Visualization of Intracellular Hydrogen Peroxide with HyPer, a Genetically Encoded Fluorescent Probe

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Abstract

The fluorescent sensor HyPer allows monitoring of intracellular H2O2 levels with a high degree of sensitivity and specificity. Here, we provide a detailed protocol of ratiometric imaging of H2O2 produced by cells during phagocytosis, including instructions for experiments on different commercial confocal systems, namely, Leica SP2, Leica SP5, and Carl Zeiss LSM, as well as wide-field Leica 6000 microscope. The general
1. INTRODUCTION

Cells use oxygen not only for energy demands but also to produce reactive oxygen species (ROS). Superoxide anion radical, hydroxyl radical, hydrogen peroxide, and nitric oxide are the most investigated ROS in biological systems (Dröge, 2002). ROS are capable of generating nonspecific damage to biomolecules under pathological conditions, a process summarized as oxidative stress. At the level of physiological regulation, subtoxic production of ROS, namely H$_2$O$_2$, is used by cells to modify signaling cascades via selective oxidation of a relatively small number of protein thiol groups (Winterbourn, 2008). Since the 1960s, ROS production was mainly investigated in the context of pathogen killing by phagocytes. Phagocytes, namely neutrophils and macrophages, represent the first line of host defense against pathogens such as fungi and bacteria. Phagocytes engulf opsonized microbial cells into endocytic phagosomes, ultimately leading to pathogen destruction. The main mechanism of intraphagosomal killing is associated with ROS produced by NADPH oxidase (Nox) at the phagocyte plasma membrane and other membrane compartments (Rada & Leto, 2008).

The appearance of the first data on specific roles of oxygen radicals in microbial destruction determined the view on ROS as poisoning substances during the next decades. However, a growing set of the data supports now a second messenger role of ROS (D’Autréaux & Toledano, 2007). Particularly, H$_2$O$_2$ serves as a regulator of intracellular signaling cascades in non-phagocytosing cell types. Further studies of H$_2$O$_2$-dependent signaling reveal its participation in the process of phagocytosis as well (Forman & Torres, 2002). Apparently, almost all eukaryotic cell types express Nox family enzymes, which are the main source of H$_2$O$_2$ in cells upon stimulation with various growth factors and cytokines (Bae et al., 2000; Bedard & Krause, 2007; Lange et al., 2009; Leto, Morand, Hurt, & Ueyama, 2009; Mishina et al., 2011; Tabet et al., 2008). The NADPH oxidase complex consists of a membrane-bound cytochrome b558 (Nox1-5) associated with p22 and several cytosolic subunits including p40phox, p47phox, p67phox, and the small GTPases Rac1 or Rac2 (Bedard & Krause, 2007).
H₂O₂ is much more stable than the other ROS, mainly because it is less reactive. H₂O₂ has been postulated to be able to diffuse over a long distance within or between cells. Although the properties of H₂O₂ make this molecule a good candidate for the “second messenger” role, a prerequisite for this function is the precise control of H₂O₂ in time and space.

Since the discovery of ROS, many methodologies for their detection have been developed. Most of them allow very selective and sensitive detection of ROS produced in vitro by isolated enzymes or organelles (Gomes, Fernandes, & Lima, 2005). Some of these techniques detect ROS released by cultured cells to the extracellular media (Wardman, 2007). However, the problem of intracellular ROS detection was not solved until recently. The most widely used approach to ROS detection is based on using the cell-permeable low molecular weight compound dichlorodihydrofluorescein diacetate (H₂DCFDA) and its analogues (Invitrogen, Molecular Probes). However, this compound has several drawbacks that make its use too complicated and the results are often uninterpretable (Bonini, Rota, Tomasi, & Mason, 2006; Crow, 1997; Marchesi, Rota, Fann, Chignell, & Mason, 1999; Rota, Chignell, & Mason, 1999; Rota, Fann, & Mason, 1999; Wardman, 2007). Promising improvement in ROS imaging via synthetic small molecules was recently achieved by the introduction of Peroxy Green 1 and Peroxy Crimson 1 dyes (Miller, Tulyathan, Tulyanthan, Isacoff, & Chang, 2007). However, these dyes have several caveats: (i) they are not ratiometric and therefore require a control dye for proper quantification and (ii) they are irreversibly oxidized with ROS. Therefore, they are often less well suitable for dynamic real-time imaging of ROS-dependent events in live cells. However, due to the intrinsic memory effect, they are useful to report on ROS changes in a global fashion and in cases where low levels of ROS are produced over extended periods of time.

