In vivo oxygen imaging using green fluorescent protein

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Takahashi, Eiji, Tomohiro Takano, Yasutomu Nomura, Satoshi Okano, Osamu Nakajima, and Michihiko Sato. In vivo oxygen imaging using green fluorescent protein. Am J Physiol Cell Physiol 291: C781–C787, 2006.—In vivo oxygen measurement is the key to understanding how biological systems dynamically adapt to reductions in oxygen supply. High spatial resolution oxygen imaging is of particular importance because recent studies address the significance of within-tissue and within-cell heterogeneities in oxygen concentration in health and disease. Here, we report a new technique for in vivo molecular imaging of oxygen in organs using green fluorescent protein (GFP). GFP-expressing COS-7 cells were briefly photoactivated with a strong blue light while lowering the oxygen concentration from 10% to <0.001%. Red fluorescence (excitation 520–550 nm, emission >580 nm) appeared after photoactivation at <2% oxygen (the red shift of GFP fluorescence). The red shift disappeared after reoxygenation of the cell, indicating that the red shift is stable as long as the cell is hypoxic. The red shift of GFP fluorescence was also demonstrated in single cardiomyocytes isolated from the GFP knock-in mouse (green mouse) heart. Then, we tried in vivo molecular imaging of hypoxia in organs. The red shift could be imaged in the ischemic liver and kidney in the green mouse using microscopic optics provided that oxygen diffusion from the atmosphere was prevented. In crystallloid-perfused beating heart isolated from the green mouse, significant spatial heterogeneities in the red shift were demonstrated in the epicardium distal to the coronary artery ligation. We conclude that the present technique using GFP as an oxygen indicator may allow in vivo molecular imaging of oxygen in organs.

IN VIVO MEASUREMENT OF THE TISSUE OXYGEN LEVEL IS CRUCIAL FOR UNDERSTANDING HOW BIOLOGICAL SYSTEMS DYNAMICALLY ADAPT TO REDUCTIONS IN OXYGEN SUPPLY AND INCREASES IN OXYGEN DEMAND. RECENTLY, THE EXISTENCE OF MACROSCOPIC (WITHIN-TISSUE) AND MICROSCOPIC (WITHIN-CELL) HETEROGENEITIES IN OXYGEN CONCENTRATION IN TISSUES HAS DRAWN CONSIDERABLE ATTENTION, BECAUSE THESE SHOULD BE RELEVANT TO THE PHYSIOLOGY AND PATHOPHYSIOLOGY OF GENE EXPRESSION AND ORGAN FUNCTION IN HYPOXIA (7, 13, 15, 21, 25, 34). THUS A METHOD FOR OXYGEN MEASUREMENT THAT CAN RESOLVE SUCH SPATIAL HETEROGENEITIES (I.E., HIGH SPATIAL RESOLUTION IMAGING) IS MOST DESIRABLE. AS COMPARED BY SWARTZ AND DUNN (24), CURRENT TECHNIQUES FOR IN VIVO OXYGEN MEASUREMENT, INCLUDING THE OXYGEN MICROELECTRODE, NEAR-INFRARED SPECTROSCOPY, PHOSPHORESCENCE QUENCHING BY OXYGEN, ELECTRON PARAMAGNETIC RESONANCE OXIMETRY, AND NMR SPECTROSCOPY, DO NOT NECESSARILY FULFILL THIS REQUIREMENT.

