Chapter 16

Imaging of Cellular Oxygen and Analysis of Metabolic Responses of Mammalian Cells

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Abstract

Many parameters reflecting mitochondrial function and metabolic status of the cell, including the mitochondrial membrane potential, reactive oxygen species, ATP, NADH, ion gradients, and ion fluxes (Ca^{2+}, H^{+}), are amenable for analysis by live cell imaging and are widely used in many labs. However, one key metabolite – cellular oxygen – is currently not analyzed routinely. Here we present several imaging techniques that use the phosphorescent oxygen-sensitive probes loaded intracellularly and which allow real-time monitoring of O_2 in live respiring cells and metabolic responses to cell stimulation. The techniques include conventional wide-field fluorescence microscopy to monitor relative changes in cell respiration, microsecond FLIM format which provides quantitative readout of O_2 concentration within/near the cells, and live cell array devices for the monitoring of metabolic responses of individual suspension cells. Step by step procedures of typical experiments for each of these applications and troubleshooting guide are given.

Key words: Cell metabolism, mitochondrial function, cellular oxygen, phosphorescent oxygen-sensitive probe, phosphorescence quenching, live cell imaging, microsecond FLIM.

1. Introduction

Molecular oxygen (O_2) is the key substrate of aerobic organisms and the terminal acceptor of the electron transport chain, which can serve as an informative marker of cell metabolism and mitochondrial function (1–6). The development of a number of fluorescence and phosphorescence based O_2-sensitive probes and assays (7–10) has provided relatively simple, non-chemical and

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non-invasive means to measure O_2 in samples containing respiring cells. Based on the collisional (i.e., non-chemical) quenching by O_2 of certain photoluminescent structures (8), these probes combine good selectivity and reliable work across the whole physiological range of O_2 (0–250 μM) and particularly at low O_2 concentrations. They allow contact-less "sensing" of O_2 in complex biological samples and particularly in samples containing respiring mammalian cells. Using extracellular O_2-sensitive probes, optical O_2 sensing and respirometry were initially applied to the imaging of tissue oxygenation (9) and analysis of bulk consumption of O_2 by populations of cells. A panel of high-throughput O_2 consumption assays performed in standard microtiter plates with detection on a fluorescent plate reader have been developed and successfully used in a number of useful applications (10, 11–15).

More recently, O_2 probes suitable for intracellular use have been developed (16, 17–22), which enable the analysis of localized O_2 gradients within the cells and a number of additional measurement tasks. In particular, real-time monitoring of fast, transient respiratory responses of individual cells and cell populations (16, 20, 23, 24), which thus far was outside the scope of traditional O_2-sensing probes and techniques, has been achieved. Some of these probes are compatible for fluorescence microscopy imaging. It has been shown that using these intracellular O_2 probes and relatively simple cell-handling procedures, sensing of intracellular O_2, and real-time monitoring of metabolic and respiratory responses of live mammalian cells and tissue can be performed (15, 21, 23). Several different cell types, particularly PC12, HepG2, HCT116, HeLa, SH-SY5Y, and A549 cells were examined so far by O_2 imaging, which has been shown to provide information-rich data about cell metabolism and good spatial and temporal resolution (16, 22).

Application of the O_2 imaging approach to suspension cells has been limited due to cell mobility and difficulties with sample manipulation and effector addition. These limitations have been overcome by the use of Live Cell Array™ (LCA) – a convenient platform developed for imaging of suspension cells (24). LCA provides capturing of cells within an array of specially designed microwells, whereby each well retains a single cell while maintaining cell viability. LCAs facilitate high content image analysis of large number of individual suspension cells with effector treatments.

In this chapter we describe the use of intracellular O_2-sensing probes based on phosphorescent platinum(II)-porphyrin dyes, in conjunction with fluorescence imaging for the assessment of mitochondrial function and real-time monitoring of responses of mammalian cells to metabolic effectors. These probes have been developed in our lab and commercialized by Luxcel Biosciences (Cork, Ireland). Standard wide-field fluorescence intensity

imaging in time-lapse mode, as well as microsecond FLIM with pulsed LED excitation are described and applied to commonly used cell models including the adherent HCT116 cells, differentiated PC12 cells, and suspension Jurkat T cells.