To overcome the drawbacks of chemical probes, we designed and developed HyPer, a genetically encoded ratiometric fluorescent sensor for H₂O₂ (Belousov et al., 2006). The properties of the H₂O₂-sensing domain of HyPer, derived from bacterial OxyR protein, dictate perfect selectivity of the probe, high sensitivity and, importantly, reversibility. HyPer may be expressed in any cell compartment by transfecting cells with DNA encoding HyPer fused with a subcellular localization tag. HyPer has two excitation peaks corresponding to protonated (420 nm) and charged (500 nm) forms of Tyr residue of the YFP chromophore. Both forms can be easily visualized by laser excitation of a confocal system or with wide-field fluorescent microscopy. Imaging the emission after excitation at both peaks
ratiometrically avoids artifacts associated with cell movement or differences in the sensor expression level between cells. However, for cells that do not move significantly and do not change the shape in course of the experiment, single wavelength monitoring is possible (Belousov et al., 2006; Markvicheva, Bogdanova, Staroverov, Lukyanov, & Belousov, 2009).

2. EXPERIMENTAL DESIGN

2.1. Choosing transfection method

For cell lines that are easy to transfect (such as HEK, HeLa, NIH-3T3), we recommend the use of liposome transfection. We obtain the best transfection efficiency with FuGene6 transfection reagent (Jacobsen, Calvin, Colvin, & Wright, 2004). For hard-to-transfect cell types (in our studies—RAW 264.7 line that gives ~5–10% transfected cells with liposome transfection), lentiviral transduction is preferred (Tiscornia, Singer, & Verma, 2006).

2.2. Parameter selection for image acquisition

2.2.1 Choosing excitation and emission wavelengths

HyPer is the probe of choice for time-lapse experiments. To record ratiometric time series, the 420-nm peak is excited with the 405-nm laser on a confocal microscope, or by using the filter set of a wide-field microscope enabling excitation in the 400–430 nm range. Emission is usually detected in the 510–550 nm range. The 500-nm peak can be excited with the 488-nm or 496-nm laser lines or with a wide-field filter set enabling excitation in the 470–500 nm range. The detection settings are the same as for the 420-nm peak. The YFP chromophore is photoconvertible (Dickson, Cubitt, Tsien, & Moerner, 1997). Excitation of the 500-nm peak converts part of the chromophores to a dark state in a light intensity-dependent manner. Excitation of the protonated form of the chromophore (420-nm peak) converts it to the fluorescent state. Therefore, to obtain maximal brightness, each frame should be first imaged with the excitation of the 420-nm peak, and then 500 nm. When using a laser scanning confocal system, we suggest changing excitation lasers between lines rather than between frames, because this produces less delay between excitation lasers in each “point” of the imaged cell.
2.2.2 Choosing light intensity, resolution, and time delay between frames

Light of the laser or lamp may cause phototoxic effects in the imaged cells. Therefore, in continuous time-lapse experiments, settings should aim not only in getting signals from the probe but also to do this with minimal light irradiation. First, maximize the time delay between frames (in our case, “frame” means microscopic field imaged at both excitation wavelengths). \( \text{H}_2\text{O}_2 \) is usually produced on a minutes scale. Thus, there is no need for fast acquisition rates. For phagocytosis experiments, the delay in producing relevant series without cell damage and HyPer bleaching should be 10–60 s. Try to minimize laser power which can be partially compensated by increasing PMT voltage. When imaging with a Leica SP2 or a Carl Zeiss 510/510 Meta, use as low resolution settings as possible, because this decreases the frame acquisition time, thus lowering the amount of illuminating light.