ENDOGENOUS CHROMOPHORES THAT CHANGE THEIR OPTICAL PROPERTIES ACCORDING TO OXYGEN AVAILABILITY ARE PARTICULARLY SUITABLE FOR IN VIVO MOLECULAR IMAGING OF OXYGEN. FOR INSTANCE, MYOGLOBIN OCCURS IN THE CYTOSOL OF RED MUSCLES AND CHANGES ITS LIGHT ABSORPTION ACCORDING TO THE OXYGEN ABUNDANCE, WITH A P50 OF ~2 mmHg (33). BECAUSE THE OXYGEN AFFINITY OF MYOGLOBIN OVERLAPS THE REGULATORY PO2 RANGE OF MITOCHONDRIAL RESPIRATION (11), OPTICAL OR NMR SPECTROSCOPY OF MYOGLOBIN IN SITU IS AN EXCELLENT TECHNIQUE TO DEFINE PHYSIOLOGICALLY MEANINGFUL HETEROGENEITIES OF TISSUE OXYGEN CONCENTRATION IN ORGANS (14, 16, 28) AND IN ISOLATED CELLS (27, 32). ONE APPARENT LIMITATION OF THIS TECHNIQUE IS THAT IT IS ONLY APPLICABLE TO RED MUSCLES SUCH AS SKELETAL AND CARDIAC MUSCLES.


GREEN FLUORESCENT PROTEIN (GFP) HAS BEEN WIDELY USED AS A FLUORESCENT MARKER FOR GENE EXPRESSION AND FOR THE INTRACELLULAR LOCALIZATION OF PROTEINS IN LIVING CELLS. SEVERAL GFP VARIANTS HAVE BEEN GENERATED IN WHICH THE FLUORESCENCE SPECTRUM RANGES FROM BLUE TO YELLOW, BUT THE GROUP OF ENHANCED GFPs DOES NOT DISPLAY SIGNIFICANT RED FLUORESCENCE. IN 1997, ELOWITZ ET AL. (6) REPORTED THAT IN Escherichia coli TRANSFORMED WITH A PLASMID THAT EXPRESSES A GFP, THE GFP FLUORESCENCE MAY TURN REDDISH AFTER A BRIEF (2–60 s) ILLUMINATION WITH 475–495 nm LIGHT IF THE BACTERIA ARE SEALED BETWEEN MICROSCOPE SLIDES AND LEFT FOR A PROLONGED TIME (~25 min). THE RED SHIFT OF GFP FLUORESCENCE

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was also demonstrated in the transformed E. coli when photoactivation was conducted after addition of an oxygen scavenger to the suspension medium. These results indicate that the fluorescent characteristics of enhanced GFP may be modified according to the oxygen moiety of the molecule and, from the standpoint of oxygen measurement, suggest the possibility that GFP might be used as an in vivo oxygen indicator.

In the present study, we first determined the characteristics of enhanced GFP as an intracellular oxygen indicator in cultured COS-7 cells. Then, toward the goal of in vivo molecular imaging of oxygen in tissues, we attempted a high spatial resolution imaging of hypoxia in adult single cardiomyocytes freshly isolated from the GFP knock-in mouse (green mouse) and in organs (kidney, liver, and heart) in the green mouse. We photoactivated GFP in vivo to see whether the green fluorescence of GFP turns reddish under anaerobic conditions.

METHODS AND RESULTS

Detection of hypoxia by GFP fluorescence in cultured COS-7 cells. The appearance of a red fluorescence in GFP fluorescence following photoactivation under anaerobic conditions (the red shift of GFP fluorescence) has been characterized in great detail by Elowitz et al. (6) in GFP solutions and in GFP-expressing E. coli. Here, we examined whether the red shift of GFP fluorescence could be demonstrated in mammalian cells.

Cultured COS-7 cells were transfected with expression vector pCMX-SAH/Y145F (pCMX-humanized GFP-S65A/Y145F) by using a linear polyethyleneimine reagent (jetPEI; Polyplus-transfection, Illkirch, France), and cultured for 48 h on a 13.5-mm-diameter low-fluorescence plastic disk (Cell Disk LF; Sumilon, Tokyo, Japan). Cultured cells were washed in PBS three times and placed in an airtight measuring cuvette at 37°C and superfused with humidified gas at various oxygen concentrations. A 16-bit charge-coupled device (CCD) camera (model SV512; Pixelsvision, Tigard, OR) captured green and red fluorescent images of cells by using NIBA (excitation 470 – 490 nm, emission 515–550 nm) and WIG (excitation 520 – 550 nm, emission 580 nm) filter sets, respectively, in an inverted microscope with a ×40 long-working-distance objective lens (model IX70; Olympus, Tokyo, Japan). The exposure duration was 2 s while a 6% neutral density filter was inserted in the light path. Photoactivation was carried out with 480 – 500 nm light for 120 s. Cells in the measuring cuvette were superfused with gas containing either 0.001, 1,
2, 3, or 5% oxygen at 4 ml/min. To quantify the magnitude of the red fluorescence, the increase in the red fluorescence after a photoactivation (ΔR) was normalized to the green fluorescence level before photoactivation (G) and designated as ΔR/G. Figure 2 shows that a significant red shift of GFP fluorescence was induced by photoactivation when the oxygen concentration was lowered to 2%. The magnitude of the red fluorescence reached ~30% of the green fluorescence level under anaerobic conditions (Fig. 2).

