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Intracellular pH and pCa Measurement

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1. Introduction

Recent improvements in confocal technology permit the use of a confocal microscope as an effective tool to both measure concentration and visualize distribution of several ions. Effective discrimination against out-of-focus information in the confocal microscope permits for mapping of ion distribution at the subcellular level ***(1)***. To generate such a map with any degree of accuracy it is necessary to compensate for inherent cellular inhomogeneities in optical path, probe distribution, etc. To do so ratiometric measurements are usually employed ***(2,3)***. Fluorescence ratiometry takes advantage of a differential spectral sensitivity of a ratio probe to the measured parameter (e.g., ion concentration). Whereas fluorescent properties of the ratio probe at one wavelength are parameter sensitive in one manner, the emission (or excitation) of the probe at a distinct, well-separated wavelength must be either parameter insensitive, inversely sensitive, or exhibit a different sensitivity profile. Emission intensities for the two wavelengths are then divided by each other and thus the resulting ratio becomes normalized for inhomogeneities in probe distribution and concentration and in the system geometry. To obtain meaningful data the ratio values must be calibrated against a standard, which usually is a graded change in the parameter. Modern day confocal microscopy depends on lasers as light sources which causes a severe limitation in number of wavelengths available for fluorochrome excitation. Several H^+-sensitive fluorochromes are excited at wavelengths close to those emitted by standard lasers, however, this is not the case with Ca^{2+}-sensitive fluorochromes ***(4)***.

This chapter provides protocols for measurement of intracellular pH (pH_i) and intracellular pCa (pCa_i) with a typical confocal microscope of the present day. Such a microscope consists of a scanner capable of simultaneous dual

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excitation as well as simultaneous collection of dual emissions. The scan time for a full frame is about 1 second in a 'standard' mode or 2 s in a "slow" mode where the pixel dwell time is greater and, consequently, the signal-to-noise ratio is higher. The simultaneous dual excitation/dual emission mode of operation requires a FITC/Texas Red-type filter module in which the two emission wavelengths are separated at 560 nm with a dichroic mirror. The light source is likely to be a mixed gas krypton/argon laser. The fluorochromes, use of which is described herein, are seminaphthofluorescein-calcein acetoxylmethyl ester (SNAFL-calcein AM) for pH_i measurement (***5,6*** and *see* **Note 1**) and, for pCa_i measurement, a mixture of Calcium Green and Fura-Red based on a mixture of fluo-3 and fura-red, first described by Lipp and Niggli ***(7)***. SNAFL-calcein seems to be a good choice for pH_i measurements with the confocal microscope as it loads easily, is well retained in cells, and can be used without any modification to the currently most popular hardware. The Calcium Green/Fluo-3 and Fura-Red dye mixture is a necessity due to the lack of commercially available indicators that exhibit a spectral shift upon Ca^{2+} binding (necessary for ratioing) and are excited with visible light.

2. Materials

1. SNAFL-calcein AM, Calcium Green AM, Fura-Red AM, calcium calibration Kit 1
2. Pluronic F-127 can be purchased from Molecular Probes (Eugene, OR)
3. Ionomycin from Calbiochem (La Jolla, CA).
4. Dimethylsulfoxide (DMSO) and nigericin from Sigma Chemical Co. (St. Louis, MO).
5. Trypsin/EDTA.
6. α-minimal essential medium (α-MEM).
7. α-MEM with 20 m*M* HEPES (MEM-HEPES, pH 7.36)
8. Heat-inactivated fetal bovine serum (FBS) is from Gibco (Canadian Life Technologies, Burlington, ON, Canada). All MEM-HEPES media should be $NaHCO_3^-$ free.
9. Amino acid-free MEM-HEPES or a buffers shown in **Tables 1** and **2** are used for dye loading.
10. Nigericin-containing calibration solutions: Five solutions with pH values of 6.4, 6.7, 7.0, 7.3, and 7.6. made of K^+-MEM-HEPES supplemented with 10 μ*M* of nigericin.

