

METHODS PAGE

Measuring Ca²⁺ dynamics in pancreatic acini using confocal microscopy

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Introduction

The pancreatic acinar cell is the main parenchymal cell of the exocrine pancreas and plays a primary role in the secretion of pancreatic enzymes into the pancreatic duct (15). It is also the site for the initiation of pancreatitis (3, 14). Dynamic changes in cytosolic Ca²⁺ are necessary for both physiological and pathological acinar cell events (11). These divergent effects of Ca²⁺ are thought to result from distinct spatial and temporal patterns of Ca²⁺ signaling. For example, enzyme and fluid secretion from the acinar cell are linked to Ca²⁺ spikes from a restricted region of the apical pole where zymogen granules are localized (6). In contrast, a global Ca²⁺ wave followed by intense non-oscillatory Ca²⁺ signals is associated with pathologic intra-acinar protease activation (4, 7, 12).

The information below is intended to provide detailed instructions for detecting these Ca²⁺ signals in real time from freshly isolated

pancreatic acinar cells using confocal imaging (see **Note 1** about the different types of microscopy that are available for Ca²⁺ imaging). This provides a powerful tool to characterize acinar cell physiology and pathology.

1. Materials

1.1 Preparation of Pancreatic Acinar Cells

1. C57BL/6 mice (NCI) weighing between 20-30 gm or Sprague Dawley rats (Charles River) weighing between 50-60 gm. The pancreas from younger rodents appear to yield more evenly distributed acini, possibly because they contain less fibro-fatty tissue.
2. HEPES incubation buffer: 20 mM HEPES (Sigma #H4034), 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl₂, 1.3 mM CaCl₂, 10 mM glucose, 2 mM glutamine, and 1 × minimum Eagle's medium non-essential amino acids (Gibco #11140-050). Oxygenate for 20 min with rapid bubbling and pH to 7.4 with NaOH.

3. Bovine serum albumin (BSA) incubation buffer: HEPES incubation buffer (from (2)) plus 1% BSA (Sigma #A7906)
4. Collagenase digestion buffer: BSA incubation buffer (from (3)) plus 200 units/ml type-4 collagenase (Worthington #4188), and 1 mg/ml soybean trypsin inhibitor (Sigma #T-9003)
5. 125 mL Erlenmeyer plastic flask
6. P1000, P100, P10 pipettes
7. Dissection scissors and fine forceps (Fine Science Tools #14161-10)
8. 70% ethanol
9. Plastic transfer pipettes (USA Scientific #1020-2500)
10. 15 ml centrifuge tubes (Fisherbrand #05-527-45, polystyrene graduated conical)
11. Water bath with shaker (Precision Scientific #51220076)

1.2 Confocal Imaging of Pancreatic Acinar Cells

1. Acid-washed 22X22 mm glass coverslips (Fisherbrand #032811-9): Place coverslips in a solution of 2 parts HNO₃ (16.4 M stock) and 1 part HCl (11.6 M stock) for 2 hrs, then wash with deionized (DI) water and decant. Store in 70% ethanol until needed. (**Note 2**)
2. 18X18 mm glass coverslips (Fisherbrand #021510-9)
3. Laboratory film (Parafilm #PM-996)
4. Ca²⁺ indicator: We frequently use Fluo-4 (Invitrogen #F14201) or Fluo-5F (Invitrogen #F14222); final concentration range of 3-6 uM, reconstituted in DMSO. See Table 1 for a list of other commonly used Ca²⁺ indicators.
5. Perfusion setup: A luer lock (Becton Dickinson #932777) is fastened to a 60 ml syringe (BD Bioscience #DG567805), and a 23 $\frac{3}{4}$ gauge needle (Becton Dickinson #9328270) is secured to the luer lock. Three feet of PE50 tubing (Clay Adam PE50 427411) is attached to the needle and will run directly into the perfusion chamber. (**Note 3**)
6. Ring stand with clamps
7. Perifusion chamber (**Note 4**)
8. Vacuum line (with flask and tubing)