Finally, we questioned whether the red shift of GFP fluorescence could be reversed upon reoxygenation. For this, anaerobic photoactivation was followed by superfusion of the cell with humidified gas containing 15% oxygen. As shown in Fig. 3, the red fluorescence persisted after the photoactivation (at least 30 min) as long as the oxygen concentration was kept low. In contrast, the red fluorescence induced by an anaerobic photoactivation quickly disappeared upon reoxygenation of the cell (Fig. 3, bottom). Thus the red fluorescence of GFP is stable only under anaerobic conditions.

Detection of hypoxia by GFP fluorescence in single adult cardiomyocytes. To further examine the feasibility of GFP as an in vivo oxygen probe, we used single cardiomyocytes freshly isolated from an adult GFP knock-in mouse. Transgenic mice stably expressing enhanced GFP (green mice) were obtained from the Research Laboratory for Molecular Genetics at Yamagata University. Use of the green mouse conformed to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, established by the Physiological Society of Japan. Prior approval for the experiment was obtained from the Animal Research Committee, Yamagata University School of Medicine. After intraperitoneal injection of pentobarbital sodium (50 mg/kg), the heart was excised and Langendorff perfusion was conducted. Single cardiomyocytes were isolated from the heart using collagenase (Blendzyme 4; Roche Diagnostics, Indianapolis, IN) and suspended in a HEPES-Tyrode solution containing (in mM) 130 NaCl, 6 HEPES, 10 glucose, 5.4 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.2 CaCl₂ (pH 7.4 at 37°C). These cells were placed in an airtight measuring cuvette on the stage of an inverted microscope. The temperature was regulated at 37°C. For the fluorescence measurements, we used the same optical system as mentioned above. The durations of exposure for the green and red fluorescence measurements were 1 and 3.3 s, respectively. The duration of exposure for photoactivation was 120 s.

The intensity of the green fluorescence was 7 ± 2 (in arbitrary units, mean ± SD, n = 10) and 269 ± 70 (n = 10) in single cardiomyocytes isolated from wild-type and green mice, respectively. Although the ~38-fold increase in the green fluorescence certainly indicates stable expression of GFP in the green mouse heart, it was substantially lower than the intensity of the green fluorescence in transiently GFP-expressing cultured COS-7 cells (19,600 ± 12,000, n = 9).

Because of the considerably lower expression of GFP in cardiomyocytes in the green mouse, the red autofluorescence in cardiomyocytes now could not be ignored (Fig. 4). The levels of the red autofluorescence under our experimental conditions...
were 86 ± 30 (n = 10) and 125 ± 20 (n = 10) in single cardiomyocytes isolated from wild-type and green mice, respectively. Thus the level of nonspecific red fluorescence was comparable to 46% of the GFP green fluorescence. To abolish the effects of the red autofluorescence, the difference in the red fluorescence images before and after photoactivation (ΔR) was calculated, with the assumption that photoactivation per se does not affect the red autofluorescence. Finally, the image was normalized to the corresponding green fluorescence image (ΔR/G) to compensate for variations in fluorescence arising from the geometry of the cell (for instance, cell thickness). These image-related calculations were performed using IPLab software (Scanalytics, Rockville, MD). As a result, the red fluorescence, with a magnitude of ~20% of the green GFP fluorescence, could be demonstrated (Fig. 4).

