyruvate. Furthermore, addition of D-alanine in media does not cause pH changes inside and outside cells. Other D-amino acids can be used as well, however, addition of some of them, for example D-Arg, can substantially change pH. Chromophores of HyPer and HyPerRed are pH-sensitive. The spectral shift that occurs upon elevation of pH mimics that during oxidation whereas acidification resembles reduction. Therefore, we advise using SypHer (Belousov et al., 2006; Addgene plasmid #42213), or SypHer2 (Matlashov et al., 2015), or HyPerRed-C199S (Ermakova et al., 2014; Addgene plasmid #48252) genetically encoded pH probes to monitor pH in parallel experiments. SypHer and SypHer2 are C199S mutants of HyPer and HyPer2, respectively. They are not sensitive to H_2O_2, but retain pH sensitivity of the parental H_2O_2 sensors.

Choice of localization signal

Careful consideration must be given to the choice of localization signals. Small targeting peptides can be easily added to the protein sequence by general molecular biology methods. In some cases (NLS), better localization can be achieved by multiplying the same localization signal several times. Fusing biosensor or DAAO with strictly localizing proteins should be done keeping in mind cellular sorting mechanisms, natural interactions of the protein with other molecules and its topology that is critical for transmembrane proteins.

Troubleshooting

See troubleshooting Table 1

Anticipated Results

This Basic Protocol allows local production of H_2O_2 and visualization of its dynamics in two compartments simultaneously. Depending on localization of DAAO and cell line chosen different patterns of H_2O_2 distribution may be observed. A wide range of H_2O_2 concentration (from low nanomolar to submicromolar) can be created at different