3. MATERIALS

Reagents
- DMEM (Invitrogen, cat. no. 41965-039)
- Minimum Essential Medium Eagle (EMEM) (Sigma–Aldrich, cat. no. M3024)
- OPTI-MEM (Invitrogen, cat. no. 31985-047)
- RPMI-1640 (Invitrogen, cat. no. 21875-034)
- Hanks Balanced Salt Solution (HBSS) (Invitrogen, cat. no. 14025-100)
- Dulbecco–PBS (Invitrogen, cat. no. 14040-091)
- Fetal calf serum, FCS (Invitrogen, cat. no. 16000-044)
- Low-endotoxin FCS (PAA, cat. no. A11-151)
- Bovine serum (Invitrogen, cat. no. 16170-078)
- Penicillin/streptomycin (Invitrogen, cat. no. 15070-063)
- L-Glutamine (Invitrogen, cat. no. 25030-024)
- Trypsin/EDTA solution (Invitrogen, cat. no. R-001-100)
- HEPES (Promega, cat. no. H5302)
- \( \text{H}_2\text{O}_2 \) (Sigma–Aldrich, cat. no. 516813)
  \text{Caution}. May cause severe skin burns and eye damage. Follow the safety guide.
- BSA (Sigma–Aldrich, cat. no. 9048-46-8)
- FuGene6 transfection reagent (Roche Diagnostics, cat. no. 1198887001)
- Mammalian cells
- Sodium bicarbonate
- Zymosan A (Sigma–Aldrich, cat. no Z4250)

**Equipment**
- Glass-bottom dishes (World Precision Instruments, cat. no. FD35-100)
- Standard equipment for eukaryotic cell culturing
  
  *Critical*. For hard-to-transfect cell cultures, when using lentiviral vectors for delivery of HyPer cDNA, S2 safety level cell culture facility is required.
- Confocal microscope or wide-field fluorescent microscope both equipped with a thermostating box
  
  *Critical*. For ratiometric HyPer imaging, confocal microscopes should be equipped with lasers providing violet (405 nm) and blue (488 or 496 nm) laser lines. Alternatively, mercury or xenon arc lamps can be used for the HyPer imaging.

**Reagent setup**
- Complete growth medium: For HeLa-Kyoto cells, we use DMEM supplemented with penicillin/streptomycin, L-glutamine, and 10% FCS; for RAW 264.7 cells, RPMI-1640 supplemented with penicillin/streptomycin, L-glutamine, and 10% heat-inactivated low-endotoxin FCS.
  
  *Critical*. For RAW 264.7 cells maintaining, it is critical to use heat-inactivated serum with low endotoxin (ideally endotoxin free) in order to obtain appropriate phagocytic activity.
- Cell starvation and imaging medium: EMEM supplemented with either 2.2 g/l sodium bicarbonate and 20 mM HEPES (if CO₂ supply for thermostating box available) or just 20 mM HEPES (use bicarbonate-free media in case no CO₂ supply for thermostating box is available in order to prevent alkalinization of the imaging media), penicillin/streptomycin, L-glutamine, and 0.5% BSA.
- Preparation of serum-opsonized zymosan A suspension: We used adult bovine serum to opsonize zymosan A. Mix the serum (1:1) with PBS. To make a 30 × stock, incubate 1.5 ml of PBS–serum mixture with 5 mg of zymosan A powder at 37 °C on a shaker for 30–60 min. Avoid stirring, shake gently. After incubation, wash zymosan with PBS for three times and finally resuspend in 500 μl of PBS. Opsonized zymosan can be stored at −70 °C. For experiment, keep an aliquot on blue ice.
  
  *Critical*. It is important to use adult nonheat-inactivated serum for opsonization. Fetal and heat-inactivated sera contain reduced amount of complement proteins, which are required for zymosan particle opsonization.
3.1. Equipment setup

3.1.1 Inverted fluorescence microscope equipped for rapid ratio imaging

We used a Leica DMI6000 microscope equipped with an external filter wheel CFP/YFP FRET set (11522073). CFP/YFP FRET set includes excitation filters CFP\textsubscript{ex} (BP 427/10), YFP\textsubscript{ex} (BP 504/12), and emission filters CFP\textsubscript{em} (BP 472/30), YFP\textsubscript{em} (BP 542/27). This set allows high-speed HyPer acquisition (CFP\textsubscript{ex}/YFP\textsubscript{em} for the 420-nm peak and YFP\textsubscript{ex}/YFP\textsubscript{em} for the 500-nm peak). The FRET set was used with a “FRET” filter cube. The cube was equipped only with a dichroic mirror (440/520 nm) but neither contained an excitation filter nor an emission filter. Alternatively, any confocal fluorescent microscope, either scanning or spinning disc, equipped with 405-nm and 488 (496)-nm lasers may be used. In this protocol, we describe settings for scanning confocal systems.