2. Materials

1. HCT116 human colorectal carcinoma, PC12 rat pheochromocytoma, and Jurkat human T-lymphoma cell lines (American Tissue Culture Collection, ATCC) (*see* **Note 1**).
2. Cell culture media: McCoy's 5A modified medium (McCoy), Roswell Park Memorial Institute 1640 (RPMI) (Sigma-Aldrich), Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) (*see* **Note 2**).
3. Fetal bovine serum (FBS) and horse serum (HS) (Sigma).
4. Tissue culture grade dimethylsulfoxide (DMSO) and ethanol.
5. Penicillin/streptomycin (antibiotic/antimycotic) stock solution (Sigma)
6. Collagen IV and poly-D-lysine (Sigma) – prepared as 5 μg/mL solution in growth medium for coating the dishes for cell adhesion.
7. Nerve growth factor (NGF) (Sigma) for differentiation of PC12 cells.
8. MitoXpress™ and IC60N phosphorescent oxygen-sensitive probes (Luxcel Biosciences, Ireland) (*see* **Note 3**).
9. EndoPorter™ reagent (Gene Tools, USA) for probe transfection – prepared as 1 mM stock in DMSO (*see* **Note 4**).
10. Glass-bottom imaging dishes (MatTek, USA).
11. Custom-made glass cylinders, approximately 9 mm outer diameter, 7 mm inner diameter, 15 mm long – one per dish (*see* **Note 5**).
12. Vacuum grease Apiezon L (Apiezon Corporation, USA).
13. Metabolic effectors (Sigma): mitochondrial uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and complex III inhibitor antimycin A – prepared as 1 mM stock solutions in DMSO; extracellular Ca^{2+} chelator ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) – prepared as 0.5 M solution in water, pH 7.4.

14. Live Cell Array^{TM} devices (Nunc) for imaging of suspension cells.
15. Standard tissue culture equipment: CO_2 incubator, laminar flow cabinet, Gilson pipettes, centrifuge, vacuum aspirator, inverted transmission microscope, hemocytometer.

2.1. Imaging Equipment

1. Standard wide-field fluorescent microscope Axiovert 200 with heated stage and objective (Carl Zeiss, Germany).
2. Fast gated CCD camera (LaVision, Germany) coupled to the microscope.
3. Custom-made LED excitation module (LaVision) coupled to the microscope, synchronized with CCD camera and controlled by the microscope software (LaVision). The module contains LED driver and a set of bright 397, 470, and 590 LEDs which are selected manually.
4. Filter cubes for the O_2-probe: 395 nm bandpass excitation, 450 nm dichroic mirror, 650 nm longpass emission (Carl Zeiss) (*see* **Note 6**). Filter cubes for the other fluorescent probes (e.g., 488/525, 535/550 nm – Carl Zeiss), as required.
5. Incubation system for cell culture (PeCon, Germany) consisting of a heating insert mounted on the microscope stage, low-volume incubation chamber, temperature, and O_2 and CO_2 controllers. This system allows rapid adjustment of temperature, gas composition (O_2, CO_2), and humidity in the sample compartment and changing of O_2 concentration.
6. Perfusion pump (Ismatec SA, Glattbrug, Switzerland) with 0.02 mm i.d. tubing (Gradko International Limited, Winchester, England) and a standard 22.5G hypodermic needle which are connected to the incubation chamber (*see* details in **Section 16.3**). The set-up provides effector addition during time-lapse imaging experiments, without disturbing the sample, gas composition, temperature, and focus of the region of interest (ROI).
7. ImSpector software (LaVision) for instrument control, FLIM and time-lapse measurements, image analysis, and data processing.
8. Wide-field fluorescence microscope IX51 (Olympus) equipped with a long distance 40 × objective LUCPFLN (Olympus), climate control chamber for imaging in Live Cell Array devices. Equipped with a 395 nm excitation filter and a 600 nm cut-off emission filter.

3. Methods

3.1. Culturing and Preparing the Cells for Imaging Experiments

1. All cell culture work is performed under a biological safety cabinet. HCT116 cells are cultured as adherent cells; PC12 cells are initially cultured in suspension and then adhered on pre-coated surfaces and differentiated (*see* **Notes 1** and **2**). Jurkat cells are cultured in suspension.
2. HCT116 cells are cultured in 75 cm^2 cell culture flasks with 0.2 μm vent cap in McCoys 5A medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (P/S), 10% FBS, maintaining them in CO_2 incubator at 37°C and 5% CO_2. Cells are grown to a density of 1–2 × 10^6 cells/mL, regularly split, and reseeded at 1.5 × 10^5 cells/mL in 10 mL of medium. Cells are harvested from the flask using trypsin/EDTA, counted on a haemocytometer, and plated on imaging dishes by dispensing 0.2 mL of cell suspension (6 × 10^4 cells/mL) in the medium. Cells are then grown in minidishes to a confluence of approximately 90% (*see* **Note 7**) changing growth medium every 2 days.
3. PC12 cells are cultured in 75-cm^2 flasks in RPMI medium supplemented with 2 mM L-glutamine, 10% horse serum (HS), 5% fetal bovine serum (FBS), P/S at 5% CO_2 with regular passages in 30 mL at 0.5–1 × 10^5 cells/mL. For seeding, cells are harvested, centrifuged at 200*g* for 5 min, separated from the supernatant, washed in PBS, centrifuged again, and resuspended in 1 mL of trypsin/EDTA solution. Cells are then incubated for 90 s at 37°C, diluted with 10 mL of RPMI supplemented with 1% HS, and gently passed ten times through 23G needle to separate individual cells. The cells are counted and seeded in MatTek dishes pre-coated with a mixture of collagen IV (0.007%) and poly-D-lysine (0.003%) at 2.5 × 10^4 cells/dish. When the cells reach the confluence of ~50%, they are differentiated for 3–5 days in RPMI supplemented with 1% horse serum, P/S, and 100 ng/mL NGF. After differentiation cell layer becomes confluent and cells remain active for 2–4 days (*see* **Notes 7** and **8**).
4. Jurkat cells are cultured in 75-cm^2 flasks in RPMI containing 10% FBS, P/S to a density of 1–2 × 10^6 cells/mL at 37°C and 5% CO_2. Cells are split regularly and reseeded at 1.50 × 10^5 cells/mL in 20 mL of medium (*see* **Note 8**). Cells are then harvested from the flask, counted on a hemocytometer, centrifuged at 200*g*, resuspended in medium, adjusted to a concentration 1 × 10^6 cells/mL, and kept at 37°C.