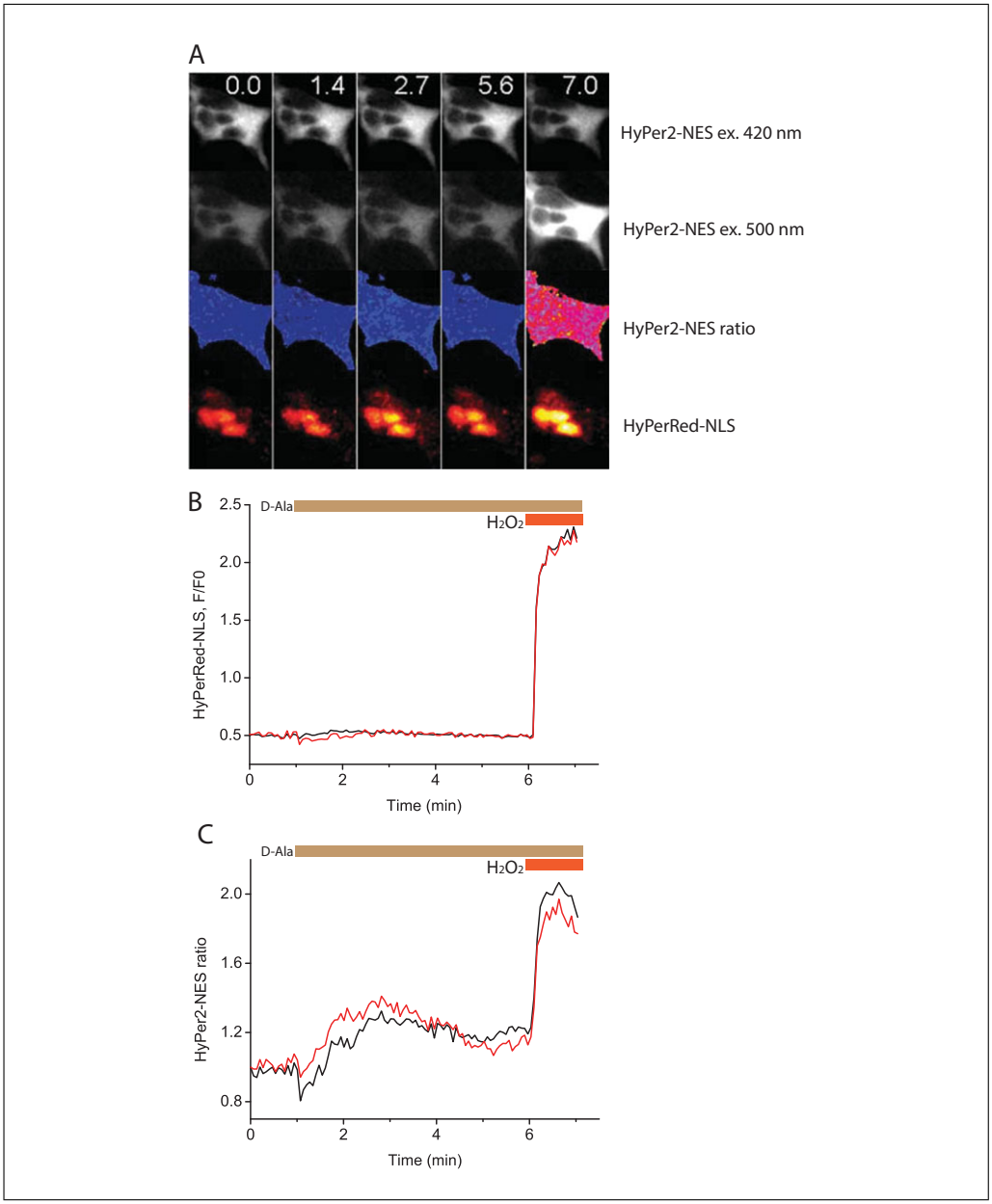

Figure 2 DAAO-NLS driven H_2O_2 production in HeLa-Kyoto cells imaged using HyPerRed-NLS and HyPer2-NES. (**A**) Series of images of the cells in two HyPer2 imaging channels, in HyPer2 ratio channel and in HyPerRed channel. Numbers indicate time in minutes. Scale bar 10 μm. (**B**) Sample traces of HyPer2-NES fluorescence ratio in response to D-Ala and H_2O_2. (**C**) Sample traces of HyPerRed-NLS fluorescence in response to D-Ala and H_2O_2.

compartments with various impacts on cell physiology. Figures 2 and 3 show sample results of the experiments carried out according to the Basic Protocol. Figure 2 demonstrates response of the cells expressing DAAO-NLS to the addition of 0.25 mM D-Ala. This amount of the substrate leads to generation of low amount of H_2O_2 that cannot exit the nucleus. Therefore, only HyPerRed-NLS becomes partially oxidized. Figure 3 demonstrates the response of the cells expressing DAAO-NES to the addition of 0.6 mM D-Ala. This amount of D-Ala is sufficiently high to elicit responses of both HyPer2-NES and HyPerRed-NLS.

Time Considerations

Once all plasmids encoding localized DAAO and HyPer are available, two incubation points during cell culturing procedures will take 2 days, imaging can be performed on the third day and could be completed within a day of work or less. However, up to one week is needed for transfection optimization and localization validation.

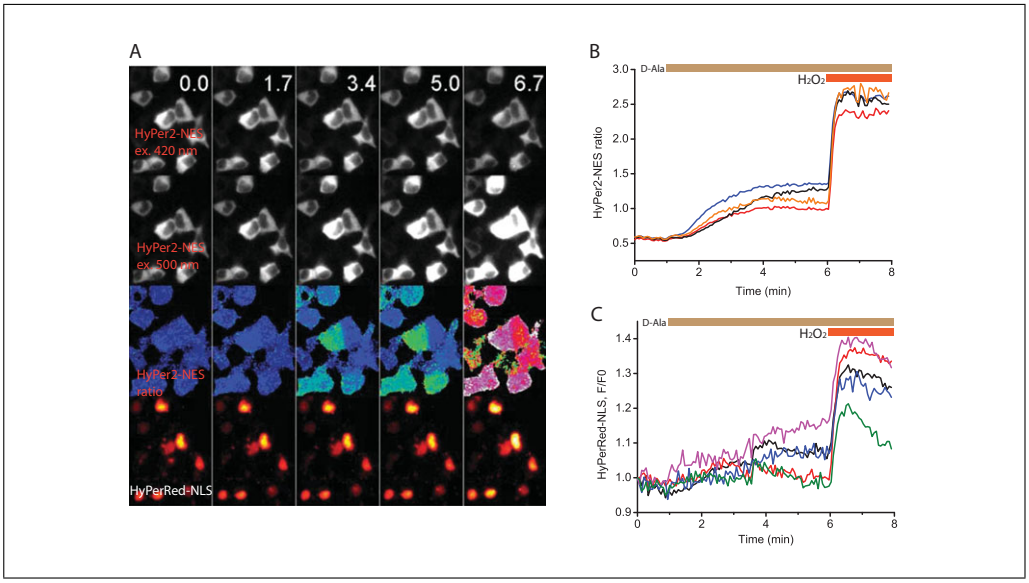

Figure 3 DAAO-NES driven H_2O_2 production in HeLa-Kyoto cells imaged using HyPerRed-NLS and HyPer2-NES. (**A**) Series of images of the cells in two HyPer2 imaging channels, in HyPer2 ratio channel and in HyPerRed channel. Numbers indicate time in minutes. Scale bar 30 μm. (**B**) Sample traces of HyPer2-NES fluorescence ratio in response to D-Ala and H_2O_2. (**C**) Sample traces of HyPerRed-NLS fluorescence in response to D-Ala and H_2O_2.

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