3. Methods

3.1. Measurement of pH_i

These conditions have been optimized for near-confluent MDCK cells. **Conditions for each cell type have to be established experimentally**.

1. Load cells with 5 μ*M* SNAFL-calcein AM in serum-free and amino-acid-free medium for 30–40 min. Higher dye concentrations and/or longer incubation times will cause compartmentalization of the dye, which may cause measurement

Table 1
Composition of the Media Used for Dye Loading (A), pH_i Measurement (B), and Calibration (C)

	A	B[a]	C[b]
NaCl, m*M*	118	118	0
KCl, m*M*	5.3	5.3	123.3
$CaCl_2$, m*M*	1.8	1.8	1.8
$MgSO_4$, m*M*	0.8	0.8	0.8
$NaH_2PO_4 \cdot 2H_2O$, m*M*	1	1	1
HEPES, m*M*	20	20	20
D-Glucose, m*M*	10	10	10
Phenol red, m*M*	0.03	0.03	0.03
Lipoic acid, m*M*	1	1	1
Sodium pyruvate, m*M*	1	1	1
Set of amino acids	–	+	+
Set of vitamins	+	+	+
Fetal bovine serum, %	0	3	0
Nigericin, µ*M*	0	0	10

[a]Composition of the medium for pH_i measurement is essentially the same as that of the culture medium (α-MEM), in which HEPES was replaced by sodium bicarbonate and 10% FBS was added.

[b]pH of the calibration medium was adjusted with KOH or HCl.

The + or – represents the presence or absence of a component in the medium, respectively.

Table 2
Composition of the Buffers Used for Dye Loading (A), pCa_i Measurement (B), and Calibration (C)

	A	B	C
NaCl, m*M*	140	140	–
KCL, m*M*	4	4	100
$CaCl_2$, m*M*	1	1	–
$MgCl_2$, m*M*	1	1	–
HEPES, m*M*	10	10	–
Glucose, m*M*	10	10	–
Ionomycin, µ*M*	–	–	2
pH	7.2	7.2	7.2
MOPS, m*M*	–	–	10
EGTA/CaEGTA, m*M*	–	–	0–10
Pluronic F = 127, 0.02%	+	–	–

The – represents the absence of a component in the medium.

errors. (*see* **Notes 2** and **3**). We have never had any need to use Pluronic F-127 to aid loading of SNAFL-calcein. This may, however, be necessary with some cell types. For loading of calcium probes we use 0.02% Pluronic F-127 (*see* **Table 2**).

2. Incubate cells for 15–20 min in α -MEM with 20 m*M* HEPES containing 2% serum to deestrify the probe and let cells recover. After this incubation is completed rinse cells twice with K^+-MEM-HEPES (*see* **Note 4**).
3. Place the cell under the microscope. An inverted microscope is the configuration of choice as it is possible to use one of many types of cell culture chambers designed for microscopy. We use a Bio-Rad MRC 600 on an upright microscope, therefore the coverslips with cells are placed in MEM-HEPES containing 2% FBS in a 60-mm culture dish. Images of cells (20 cells per image) are collected with a Zeiss Plan-Neofluar objective (40x, multiimmersion). Measurements can be made at either room temperature or 37°C.
4. Using **simultaneously** 488 **and** 568 nm laser bands, the FITC/Texas Red-type filter set (e.g., K1 and K2 in a Bio-Rad MRC 600) and simultaneous dual-excitation **and** simultaneous dual-emission recording feature of a confocal microscope collect intracellular emission of SNAFL-calcein from the green channel (excitation: 488 nm, emission: 525 nm) and from the red channel (excitation: 568 nm, emission: 615 nm). Be sure that the gain ranges of both channels cover the expected dynamic range of both emissions, i.e., while the weakest emission is still recorded, the strongest emission does not saturate the photomultiplier. This is done on a Bio-Rad MRC 600 by manual adjustment of gain control ("auto" gain control in OFF position) and confocal aperture (the pinhole).