2. Methods

2.1 Preparation of Pancreatic Acinar Cells

1. Each experiment begins with the preparation of fresh HEPES incubation buffer (see Materials 1.1). Subsequently, the BSA incubation buffer and collagenase digestion buffer are prepared. All buffers are made fresh and stored at room temperature, with the exception of the collagenase buffer which is kept at 37°C.
2. One C57BL/6 mouse or one Sprague Dawley rat is euthanized by CO₂ asphyxiation. The cervical vertebrae are severed, the rodent is fixed in the supine position, and the abdominal surface is prepared using 70% ethanol. A laparotomy is performed to expose the abdominal cavity. The pancreas is dissected out and immediately placed into a 15 ml conical tube containing 6 ml of collagenase digestion buffer.
3. The collagenase digestion buffer containing the whole pancreas is poured into a small weighing boat. Large pieces of fat and blood vessels are removed, and 5 ml of collagenase digestion buffer are poured back into the 15 ml conical tube.
4. Using fine dissection scissors, the pancreas is minced in the small weighing boat containing 1 ml of collagenase digestion buffer. Mincing should continue for 5 min or until the resulting solution appears evenly dispersed.
5. The minced product is transferred to a 125 ml Erlenmeyer plastic flask, and the remaining 5 ml of collagenase digestion buffer is added to the container.
6. The flask is placed in a 37°C water bath and rocked at 90 rpm for 30 min. During this waiting period, Ca²⁺ activating agonists (e.g.

- caerulein, carbachol) are freshly prepared, Ca^{2+} indicators are reconstituted, and 22x22 mm acid-washed coverslips (see Materials 1.2.1) are rinsed with DI water, dried, and then placed on top of a flat surface lined by laboratory film.
7. After the 30 min digest is complete, the suspension is transferred back to the 15 ml conical tube and the cells are allowed to settle. The collagenase digestion buffer is removed and replaced with 6 ml of BSA incubation buffer. The tube is then vigorously shaken by hand for 10 seconds in order to break the cells into smaller clusters. Immediately after shaking, a transfer pipette is used to draw off large, floating debris from the unsettled media.
 8. The remaining cells are allowed to settle, and the media is exchanged with fresh BSA incubation buffer.
 9. Step 8 is repeated 1-2 times until the suspension is comprised of only small clusters that are barely visible to the naked eye.
 10. The BSA incubation buffer is removed and replaced with HEPES incubation buffer.
 11. Fluo-4 or Fluo-5F is added to the media at a final concentration of 3-6 μM , and the cells are resuspended. 500 μl of this suspension is plated onto each 22x22 mm coverslip and remains in the dark at room temperature for 20 min to allow the Ca^{2+} indicator to permeate the cells. See Table 1 for a list of commonly used Ca^{2+} indicators.

Table 1. Commonly used fluorescent Ca²⁺ indicators[#]