4. PROCEDURE

4.1. Transfection

1. Plate the cells on glass-bottom dishes.

\textit{Pause point}. After plating the cells, wait at least 12 h.

2. Transfect the cells with the HyPer encoding vector, using FuGene6 transfection reagent or by any other appropriate method. Useful tips for transient transfection are highlighted in the manufacturer’s protocols and in other sources (Dalby et al., 2004; Jacobsen et al., 2004; Jordan & Wurm, 2004).

3. Culture the cells for 12–48 h at 37 °C, 5% CO\textsubscript{2}.

\textit{Critical step}. HyPer maturates quickly at 37 °C. However, within a particular experimental system, the signal brightness depends on many parameters, including expression activity of the chosen promoter in a particular cell line, stability of mRNA, effectiveness of a protein folding, and protein turnover rate (degradation rate) of the fusion construct. Depending on these parameters, it can be necessary to incubate transfected cells for 24–48 h to get a reliable signal for easy-to-transfect cell lines (HeLa, HEK, etc.) HyPer fluorescence can be detected after 6–12 h posttransfection. After lentiviral transduction, 36–48 h may be required to detect fluorescence.

\textit{Pause point}. Incubate cells on a glass-bottom dish for 12–48 h at 37 °C, 5% CO\textsubscript{2}.
4.2. Preparation of transfected cells for the experiment

4. A. For experiment with H$_2$O$_2$ addition: Replace complete medium with 1.5 ml preheated (37 °C) HBSS.
B. For induction of phagocytosis: Replace complete medium with 1.5 ml preheated (37 °C) imaging medium supplemented with 0.1% (wt/vol) BSA. Incubate macrophages for 30 min at 37 °C. Keep the cells out of the CO$_2$ incubator if using bicarbonate-free imaging media (Table 3.1).

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Low number or no transfected cells</td>
<td>Nonoptimal transfection conditions</td>
<td>Optimize transfection protocol or use lentiviral transduction system</td>
</tr>
<tr>
<td>5</td>
<td>Low signal/background ratio</td>
<td>Low expression level of HyPer</td>
<td>Wait for 24 h to get higher protein expression level</td>
</tr>
<tr>
<td>6 (A) and (B)</td>
<td>Rapid “bleaching” of the probe under blue light excitation</td>
<td>Photoconversion of the chromophore into the dark state</td>
<td>Irradiate the sample with a pulse of violet light or wait for several minutes</td>
</tr>
<tr>
<td>6 (A) and (B)</td>
<td>Fluorescence signal is saturated</td>
<td>(i) Too long exposure time</td>
<td>Decrease the exposure time/PMT voltage/light intensity. It is also helpful to decrease binning of the CCD camera</td>
</tr>
<tr>
<td>6 (A) and (B)</td>
<td>Fluorescence signal is saturated</td>
<td>(ii) Too high PMT voltage/excitation light intensity</td>
<td></td>
</tr>
<tr>
<td>7 (A) and (B)</td>
<td>No phagocytosis</td>
<td>(i) Nonheat-inactivated FCS or serum with high concentrations of endotoxins is used for culturing the cells</td>
<td>(i) Change serum for heat-inactivated low-LPS FCS</td>
</tr>
<tr>
<td>7 (A) and (B)</td>
<td>No phagocytosis</td>
<td>(ii) High passage number</td>
<td>(ii) Thaw fresh aliquot of the cells, use after passage 3</td>
</tr>
<tr>
<td>7 (A) and (B)</td>
<td>Weak phagocytosis (10–50% of the cells phagocyte) or no H$_2$O$_2$ production upon phagocytosis</td>
<td>(i) Weak opsonization</td>
<td>(i) Change lot or origin of serum used for opsonization</td>
</tr>
<tr>
<td>7 (A) and (B)</td>
<td>Weak phagocytosis (10–50% of the cells phagocyte) or no H$_2$O$_2$ production upon phagocytosis</td>
<td>(ii) No or too short time of serum starvation</td>
<td>(ii) Increase the serum starvation time up to 60 min</td>
</tr>
<tr>
<td>7 (A) and (B)</td>
<td>Weak phagocytosis (10–50% of the cells phagocyte) or no H$_2$O$_2$ production upon phagocytosis</td>
<td>(iii) High passage number</td>
<td>(iii) Thaw fresh aliquot of the cells, use after passage 3</td>
</tr>
</tbody>
</table>
4.3. Primary visualization