**Macroscopic detection of hypoxia by GFP fluorescence in whole organs.** Finally, we applied this technique to visualize hypoxic regions in whole organs, blood-perfused kidney and liver, and crystalloid-perfused heart. We used a macroscopic fluorescence imaging system (Fig. 5; Keyence, Osaka, Japan) equipped with two 120-W mercury arc lamps. The fluorescent image of the organ was captured with a 12-bit CCD camera (MicroMax, Princeton Instruments, Trenton, NJ). Green and red fluorescence was measured by using 450–490/ >510 nm (excitation/emission) and 527.5–552.5/ >572 nm (excitation/emission) filter sets, respectively. For the photoactivation, we used the same excitation filter as used for the green fluorescence measurement. The duration of photoactivation had to be prolonged to >10 min because the intensity of the light was low (0.9 mW/mm² for a single light source) compared with the fluorescence microscope system (5.1 mW/mm²) mentioned above.

A green mouse of either sex was anesthetized with pentobarbital sodium (50 mg/kg ip) and placed on a warming plate (37°C). The liver was exposed and, after an aliquot of heavy mineral oil was applied, covered with a thin polyvinyl chloride film (Saran Wrap) to avoid oxygen diffusion from the atmospheric air. The autofluorescence of Saran Wrap was negligible before and after the photoactivation. The optics were then adjusted so that a spot with a diameter of ~5 mm illuminated the surface of the organ. Ten minutes after ligation of the hepatic artery and the portal vein, the first fluorescence measurements were conducted. Photoactivation was then conducted and was immediately followed by the second fluorescence measurements.

**Figure 6** shows the appearance of the red fluorescence in the ischemic liver after a photoactivation. It must be noted that the red shift of the GFP fluorescence was consistently observed only in regions where the surface of the liver was covered with Saran Wrap. These changes in the red fluorescence were not observed in the normoxic blood-perfused liver (data not shown). Because the hepatic artery and the portal vein were occluded, the oxygen supply to the tissue should have been almost completely cut off. Therefore, the finding that the red shift was not observed in regions without Saran Wrap suggests that a significant amount of oxygen might have diffused from the atmosphere into the tissue. Similar observations were made in the blood-perfused kidney (data not shown).

Finally, we attempted imaging of hypoxic regions using the red shift of GFP fluorescence in Langendorff-perfused heart isolated from the green mouse. The heart was isolated from an anesthetized green mouse (50 mg/kg ip pentobarbital sodium), and constant pressure Langendorff perfusion was started with HEPES-Tyrode solution containing 2.0 mM Ca²⁺. The left anterior descending coronary artery was ligated with a 6-0 suture and the heart was wrapped with Saran Wrap. We used
two independent light sources for GFP excitation/photoactivation (Fig. 5) because the light intensity was lower in our macroscopic fluorescence system compared with the fluorescence microscope. Two light guides were arranged so that the surface of the heart was uniformly illuminated. The coronary artery-ligated beating heart was photoactivated for 10–20 min. Because the heart was spontaneously beating at ~200 beats/min and fluorescence measurements required prolonged durations of exposure, motion blurring in the fluorescent image was a serious problem. To avoid the motion artifact associated with spontaneous heart beating, the Langendorff perfusion was temporarily discontinued (for <1 min) after a photoactivation. After verifying that the heartbeat was spontaneously diminished and finally stopped, the fluorescence measurement for the red shift was conducted. This protocol was based on the finding that the red shift persists as long as the heart is kept hypoxic (Fig. 3). Finally, to compensate for heterogeneities in GFP fluorescence in the heart, the red fluorescence was normalized to the GFP (green) fluorescence. The red shift was evident in regions where the blood supply appeared to have depended on the ligated coronary artery (Fig. 7), while significant macroscopic heterogeneities were observed. Thus visualization of hypoxic regions by the red fluorescence of GFP was possible in the beating crystalloid-perfused heart.