Ca ²⁺ indicator	Cat. #*	Excitation λ (nm)	Emission λ (nm)	K _d (nM)	Comments
HIGH AFFINITY					
Fluo-3	F1244	506	525	390	<ul style="list-style-type: none"> Single wavelength excitation and emission dye that is excited by a 488 nm Argon laser from a confocal microscope
Fluo-4	F14201	494	520	345	<ul style="list-style-type: none"> More sensitive than Fluo-3 due to a doubled efficiency of fluorescence excitation, meaning that the same amount of energy emits twice as much fluorescence
Fura-2	F1201	340/380	510	145	<ul style="list-style-type: none"> Ratiometric dye, thus the fluorescence ratio is independent of dye loading or dye loss from photobleaching Excitation wavelengths are in the UV range
Fura-Red	F3021	488	660	140	<ul style="list-style-type: none"> Fluorescence is inversely proportional to [Ca²⁺] Can be used along with Fluo-4 for ratiometric imaging at a visible wavelength The longer emission wavelength allows for simultaneous imaging with dyes that emit in the shorter 500-600 nm range or with GFP tagged proteins (460-509 nm)
LOW AFFINITY					
Benzothiazole Coumarin (BTC)	B6791	400	480	7000	<ul style="list-style-type: none"> Used by Kasai and colleagues in studies examining large amplitude Ca²⁺ micro-domains (6) Poorly water soluble AM ester form is fluorescent and, therefore, a potential source of error
Fluo-5F	F14222	494	520	2300	<ul style="list-style-type: none"> The higher K_d allows for measurement of micromolar Ca²⁺ in the cytosol
Fluo-4FF	F23981	494	516	9700	
Fluo-5N	F14204	494	520	90000	
Mag-Fluo-4	M14206	490	520	22000	<ul style="list-style-type: none"> Low affinity dye used to measure Ca²⁺ changes in intracellular Ca²⁺ pools where free [Ca²⁺] is high, such as in the ER (2)
Rhod-2	R1244	553	580	570	<ul style="list-style-type: none"> A cationic indicator used to measure mitochondrial Ca²⁺ signals due to the negative charge of mitochondria (1)

[#]Modified from Table 19.1 of the Molecular Probes website and from the Online Handbook (www.invitrogen.com)

Invitrogen recommends cell-permeant esters wherever feasible.

2.2 Confocal Imaging of Pancreatic Acinar Cells

1. Each perfusion setup is thoroughly rinsed with DI water and filled with an appropriate amount of buffer or agonist. Each syringe is then primed with buffer or agonist to insure proper flow. Syringes are clamped to a ring stand approximately 1-2 feet above the microscope stage.
2. Fine tweezers are used to grasp a single coverslip containing the cell suspension. The coverslip is tilted 45°, and the excess buffer is allowed to run off the coverslip. The coverslip is placed on top of the rubber gasket of the perfusion chamber with the cells facing upwards and the chamber is assembled. Step 2 should take no more than 30 sec.
3. The perfusion chamber is secured to the stage of an inverted microscope, and tubing containing buffer is inserted into the first inlet of the chamber (Fig. 1). The syringe containing buffer is turned on, and the buffer is allowed to perfuse over the entire surface of the coverslip. Once the buffer has reached the opposite end of the chamber, a vacuum line is inserted into the vacuum inlet. (**Note 5**) Other tubes containing agonist are inserted into their appropriate position along the chamber.
4. The cells are visualized using a 40X, 1.4 numerical aperture objective. An argon laser, whose power setting varies depending on the innate laser power of the photomultiplier tube, is used to excite the Fluo dye at a wavelength of 488 nm. Long-pass emission signals of >

515 nm are collected at frame speeds of 2-5 sec/frame for visualizing oscillatory patterns and 0.2-0.3 sec/frame for visualizing global Ca^{2+} waves. (Figs. 2,3; **Note 6**) Ca^{2+} waves are best observed using high speed collection systems such as the spinning disk system.

5. After imaging is complete the syringes are closed and all tubing is removed. The chamber is disassembled, and steps 2-4 are repeated.

2.3 Reporting Data

1. Data are initially collected as numerical values of fluorescence intensity over time using the software LSM Imager (used exclusively for Zeiss microscopes).
2. The data are transferred to Image J software (provided as freeware by the National Institutes of Health).
3. Using Image J, real-time cellular images can be viewed and regions of interest (ROIs) selected (i.e. apical, basolateral, nuclear, etc.)
4. The fluorescence intensities for these ROIs are acquired, and data are represented as fluorescence intensity/baseline fluorescence intensity (i.e. F/F_0).
5. Tracings are generated by plotting F/F_0 over time. (**Note 7**)
6. In addition to tracings, representative images are commonly provided and displayed using pseudocolor.