   Specifically, for a Bio-Rad MRC 600, we use the following settings for pH_i imaging (for **both** *photomultiplier tube 1* and *photomultiplier tube 2*): **(1)** *confocal pinhole diameter*, fully open, **(2)** *enhancement control*, off, **(3)** *gain control*, manual, **(4)** *black level control*, manual, **(5)** *neutral density filter* no. 2 (neutral density = 1.5; 3% transmission), **(6)** *scanning speed* 1 s per frame.

   The measurements must be internally calibrated.
5. Calibrate pH_i immediately after the end of each experiment under the same microscopic working conditions as those for pH_i measurements. Use a fresh batch of cells loaded with SNAFL-calcein AM, equilibrated and rinsed twice with K^+-MEM- HEPES. Flush the dish with nigericin-containing K^+-MEM-HEPES of desired pH value and incubate for 3–5 min before taking the calibration measurements. Repeat for all the pH values.

   We normally take a series of images from **different** areas of the cell monolayer in the nigericin-containing K^+-MEM-HEPES with pH values of 6.4, 6.7, 7.0, 7.3, and 7.6. At least five **different** image pairs are collected for pH calibration points. We use acidic solutions first to minimize the cell blebbing. Between each calibration measurement, samples are washed twice in the buffer for the next-point calibration and allowed 3–5 min equilibration. (*see* **Note 5**).
6. Ratio the green and red emissions by performing an arithmetic division of one image by the other. This is usually followed by a multiplication by a constant factor. Any software that performs arithmetic operations on image files will do the job. The calibration data are used to generate a standard pH curve for each experiment.

7. Be aware that useful pH range of SNAFL-calcein AM is 6. 2–7. 8, as the dye is difficult to calibrate outside this pH range according to Zhou et al. (***6*** and *see* **Notes 6–8**).

3.2. Measurement of pCa_i

Conditions optimized for sparse retinal pigment epithelial cells of the chick embryo. **Conditions for each cell type have to be established experimentally**.

1. Load cells with a mixture of acetoxylmethyl esters of Calcium Green (8 μ*M*) and Fura-Red (5 μ*M*) in the loading buffer in presence of 0.02% Pluronic F-127 (1 μL of 20% stock solution per milliliter of stain solution) for 30–40 min. Higher dye concentrations and/or longer incubation times will cause compartmentalization of the dye, which may cause measurement errors (*see* **Note 9**).
2. Incubate cells for 15–20 min in α-MEM with 10 m*M* HEPES containing 2% serum to deestrify the probe and let cells recover.
3. Place the cell under the microscope. An inverted microscope is the configuration of choice as it is possible to use one of many types of cell culture chambers designed for microscopy. We use a Bio-Rad MRC 600 on an upright microscope, therefore, the coverslips with cells are placed in measurement buffer (*see* **Table 2**) in a 60-mm culture dish. Images of cells (20 cells per image) are collected with a Zeiss Plan-Neofluar objective (40x, multiimmersion). Measurements can be made at either room temperature or 37°C.
4. Using **only** the 488-nm laser band, the FITC/Texas Red-type filter set (e.g., K1 and K2 in a Bio-Rad MRC 600) and simultaneous dual-emission recording feature of a confocal microscope collect intracellular emission of Calcium Green from the green channel (excitation: 488 nm, emission: 525 nm) and of Fura-Red from the red channel (excitation: 568 nm, emission: 615 nm). Be sure that the gain ranges of both channels cover the expected dynamic range of both emissions, i.e., while the weakest emission is still recorded, the strongest emission does not saturate the photomultiplier. **This has also to be taken into consideration while testing the proportion of the dyes in the mixture**. The system calibration is done on a Bio-Rad MRC 600 by manual adjustment of gain control ("auto" gain control in OFF position) and confocal aperture (the pinhole).
5. Calibrate pCa_i immediately after the end of each experiment under the same microscopic working conditions as those for pCa_i measurements. Use a fresh batch of cells loaded with the dye mixture, equilibrated and rinsed twice with appropriate Ca^{2+} buffer (*see* **Note 10**). Flush the dish with a series of solutions of different Ca^{2+} concentrations containing a Ca^{2+} ionophore, ionomycin. A series of measurements is taken of the dye-loaded Ca^{2+}-permeable cells flushed with the graded [Ca^{2+}] solutions to generate a standard pCa_i curve for each experiment. Repeat for all the Ca concentrations.