3.2. Imaging of Adherent HCT116 Cells Loaded with MitoXpress Probe

1. Reconstitute a 1× package of MitoXpress probe (dry sample in a plastic vial) in 0.1 mL of growth medium with additives used to culture HCT116 cells, to produce a 10-μM probe stock (*see* **Note 9**).
2. Pipette the required amount of medium (0.2 mL per dish) to a glass or plastic vial, add stock solutions of probe (100 μL/mL) and Endoporter (6 μL/mL), and incubate the resulting probe loading solution for 10–15 min at 37°C.
3. Take the dishes with HCT116 cells prepared for imaging experiments as described in **Section 3.1**, step 2, and transfer 200 μL of probe loading solution to each dish.
4. Incubate the dishes in CO_2 incubator at 37°C for 24–30 h (*see* **Note 10**).
5. After the incubation, carefully aspirate probe loading solution from the dish and discard it. Add 1 mL of fresh medium, incubate for 5 min, then aspirate and discard. Repeat washing of the cells two more times and finally add 0.2 mL of medium (*see* **Note 11**).
6. Switch on the microscope and cell incubation system and allow them to warm up and equilibrate (T, CO_2, O_2) for at least 30 min. Set up the software for image acquisition and time-lapse experiments and the required filter combination (395 nm excitation and >650 nm emission).
7. Take clean glass cylinder and apply a thin, uniform layer of vacuum grease to one of its edges.
8. Take the imaging dish with cells (prepared in **Section 3.1**), aspirate medium, and then firmly attach the cylinder with its greased side to the glass bottom of the dish where the cells are grown (*see* **Note 12**). Add 180 μL of medium to the cap and 1 mL of medium to the outer space and place the measurement unit inside the heating insert. The assembled unit with glass cap is shown in **Fig. 16.1**.
9. Assemble the incubation chamber and allow the dish with the cap and cells to equilibrate for about 20 min (*see* **Note 13**).
10. Using a 100-μL Hamilton syringe, load the solution of effector into the tubing and pump it inside the chamber to allow temperature and gas equilibration.
11. Switch the microscope in transmission mode; focus on cells inside the cap area.
12. Switch the microscope to fluorescence mode. Select ROI where the cells have relatively uniform density and good loading with probe. Representative bright field and

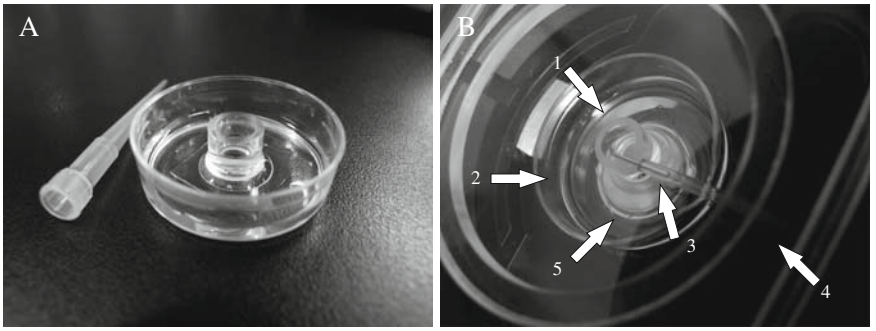

Fig. 16.1. Photographs of the MatTek dish with cap used for O_2 imaging of adherent cells. (**a**) General view of 35-mm imaging dish with attached glass cylinder mounted using vacuum grease. (**b**) Incubation chamber of the microscope with a sample prepared for O_2 measurements: 1 – glass cylinder; 2 – imaging dish; 3 – syringe needle connected to the pump for application of effector solution; 4 – heating insert with sample holder of the incubation system; 5 – microscope objective. Measurement requires restricted surface area to reduce convection and mass exchange within the sample, which is provided by a cylindrical cap attached to the bottom of the dish with cells. Effector addition should not disturb the sample. Small volume of effector stock solution is applied on top of the medium by means of perfusion pump with bended needle or gently applied onto the wall of the cap with a micropipette.

fluorescent images of HCT116 cells loaded with probe are shown in **Fig. 16.2a, b**.