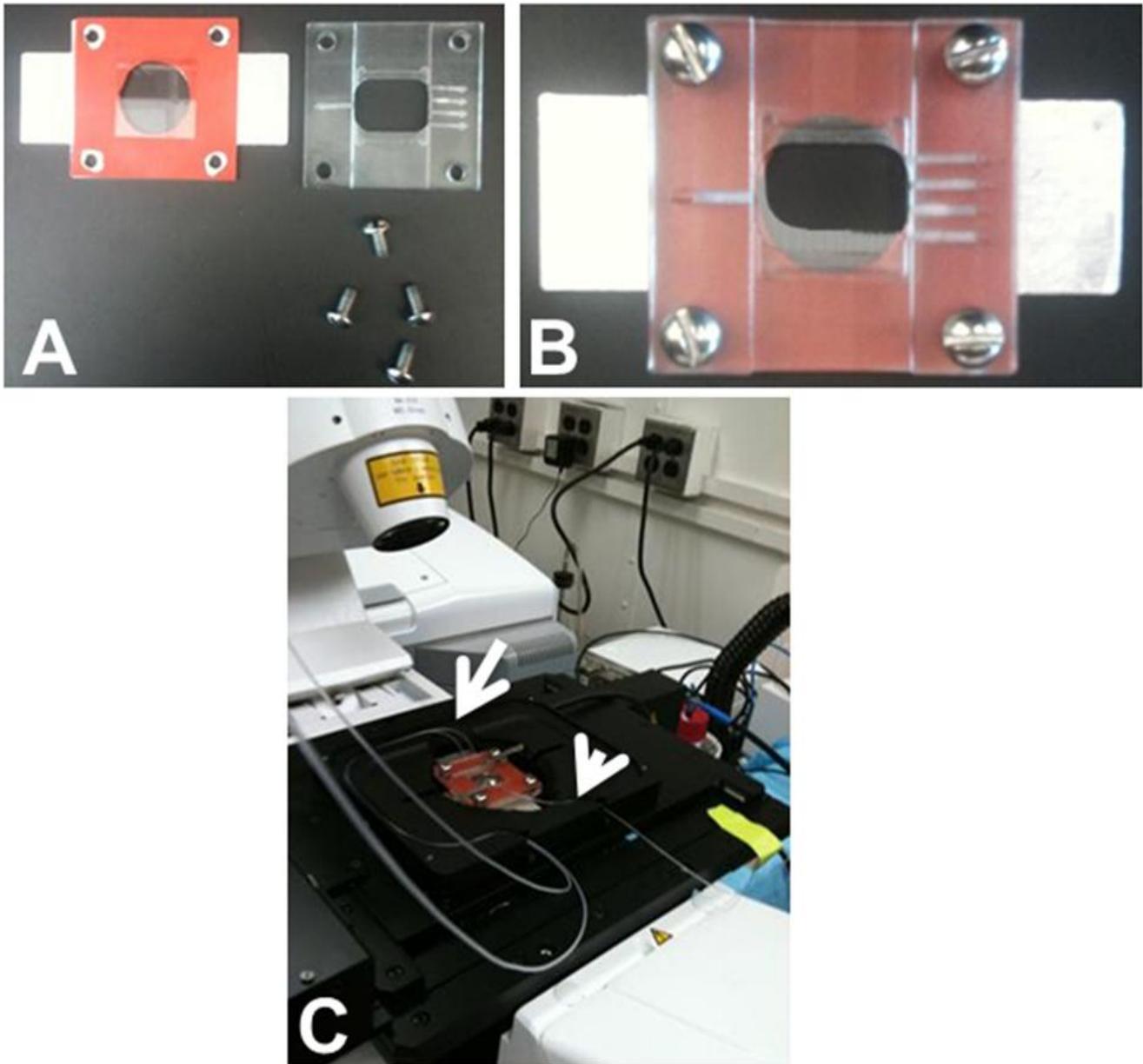


Figure 1. The perfusion chamber. **A.** The chamber consists of three layers: a metal base; a rubber gasket; and a plastic cover. **B.** The rubber gasket is placed on top of the metal base and a 22 x 22 mm coverslip containing cells is placed upwards on top of the rubber gasket. The plastic cover is screwed to the metal base and an 18 x 18 mm coverslip is placed on top. **C.** The chamber is then secured to the microscope stage. Tubing, containing buffer or agonist, is fed into inlets located on the chamber's edge (arrow). Separate tubing leads to a vacuum (arrow head).