**DISCUSSION**

Oxygen diffuses from the intravascular space to intracellular mitochondria along oxygen concentration gradients. Thus the regional oxygen concentration in a tissue is determined by the 1) vasculature geometry (oxygen source), 2) oxygen diffusion path and resistance (oxygen transport), and 3) mitochondrial respiration (oxygen sink). As a result of synergistic effects of these factors, complex oxygen concentration heterogeneities are produced within tissues. Importantly, heterogeneous oxygen supply within tissues significantly affects organ function either in normal or in disease states (3, 7, 13, 15, 19, 21, 29). In addition to the macroscopic (intercellular) heterogeneity, intracellular diffusion of oxygen produces microscopic (intracellular) gradients of oxygen concentration at elevated oxygen demand (27), although the physiological significance of the microscopic heterogeneity is less clearly understood.

Chance (3) was the first to establish the theoretical basis for the detection of tissue oxygen heterogeneities. He pointed out that the oxygen indicator must be uniformly distributed in the tissue and its oxygen affinity must be appropriate for the respiratory chain of mitochondria. If spatial heterogeneity is concerned, the measurement should be conducted with a spatial resolution as high as the order of the intercapillary distances (tens of micrometers).

The present technique using GFP as an oxygen indicator may fulfill these requirements. First, GFP is expressed in the cytoplasm of the green mouse. The expression level of GFP within a particular organ appeared to be relatively uniform, whereas the GFP fluorescence in our green mouse varied considerably according to the organ (in saline-washed organs, the GFP fluorescence levels normalized by the fluorescence in the heart were 2.8, 2.1, 34, 2.6, and 4.7 in the kidney, the spleen, the liver, the lung, and the brain, respectively). Furthermore, variations in the GFP fluorescence within an organ arising from variations in tissue thickness and heterogeneous illumination by the excitation light are effectively compensated for by introducing an image equalization technique (ΔR/G). Second, as shown in Fig. 2, the magnitude of the red shift abruptly increased when the COS-7 cells were superfused with <1% (~7 Torr) oxygen. This result certainly indicates that GFP cannot be used for quantitative determination of oxygen concentrations ranging from arterial blood Po2 to anoxia. Rather, it may be suitable for the detection of dysxia, because this threshold oxygen concentration overlaps the critical Po2 of the cell at which mitochondrial oxygen metabolism is compromised (9, 20). Third, in the green mouse, GFP is endogenously expressed in the cytoplasm, as myoglobin is in red muscles. Thus GFP reports the intracellular oxygen level at a molecular spatial resolution, and GFP can therefore be used as a probe for imaging tissue hypoxia optimal for the physiological Po2 range.

To utilize GFP as a molecular oxygen probe, the protein must be expressed in cells/tissues. Albeit the required level of expression differs according to the sensitivity of the optical system, a high level of GFP expression in a tissue is of particular importance. In cultured COS-7 cells, the conventional transient expression technique using a linear polyethyl-enamine reagent resulted in sufficient GFP expression. With the conventional fluorescence microscope, the green fluorescence was strong and the spectrum shift was evident after a brief irradiation with the activation light, enabling us to easily define the basic characteristics of the anaerobic red shift in the mammalian cell (Figs. 1–3). In contrast, efficient expression of the protein was difficult to achieve in vivo, which is a common issue concerning the in vivo introduction of exogenous genes.
We attempted in vivo transient GFP expression in the rat using a linear polyethylenimine reagent specifically designed for in vivo use but were unsuccessful at that time. We then used a genetically engineered mouse that stably expresses enhanced GFP (green mouse). Although the organs of our green mouse exhibited significant green fluorescence, the level in single ventricular myocytes was only 1/73 that in the transiently GFP-expressing COS-7 cells. Thus we had to prolong the exposure duration of the CCD camera until sufficient GFP fluorescence was detected. This procedure, at the same time, elevated the level of nonspecific red fluorescence, probably originating from endogenous flavoproteins. In fact, under our experimental conditions, the level of the nonspecific red fluorescence was almost of the same order as the GFP green fluorescence in cardiac myocytes of the green mouse. The presence of the strong background red fluorescence caused significant deterioration of the signal-to-noise ratio in the red shift detection. For this reason, we failed to detect the red shift in single red fluorescence images alone, although it was possible to do so in the COS-7 cells (Fig. 1), and somewhat complicated calculations using data from the images (ΔR/G) were indispensable (Fig. 4). Furthermore, in our macroscopic imaging device used for the organs, the magnitude of the photoactivation light was only one-third compared with that for the microscope with high numerical aperture optics. Therefore, we had to prolong the photoactivation duration to 10–15 min. This significantly restricted the time resolution of the imaging in the time-lapse measurement. For the in vivo imaging of hypoxia in organs to be practical, more efficient photoactivation should be realized by using, for example, laser scanning photoactivation optics.