5. Visualize HyPer using a mercury or xenon arc lamp. Regular GFP or FITC filter sets allow visualizing fluorescence. 

*Critical.* Irradiation of HyPer with light exciting the deprotonated form of the chromophore (500-nm peak) leads to photoconversion to the dark state. While illuminating the cells with blue light, you may see rapid pseudobleaching of HyPer. However, the chromophore can be reversed to the fluorescent state by pulse irradiation with light exciting the protonated form of the chromophore (420-nm excitation peak). We usually use 40× or 63× Plan Apochromat objectives with high numerical aperture, but other high magnitude high numerical aperture objectives may be used as well (Table 3.1).

4.4. Imaging settings

6. Setting up microscope for HyPer time-lapse imaging.

**A Setting up confocal microscope for HyPer time-lapse imaging**

i. Activate 405-nm and 488 (496)-nm lasers. Set the scanning resolution to 512 × 512 or 1024 × 1024. Usually, 512 × 512 points is enough to obtain all the necessary information both on fluorescence intensity and intracellular distribution. In the case of a Leica SP2 or Carl Zeiss LSM510 systems, lowering resolution allows increasing the scanning beam speed and decreases the so-called pixel time, thus reducing photodamage of the cells by laser light.

ii. Set pinhole size to ∼2–4 μm. Less pinhole size is usually used to obtain structural information rather than messenger or activity dynamics. Higher pinhole size may lead to a decrease in signal-to-background ratio.

iii. Set PMT voltage to 700–800. Lower value makes images less noisy, higher value allows one to use less laser power.

iv. Set the intensity of the 405-nm laser to 5–10% and the intensity of the 488-nm 10-mW Ar laser to 1–7%.

*Critical.* These numbers may vary depending on optics, lasers, laser alignment, and the equipment. Fluorescence should be clearly visible in both channels. The intensity of fluorescence should be nearly equal in both channels.

v. Set up the beam path for 420-nm peak excitation by 405-nm laser and for emission light.
vi. Set up the beam path for the 500-nm peak excitation by 488 (496 nm) laser and for emission light. Since the emission peak does not shift, upon reaction of HyPer with H₂O₂, the same detector for both channels should be used. For Leica SP2 and SP5 systems, and for the Carl Zeiss LSM710 setup, the emission range is set from 500 to 530–550 nm. For Carl Zeiss LSM510, the setup uses 505–530 or 505–550 bandpass emission filters.

vii. The “Beam expander” option available on the Leica SP2 confocal system permits increasing the irradiated volume and allows improving the fluorescence signal intensity in cells with low expression level without increasing the laser intensity. For Leica SP2, set the Beam expander to 3.

viii. Enable switching channels between lines rather than between frames. Every line in the frame should be always excited by the 405-nm laser first and then by the 488-nm (496-nm) laser.

ix. Optionally, activate the transmitted light channel. Any laser can serve as the source of the transmitted light. Set up the PMT voltage for the channel to make the cells clearly visible.

B Setting up the wide-field fluorescent microscope Leica 6000 for HyPer time-lapse imaging

i. Turn on the light source. Activate the following settings for both channels: binning (none or 2 × 2 or 4 × 4) (Note. The binning function reduces the number of pixels on the CCD chip, thus lowering resolution and increasing signal-to-noise ratio). Keep the acquisition time as low as possible.

ii. Activate filter cube “FRET” equipped with a dichroic mirror (440/520 nm).

iii. Set up excitation filter CFP (BP 427/10) to excite the 420-nm peak.

iv. Set up excitation filter YFP (BP 504/12) to excite the 500-nm peak.

v. Set up emission filter “YFP” (BP 542/27) for both channels.


viii. Optionally, activate transmitted light channel.

ix. Set up time delay between frames. Usually, it should be between 10 and 60 s to obtain a relevant time profile (Table 3.1).
4.5. Imaging

7. Proceed with time-lapse imaging for option A for confocal microscopes or option B for wide-field fluorescent microscopes.