   We normally take at least five **different** image pairs of images from **different** areas of the cell monolayer for each pCa_i calibration point. Between each calibration measurement, samples are washed twice in the buffer for the next-point calibration and allowed 3–5 min equilibration.

6. Ratio the green and red emissions by performing an arithmetic division of one image by the other. This is usually followed by a multiplication by a constant factor. Any software that performs arithmetic operations on image files will do the job. The calibration data are used to generate a standard pCa_i curve for each experiment (also *see* **Notes 11–14**).

4. Notes

1. The acidic form of SNAFL-calcein AM (Molecular Probes cat. no. S-3052) has spectral properties similar to fluorescein isothiocyanate (FITC) (excitation: Ç490 nm, emission: 535 nm) while its basic form behaves like a hybrid of TRITC and Texas Red (excitation: 540 nm, emission: 620 nm).
2. Before loading the coverslips are rinsed twice with serum free α-MEM-HEPES. They are then incubated at room temperature in amino-acid free α-MEM-HEPES containing 5 μ*M* of SNAFL-calcein AM from a stock solution in anhydrous DMSO, which was either prepared immediately before use or used from aliquot stored at –20°C. Aliquots are good for 1 mo in DMSO. The final concentration of DMSO in the culture medium should never exceed 0.1%.
3. For subconfluent cells time and the temperature of loading is very important. To prevent dye compartmentalization, with SNAFL-calcein concentration at 5 μ*M*, loading at room temperature should take no longer than 40 min. Cells could be loaded at 37°C as well, but the time of loading should then be shortened to 30–35 min. For very packed cells loading times should be slightly extended.
4. Never use antibiotics for any pH or pCa_i measurements or calibrations. NaCl is replaced with KCl in the medium for calibration only.
5. Some cell lines are very sensitive to any change in conditions of their medium (such as a difference in pH) and do not survive the complete calibration procedure very well. It might be advisable to load a few smaller coverslips under exactly the same conditions (we were doing it in one Petri dish) and use one or two of them for each pH point calibration instead of using one coverslip for the calibration of all pH points. In our experience, the entire experiment, including calibration, should be completed within ca. 3 h, otherwise fresh batch of cells must be loaded.
6. The quality and the time resolution of pH_i images are restricted by the microscopic working conditions. Samples are localized and brought into focus with fast scanning modes (1/4 s per frame) to minimize photobleaching. We routinely record images with the laser scanning speed of 1 sec per frame (512 lines), the confocal pinholes of both channels fully open and manually adjusted gain control. With the scanner *gain* on, the background noise levels of the 525–614 nm emission images and the 615 nm ones were quite different even with the same manual settings for the black level control. This is due to the gain device automatically increasing the photon/output voltage ratio ***(8,9)***.
7. This calibration of pH_i is performed according to the high $[K^+]$/nigericin method of Thomas et al. ***(10)***.