13. Initiate image acquisition in time-lapse mode and monitor basal fluorescent signal of the cells under resting conditions.
14. If the signals are sufficiently high (compared to blanks outside the cells) and stable, proceed to the experiments with effector treatments (*see* **Note 14**). Initially, it is recommended to test cellular response by treating the cells with uncoupler FCCP and/or with mitochondrial inhibitor Antimycin A (*see* **Note 15**).
15. Move the microscope stage to a new field with cells within the dish, adjust the focus, and initiate image acquisition in time-lapse mode.
16. After recording the basal signal of resting cells for 5–15 min, make the addition of FCCP between two measurements. We normally add 1/10 of the total sample volume in the cap (i.e., 20 μL–180 μL) using the lowest possible flow rate of the pump (*see* **Note 16)**. Continue image acquisition for 10–30 min after the addition of FCCP. When the response is finished, stop the acquisition and analyze signal profile(s).
17. Interpretation of signal profiles of the intracellular probe. Basal fluorescent signal prior to effector addition reflects O_2 concentration within the cell which corresponds to the resting level of respiration. An increase in probe signal after FCCP addition reflects increased respiration which

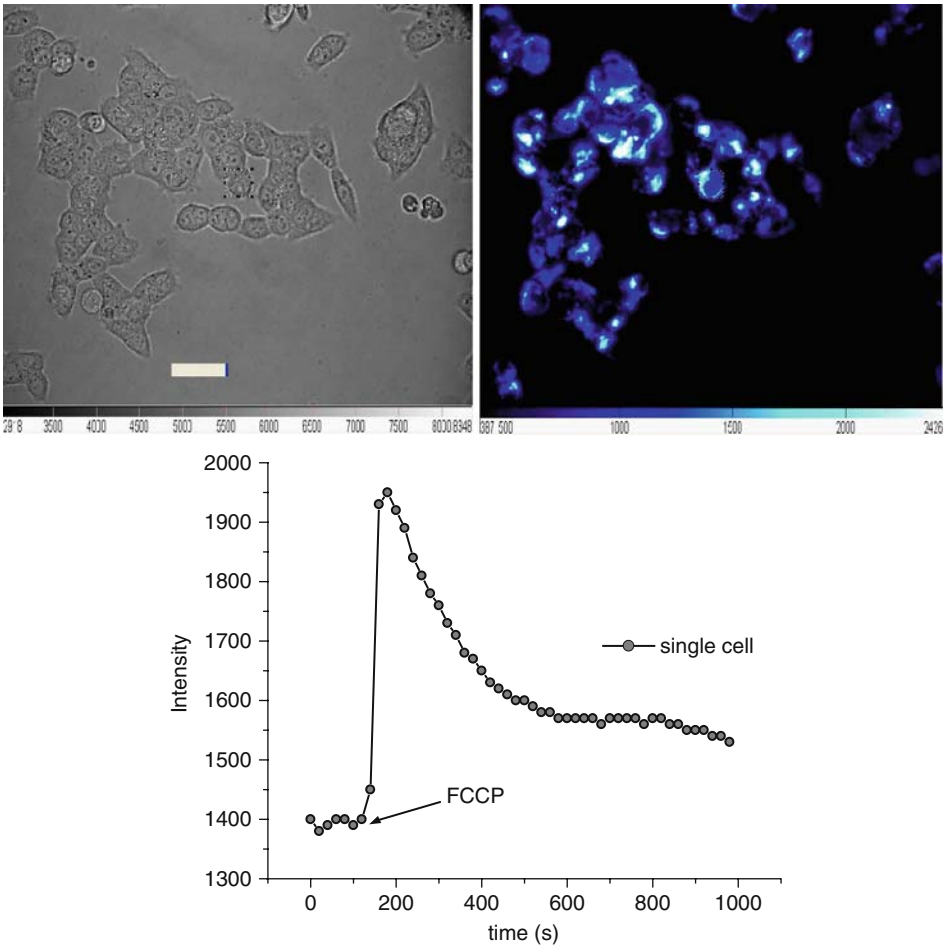

Fig. 16.2. Bright field (**a**) and fluorescent (**b**) images of HCT116 cells prepared for imaging in a MatTek dish and loaded with IC60 probe. (**c**) Respiratory response of HCT116 cells to the addition of FCCP measured by conventional imaging (390 nm bandpass/650 nm longpass). The increase in fluorescent signal reflects increased respiration due to mitochondrial uncoupling.

causes a reduction in cellular O_2. Conversely, Antimycin A is expected to inhibit cell respiration and elevate cellular O_2 to the levels present in bulk medium (i.e., air-saturated), thus bringing probe signal to a lower level (*see* **Note 17**). A characteristic profile obtained with HCT116 cells and FCCP is shown in **Fig. 16.2c**.