Recently, various fluorescent proteins that show a photoactivatable shift in the fluorescence wavelength, such as Kaede (2), or fluorescence augmentation, such as PA-GFP (18) and KFP1 (5), have been reported (see Table 1 in Ref. 18). Because distinct fluorescence can be artificially induced, these proteins have been used as endogenous molecular markers in living cells. The molecular mechanism of the red shift of GFP fluorescence demonstrated in the present study may not be identical to that in these photoactivatable proteins, because the red shift can be achieved and is stable only under anaerobic conditions (Fig. 3). The precise mechanism of the anaerobic red shift after photoactivation thus remains to be elucidated.

Irradiated light penetrates into tissues. Gandjbakhche et al. (8), using Monte Carlo simulation of photon migration in tissues, estimated that the mean penetration depth of 510–590 nm light within the epicardium of blood-free heart tissue is 0.33–0.40 mm. In fact, Seiyama et al. (22) demonstrated in blood-free liver tissue a penetration depth of ~0.6 mm at 605 nm. On the other hand, atmospheric oxygen diffuses into tissues. Stücker et al. (23) reported that atmospheric air penetrates to a depth of 0.25–0.40 mm in the skin. Thus the volume of tissue subjected to the epifluorescence measurement of oxygen is also sensitive to the oxygen concentration of surrounding medium. In the majority of physiological experiments in isolated organs, including the present one, organs are exposed and the surface is in contact with the atmospheric air. Therefore, it is not surprising that the red shift of GFP fluorescence was consistently observed only in regions where the surface was covered with Saran Wrap (Fig. 6). Thus special care should be taken in hypoxia imaging experiments using isolated organs so that atmospheric oxygen does not disturb oxygen distribution in the tissue.

The red shift occurs at <2% oxygen. It may be more relevant experimentally and clinically if GFP could detect higher level of oxygen such as 6% oxygen (venous blood oxygen level). Because the complete amino acid sequence of GFP has been determined, it would be possible to manipulate the sensitivity of the fluorescence shift to oxygen using genetic engineering technology. Then, the oxygen gradients from the capillary blood to mitochondria could be imaged in the tissue.

In addition to the in vivo molecular imaging of hypoxia, the present technique may potentially permit further applications in hypoxia research in vivo. For example, if genes responsible for adaptation to decreases in oxygen, such as HIF-1α, are cotransfected with the GFP gene (17), then the GFP-HIF-1α fusion protein allows one to determine, from the red shift of the fluorescence, whether the spatial heterogeneities in HIF-1α inductions depend on those of oxygen distribution within the tissue.

In summary, utilizing the red shift of GFP fluorescence originally described by Elowitz et al. (6), we have demonstrated that hypoxia can be detected with a subcellular spatial resolution in single cells and organs in vivo. Although the precise molecular mechanism for the red shift in anaerobic GFP is not known at present, the combination of genetic and fluorescence imaging techniques permits construction of high spatial resolution oxygen maps in organs, which should reveal the impact of oxygen heterogeneities within tissues on the physiology and pathophysiology of gene expression and organ function in hypoxia.

GRANTS

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