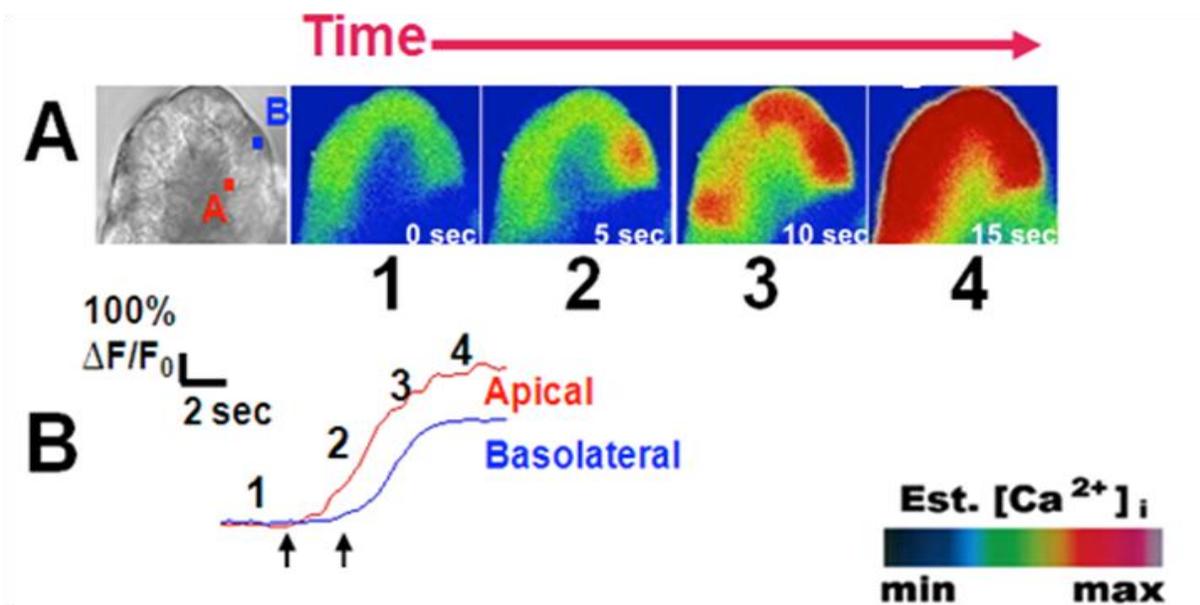


Figure 2. A typical peak-plateau Ca^{2+} signal upon stimulation with carbachol (1 μM). **A.** From left to right; Bright field view of an acinus labeled at the (A)apical and (B)asolateral regions of interest from an acinar cell. Cells were loaded with the Ca^{2+} indicator Fluo-4 (5 μM). Upon stimulation with physiologic carbachol (1 μM ; Ach analogue), subsequent images show the initiation of the Ca^{2+} signal in the apical region followed by propagation to the basal region. **B.** Each paneled image (1-4), corresponds to a frame along a representative tracing of change in fluorescence over time for each region of interest. Images are represented in pseudocolor with a color scale (bottom right). Left and right arrows show time of first Ca^{2+} rise in the apical and basal regions, respectively. This figure was originally published in the *Journal of Biological Chemistry*. Orabi AI, Shah AU, Muili K, Luo Y, Mahmood SM, Ahmad A, Reed A, Husain SZ. Ethanol enhances carbachol-induced protease activation and accelerates Ca^{2+} waves in isolated rat pancreatic acini. *The Journal of Biological Chemistry*. 2011; 286(16):14090-7. © the American Society for Biochemistry and Molecular Biology.