18. If the responses to FCCP and Antimycin A treatments are clearly seen, the samples are prepared properly, and the cells are in good condition, one can proceed to the experiments with stimulants having unknown effects on cell respiration.
19. In this case, take a new dish with cells loaded with probe and follow steps 11–17 above. Make effector addition and

monitor relative changes in intracellular O_2, which reflect the changes in cellular respiration. If required, repeat treatment.

20. Quantitative assessment. Plot signal profile for ROI with cells; adjust intensity scale as required. Select ROI outside the cells and plot the profile of blank signal. Subtract blank profile from the cell signal profile to produce the profile of probe fluorescence (*see* **Note 18**).

3.3. FLIM Analysis of Responses of Differentiated PC12 Cells Loaded with IC60 Probe

1. Prepare differentiated PC12 cells in imaging dishes as described in **Section 3.1**, step 3.
2. Take the vial containing IC60 probe, prepare stock solution and then load the cells with this probe using the same procedure as described above in **Section 3.2**, steps 1–5, for the MitoXpress probe.
3. Prepare samples and imaging system as described in **Section 3.2**, steps 6–10.
4. Start with a bright field image of the sample for prefocusing, using repetition mode with an exposure time of 5 ms.
5. Change to fluorescence mode. Using steady-state mode (continuous excitation) and exposure time of 300–1000 ms, take one fluorescence image of the sample. Fluorescent signal received by the camera should be in the region of 1000–3000 counts per pixel (*see* **Note 19**). Make adjustments of the focus and sensitivity/scale. Repeat the adjustment taking more images if required.
6. Program the software for time-lapse experiments in the FLIM mode. Using ImSpector software, set up the parameters of phosphorescence lifetime measurements: excitation pulse width, camera modulation, gate time, and modulation frequency (ratio of the gate time and repetition time) (*see* **Note 20**). General scheme of phosphorescence decay measurement implemented in our system is shown in **Fig. 16.3**. Typical settings for the IC60 probe are given in **Table 16.1**.
7. Perform one set of measurements to generate phosphorescence decay curve for the ROI containing loaded cells. Make final adjustment of the sensitivity and intensity signals generated by FLIM. Intensity at the first delay time, which is a function of the exposure time, excitation pulse length, and x-y binning, should be in the range 1000–3000 counts (*see* **Note 21**). Signal intensity can be increased by binning; however, this will decrease image resolution.
8. Move the sample to a new field with cells and commence FLIM measurements. Each time measure

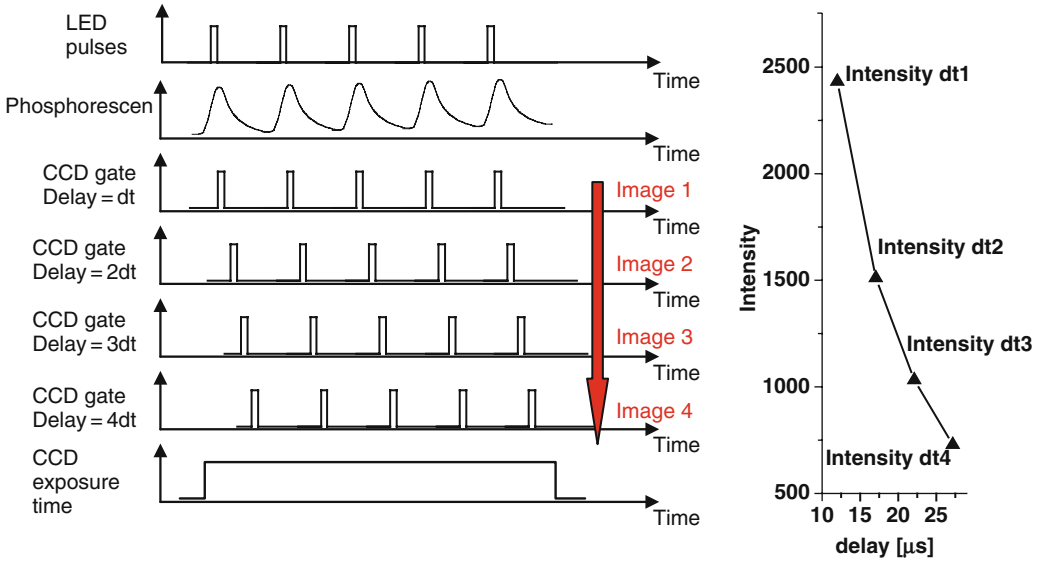

Fig. 16.3. Schematic description of FLIM measurements and the main parameters used: LED pulsing rate, gate time on the CCD camera, CCD camera exposure time, delay time, and the number of points on the curve (*left panel*). Measured phosphorescence decay curve (*right panel*).

Table 16.1
Typical settings for FLIM measurements with the O_2 probe

Parameter	Setting
Exposure time	1–3 s
Pulse length	10 µs
Integration start time	2 µs after the pulse
Integration end time	Typically 60 µs
Number of measurement points	10–20 points
Gate time	10–30 µs
Cycle time	100 µs

phosphorescence decay of the whole image, repeating this every 2–3 min.