**A Confocal microscope**

i. At least 2 h before imaging, warm up the thermal incubator box to 37 °C.

ii. Place the dish with cells onto the microscope stage. Set up focus.

iii. Switch the system to a scanning mode.

iv. Perform a single scan. Zoom selected cells with “Zoom in” function of the Leica system or the “Crop” function of Carl Zeiss system.

v. Select a region of interest (ROI) in the area of an individual transfected cell and measure the signal intensity “ROI” function of the Carl Zeiss system or the “Quantification–Histogram” function of the Leica system.

vi. Set the fluorescence intensity in each channel of ROI to be in the range between 30 and 80 by changing the light intensity and/or PMT voltage.

vii. Perform several single scans to set the final focus, adjust laser power and PMT voltage.

viii. Start time series recording. It is important to observe that the starting 405 nm/488 (496) nm excitation ratio and microscope focus are stable over this prestimulation period.

ix. Cell stimulation:

a. H$_2$O$_2$ addition: After 5 min of imaging, add drop-by-drop H$_2$O$_2$ diluted in 500 μl of prewarmed HBSS. Changes in the F500/F420 ratio are observable immediately after H$_2$O$_2$ addition. Continue data acquisition.

b. Induction of phagocytosis: After 5–10 min of imaging, add drop-by-drop opsonized zymosan suspension diluted in 500 μl of prewarmed EMEM. Continue data acquisition for 30–60 min. **Critical step.** Do not touch the microscope stage or the culture dish.

**B Wide-field fluorescent microscope**

i. Carry out the same procedures as in Step 7 (A) (i)–(iv).

ii. Perform several single scans to set the final focus, adjust light power and exposure time. The initial fluorescence intensities in both channels in a ROI should be almost equal (±5–10%) and should be in the range between 30 and 70 for an 8-bit camera.

iii. Start time series recording. It is important to observe that the microscope focus is stable over the prestimulation period.
iv. Cell stimulation:

a. H$_2$O$_2$ addition: After 5 min of imaging, add drop-by-drop H$_2$O$_2$ diluted in 500 µl of prewarmed HBSS. Change in F500/F420 ratio is observable immediately after H$_2$O$_2$ addition.

b. Induction of phagocytosis: After 5–10 min of imaging, add drop-by-drop opsonized zymosan suspension diluted in 500 µl of prewarmed EMEM. Continue data acquisition for 30–60 min.

Critical step. Do not touch the microscope stage or the culture dish (Table 3.1).

Figure 3.1 Ratio change of HyPer in HeLa-Kyoto cells upon addition of H$_2$O$_2$. (A) Images of ratio F500/F420 change in cells transfected with HyPer-cyto and exposed to 200 µM H$_2$O$_2$. The lower row of the images represents subcellular distribution of HyPer ratio. Upper and middle rows of the images show fluorescence intensity in each imaging channel. Numbers indicate timing in seconds. H$_2$O$_2$ was added between the second and third frame shown. (B) Timing of the HyPer ratio change in cells is shown in panel (A). See also Supplementary Video 1 (http://www.elsevierdirect.com/companions/9780124058835). H$_2$O$_2$—hydrogen peroxide.
4.6. Anticipated results

Typical results are presented in the figures. Figure 3.1 depicts HeLa-Kyoto cells before and after addition of H$_2$O$_2$. The increase in the HyPer ratio is clearly observed upon addition of H$_2$O$_2$ (Fig. 3.1B). Figure 3.2 shows H$_2$O$_2$ production by RAW 264.7 macrophages phagocytizing zymosan particles. A typical time course of phagocytosis-induced H$_2$O$_2$ generation profile in RAW 264.7 cells is shown in Fig. 3.2B. Panels represent pseudocolored images where pixel values reflect ratio F500/F420 (Fig. 3.2A). Immediately after the zymosan particle ingestion (at minute 1), cells begin to produce a wave of H$_2$O$_2$ peaking at minute 2 (Fig. 3.2A). In the course of the next 10–15 min, H$_2$O$_2$ levels progressively decrease to a basal level (Fig. 3.2B). Although in general cells exhibited single H$_2$O$_2$ burst, a small population of...
the cells displayed several H$_2$O$_2$ peaks after each ingestion event followed by constant and irreversible H$_2$O$_2$ production (Fig. 3.2C and D).

In summary, HyPer is a unique tool allowing very sensitive and specific H$_2$O$_2$ detection. Its main advantage is its simplicity of use that, we hope, is reflected in the present protocol.

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Competing financial interests. The authors declare that they have no competing financial interests.

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