8. Calcium Green has FITC-like spectral properties (excitation at 506 nm, emission at 530 nm) and its fluorescence intensity increases with an increase in Ca^{2+} concentration. Calcium Green has the same spectral properties as Fluo-3; however, Molecular Probes indicate that Calcium Green is five times brighter than Fluo-3 and that it bleaches more slowly. Fura-Red is a ratiometric dye that is excited at 440 and at 490 nm and emits with a huge Stokes shift at 660 nm. It is unusual in that its emission intensity decreases with an increase in Ca^{2+} concentration.
9. Conditions of loading for each cell type should be established experimentally in such a manner that proportion of the dyes will give good quality images in whole expected spectrum of pCa_i. It is important to keep in mind that Fura-Red images will get **darker** with higher pCa_i, while Calcium Green images will get **brighter** with higher pCa_i. Reported dye proportions for loading the mixture of acetoxylmethyl esters vary from 3:4 μ*M*, to 1:10 μ*M*, to 50:100 μ*M* to 100:300 μ*M* for fluo-3:Fura-Red; we load Calcium Green:Fura-Red at an 8.3:4.6 μ*M* ratio.
10. The calibration procedure based on Grynkiewicz's equation ***(11)*** is not applicable to cells loaded with a mixture of acetoxylmethyl esters of the two dyes. Even after modification of Grynkiewicz's equation to accommodate the two different Ca^{2+} K_ds of the dyes, its applicability remains highly problematic ***(12)***. Therefore, a calibration procedure similar to that used for pH_i measurements should be implemented. For calibration measurements several buffers of increasing Ca^{2+} concentrations were prepared according to Molecular Probes protocol included in their calibration kit. Molecular Probes sells two Ca buffer kits (cat. no. C-3008 and C-3009). For each pCa point we use a fresh coverslip with cells loaded under the same conditions. The coverslip is rinsed with appropriate Ca^{2+} buffer and placed in the same buffer containing 2 μ*M* of ionomycin. Several different image pairs should be collected for a given calibration point.
11. Because a single and uniform optical section can be imaged by a confocal microscope, very successful attempts have been made at measuring pCa_i in thick samples using non ratio fluorochromes ***(13,14)***.
12. Maximal recording speed ("time resolution") is limited by the scanning speed (often: 512 lines/s). While ionic changes comprise extremely fast mode of cell signaling, a standard confocal microscope takes a very long time to collect a full-field image of a decent quality. The time resolution can be further increased by reducing the image frame size which in some applications can consist of just one line. The confocal microscope in a single line scanning mode can visualize very fast (millisecond range) changes in an ion concentration along a rapidly scanned single line. Slit scanning confocal microscopes, provide an option of video-rate imaging of ionic changes.
13. Physiologically meaningful data can be collected only when the probe is where we think it is. Therefore extreme caution has to be exercised while loading cells with a fluorescent probes. Probe concentration, cell density, time of loading, temperature, the presence or absence of serum and particular amino acids in the medium, not to mention all sorts of spells, will affect cell loading with AM forms

of dyes. Overloaded probes will compartmentalize immediately, and most probes loaded into the cytosol will compartmentalize eventually, meaning that they end up in various cell compartments such as the mitochondria, endoplasmic reticulum, and all types of vesicles. This will lead to measurement errors. Unfortunately there is no ready recipe for cell loading. Dye loading conditions must be worked out for each experimental setup. The effect of presence or absence of serum on (1%) efficacy of loading and (2%) compartmentalization of loaded dye should be checked first. Next temperature of loading should be experimented with. Whereas some cell types will load quickly and well in 37°C others may need more time at lower temperature to load uniformly. To ascertain a uniform diffusion of dyes into thicker specimens (such as very packed multilayered epithelia or tissue sections) it is not unheard to use a refrigerator as an incubator! However, the presence or absence of extracellular esterases will also be a factor dictating how much time and what concentration of dye can be used to load the specimen. If an incubation in temperature lower than room temperature is required, it will be necessary to warm up the specimen for 20–30 min at 37°C to allow the dye deestrification.

14. Pluronic F-127 in the range of 1–4 μL/mL of stain solution has been often used to aid loading of AM forms of dyes. Finally, cells attempt to expel the dye by pumping it out. It has been reported that, in particular case of Fluo-3, an addition of 2.5 m*M* probenecid (Sigma) inhibits the expulsion of the dye ***(15)***.

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