9. Between two measurements of the phosphorescence decay make effector additions (refer to **Section 3.2**, steps 16 and 17 for details) and continue acquisition of decay curves. After the required number of measurements, stop the acquisition.
10. Define ROIs within the sample which contain cells loaded with probe, and generate phosphorescence decay curves for each ROI and for each time point of kinetic measurement. Extrapolate these decay curves with single-exponential fits

and determine phosphorescence lifetime value for each measurement.

11. Plot lifetime values as a function of time for each of the ROI and analyze the resulting profiles. Examples of FLIM images and respiratory response of PC12 cells to the depletion of extracellular Ca^{2+} are shown in **Fig. 16.4**. Intensity profiles can also be generated from the images (*see* **Fig. 16.4b**). If required, phosphorescence lifetime profiles produced by FLIM can be converted into O_2 concentration scale, using probe calibration which is determined separately (*see* steps 12–16 below).

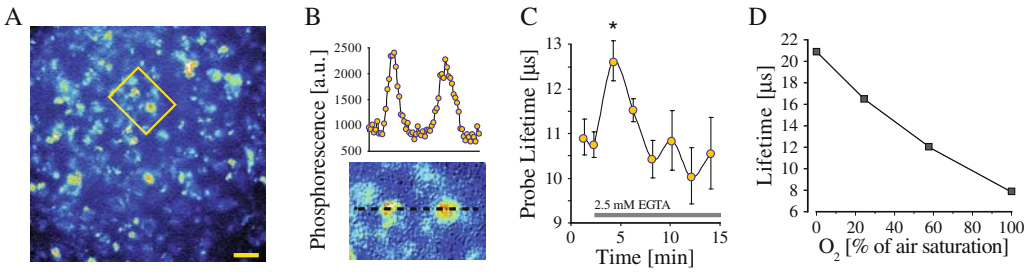

Fig. 16.4. Respiratory response of differentiated PC12 cells loaded with IC60 probe to the depletion of extracellular Ca^{2+} measured by FLIM. (**a**) Distribution of the probe intensity signal and selected ROI (*inset*). (**b**) Profile of phosphorescence intensity within ROI showing signal-to-noise ratio. (**c**) The response to the addition of EGTA (2.5 mM) with a transient increase in probe lifetime reflecting partial deoxygenation of the cell due to increased respiration ($p<0.01$ at the peak of the response). (**d**) The relationship between probe lifetime and O_2 concentration (calibration).

12. Take a fresh imaging dish (without glass cap) containing cells pre-loaded with the probe and 1–2 mL of medium and add Antimycin A to the medium at a final concentration of 10 μM. This should block cellular respiration and eliminate O_2 gradients in the sample.
13. Insert the dish in the incubation chamber with pre-set pO_2 (e.g., 3%) and incubate it for about 30 min to achieve temperature and gas equilibrium.
14. Prepare the microscope for FLIM measurements as described above (*see* steps 4–7 above). When the sample is ready, measure phosphorescence decay curve. Calculate the lifetime as described in step 10 above.
15. Change the incubation system to a new pO_2 setting and re-equilibrate the sample by incubating it for ~30 min. Move the sample/microscope stage to a new field with cells and perform lifetime measurement again. Repeat this at 3–4 other pO_2 settings.
16. Plot the relationship between phosphorescence lifetime of the probe loaded intracellularly and O_2 concentration. Adjust it to the desired O_2 scale: at 37°C and normal

ambient pressure, 20.5% O_2 in the chamber corresponds to 100% of air saturation or 200 μM O_2. Use the resulting relationship to convert measured phosphorescent lifetime profiles into profiles of cellular O_2 concentration.

3.4. Imaging in Live Cell Arrays of Suspension Jurkat Cells Loaded with the IC60 Probe

1. Reconstitute 3 × package of IC60 probe (300 nmoles dried in a vial) in 0.3 mL of medium to produce a 10-μM probe stock.
2. Take in a plastic vial 200 μL of suspension of Jurkat cells (1×10^6 cells/mL) and add 20 μL of probe and 1.2 μL of Endoporter stock solutions. Incubate at 37°C, 5% CO_2 for 24 h (*see* **Note 10**).
3. After incubation, wash the cells three times by centrifuging them at 200*g*, removing supernatant, and finally resuspend the cells in 0.1 mL of medium. Keep this suspension (approximately $\sim 2 \times 10^6$ cells/mL) at 37°C, 5% CO_2 for further use.
4. Switch on the Olympus IX51 microscope. Take a new LCA device, place it on the stage of the Olympus microscope, and allow to warm up and equilibrate at 37°C for about 20 min (*see* **Note 13**).
5. Using a P20 pipette load the LCA with 2.5 μL of suspension of cells prepared in step 3 above. Incubate for 5 min at 37°C and then flush with 15 μL of fresh medium.
6. Using transmission mode, focus on the microwells with cells within the LCA. Ensure that the device is loaded properly and the majority of the microwells contain trapped cells (*see* **Note 22**). Sample images of the LCA with cells are shown in **Fig. 16.5a, b**.
7. Switch the microscope to fluorescence mode, adjust the focus and initiate image acquisition in time-lapse mode. Take images every 20–60 s for about 10 min (*see* **Note 14**), using excitation at 395 nm and collection of emission with a 600 nm cut-off filter. Make sure that cell loading with probe is sufficient, adjust the sensitivity and scale as required.
8. After basal respiration (resting cells) is recorded, pause monitoring and apply effector treatment. Take solution of effector in medium (diluted to the required final concentration) and apply with a Gilson P20 pipette 15 μL of this solution to the loading bay of the LCA. After the effector is loaded in the LCA, resume monitoring for the required period of time (*see* **Note 23**).
9. Select several ROIs within the LCA – controls with cells – and analyze measured profiles and respiratory responses. If required, use the well without cells as a reference (blank).

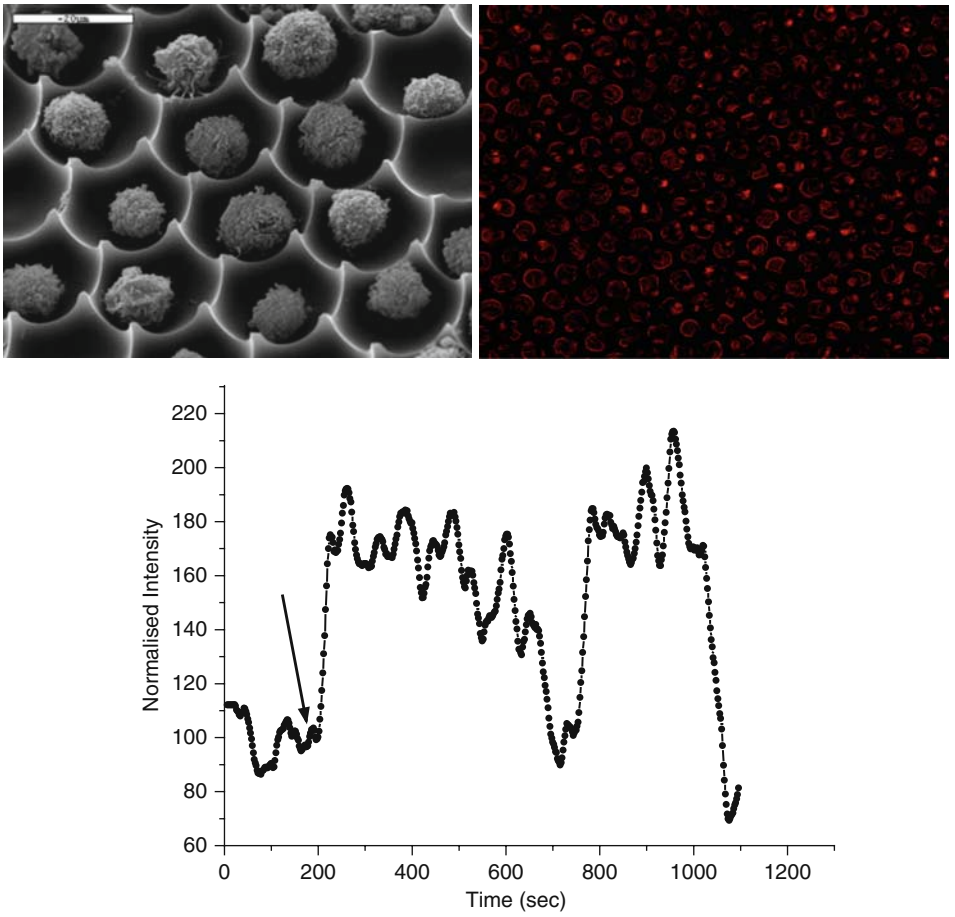

Fig. 16.5. (**a**) Photograph of a working section of Live Cell Array unit used for imaging of suspension cells (reproduced from (24) with permission of the Royal Society of Chemistry). (**b**) Fluorescent image showing individual Jurkat cells loaded with the O_2 probe trapped in the cavities of LCA. (**c**) Metabolic response of individual Jurkat cell to the addition of mitochondrial uncoupler FCCP (1 μM), measured using the LCA.

For interpretation of measured signal profiles and respiratory responses of cells, refer to **Section 3.2**, step 18. Example of response of individual Jurkat cell to FCCP treatment is shown in **Fig. 16.5c**.

4. Notes

1. A range of common cell lines can be used for imaging intracellular O_2; however, probe loading efficiency and metabolic activity may vary greatly for different cell types. Cells having well-developed mitochondrial machinery and

high rates of O_2 consumption (e.g., primary cells) are the preferred choice.

2. Growth medium, additives, and optimal culturing conditions may vary for each particular cell type.
3. Other O_2 probes for intracellular use, including those which do not require transfection reagents, are also available or in development. Contact corresponding suppliers (e.g., Luxcel Biosciences, www.luxcel.com) and research labs to get advice on the most suitable probe for your particular imaging system, measurement task, cell type.
4. In our experience, probe transfection with EndoPorter works well with many common cell types. Other loading reagents and procedures may be efficient as well. Generally, probe loading method and procedure need to be optimized for each cell type and medium.
5. Instead of the imaging dishes with cylinders, glass-bottom 96-well imaging microplates, multiwell flexiPERM inserts (Greiner Bio, Germany) can also be used. Devices with larger well size (e.g., 24-well plates) are less preferred.
6. Equivalent live cell fluorescent imaging systems, which are spectrally compatible and sensitive enough with the O_2 probes, can also be used. The MitoXpress^{TM} probe is excitable at either 370–395 or 525–545 nm, and emits around 630–700 nm (maximum at 650 nm). The IC60 probe is excitable at 380–400 as well as at 580–600 nm, and it emits in the near infrared range 720–850 nm (maximum at 760 nm). IC60 probe is more photostable; however, its very longwave emission and lower brightness (compared to the MitoXpress^{TM}) can be problematic for some imaging systems.
7. Relatively high surface density of cells and high respiratory activity are the main requirements of a successful metabolic assay using the intracellular O_2 probes. Cells having high density of mitochondria and high levels of respiration/oxidative phosphorylation such as PC12 and HCT116 produce robust responses to stimulation with various metabolic effectors, whereas slowly respiring and highly glycolytic cells would be difficult to analyze by this method.
8. Optimal conditions for culturing depend on particular cell type. Differentiation is important for many cells to display a range of functional characteristics observed in vivo.
9. Probe loading by facilitated endocytosis in the presence of Endoporter reagent does not impose special requirements on the medium used. Other loading reagents and methods may require serum-free conditions.

10. Probe loading with EndoPorter is relatively slow; however, it works well in different media and cell types and produces high fluorescent signals which are easy to measure.
11. Extreme care should be taken not to damage the cell layer and wash the cells off the surface.
12. The cylinder is required to stabilize local oxygen gradients at cell layer by reducing surface area of the medium which in turn reduces convection and mass exchange within the sample. Based on our experience, this set-up produces better quality results.
13. Efficient temperature control of the measurement system and equilibration of the sample are very important. All O_2 probes produce fluorescence response to temperature fluctuations.
14. During these preparations, sample exposure to excitation light should be kept to a minimum. Photostability of the MitoXpress probe is moderate, whereas the IC60 probe is more photostable but less bright.
15. Optimal effector concentrations which produce maximal metabolic response depend on the cell type. We normally use 0.5–5 μM for FCCP and 10 μM for Antimycin A (in the well).
16. Mixing and disturbing the sample during the addition should be avoided as this will affect local O_2 gradients at cell monolayer and, hence, the probe signal.
17. Compared to mitochondrial inhibitors, uncouplers and activators of metabolism produce strong and positive optical response which is easier to measure. If basal respiration of cell monolayer is low and unable to create local O_2 gradient, metabolic responses may not be detectable, even though they are present. This is determined by the principle of operation of the intracellular O_2 assay (10). The lack or the absence of optical response from the sample/cells is a rather common case. This can be due to low respiration rate so that the cells are unable to create measurable local O_2 gradients and changes in response to stimulation. Cell damage during sample preparation, phototoxicity, or probe photobleaching during the measurement can also occur. The following can be used to improve assay sensitivity: (i) increase cell confluence (overgrow cells); (ii) increase cell respiration by using glucose-free medium containing 10 mM of galactose and pyruvate (12); (iii) increase the height of medium layer in the cap; (iv) conduct imaging experiments under reduced pO_2 (hypoxia chamber set at 10% or 5% pO_2); (v) optimize cell culture and probe loading conditions.

18. If probe photobleaching is high, measured profiles should be corrected for it. The initial part of the profile, which corresponds to basal respiration, can be used to quantify the rate of photobleaching.
19. Try several different settings in steps 4 and 5, and select those which provide lowest photobleaching during image acquisition.
20. For our system, delay time is calculated from the ratio of the effective measurement time (between start and end of one cycle) and the number of measurement points less one point. Cycle time is the time between LED pulses. Gate is integration time of the fluorescence signal for each delay time.
21. To get approximately the same level of signal from the sample in the FLIM mode, exposure time on the camera needs to be increased approximately five times compared to the steady-state measurement mode.
22. If loading of the LCA with cells is poor, step 8 can be repeated.
23. The time of monitoring of metabolic responses of cells is determined by the mode of action of the effector. It is also limited by the changes in cell viability during the measurement (i.e., phototoxicity). Most of our imaging experiments were conducted over a period of 20–60 min. Since both MitoXpress and IC60 probes do not leak from the cells, longer experiments with reduced sampling frequency are possible.

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