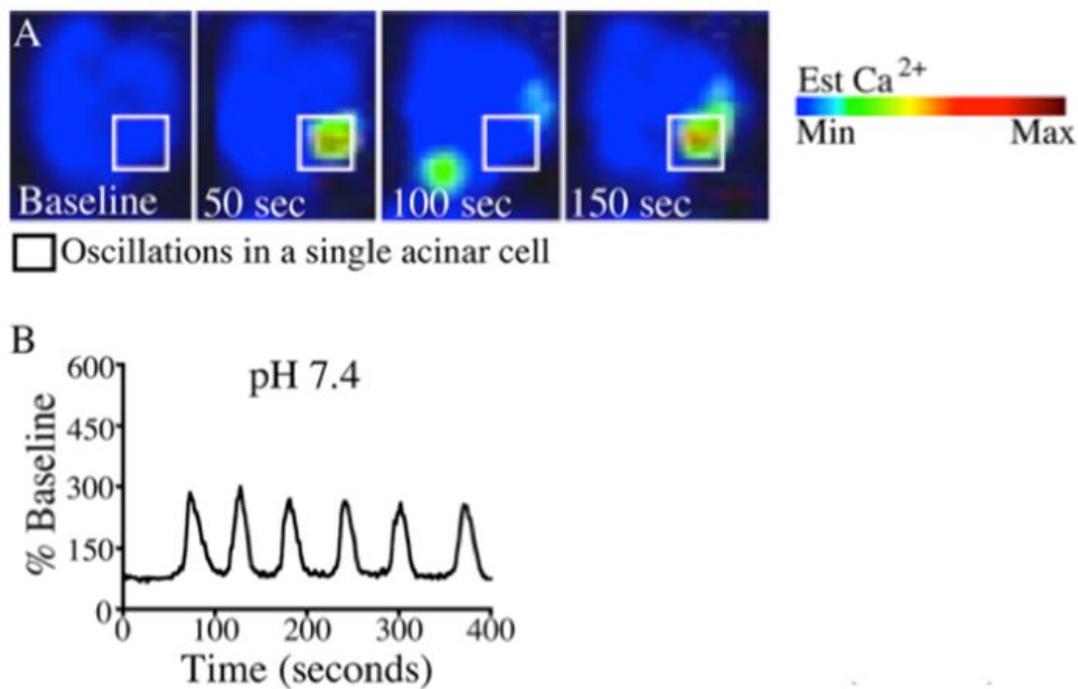


Figure 3. A typical Ca^{2+} oscillation upon stimulation caerulein (10 pM). **A.** Changes in whole cell cytosolic Ca^{2+} were measured once per second by time lapse confocal microscopy using the Ca^{2+} dye Fluo-4/ AM. Images are represented in pseudocolor with a color scale (top right). **B.** Representative plot of fluorescence over time were recorded from a single cell treated with caerulein (cholecystokinin analogue; 10 pM) at pH 7.4. This figure was originally published in the *Journal of Biological Chemistry*. Reed AM, Husain SZ, Thrower E, Alexandre M, Shah A, Gorelick FS, Nathanson MH. *The Journal of Biological Chemistry*. 2011; 286(3):1919-26. © the American Society for Biochemistry and Molecular Biology.

3. Notes

1. Confocal imaging uses a pinhole in front of a photomultiplier tube in order to eliminate light from above or below the focal plane. In contrast, wide-field imaging detects light throughout the excitation path of the specimen so that out of focus light (i.e. light above and below the desired plane of focus) contaminates the fluorescence image of the specimen. The advantage of confocal microscopy over wide-field is that it allows for thin-section imaging of a sample, as well as 3-D reconstruction of optical slices collected along a Z-axis. There are three popular types of confocal microscopes: (1) the point scanning system; (2) slit scanning; and (3) the Nipkow spinning disk. Point and slit scanners use visible wavelength lasers as the excitation source,

while spinning disk systems may use lasers or other light sources. Point scanners are slower but tend to provide better spatial resolution, whereas slit scanners and spinning disk systems are better suited for fluorescence imaging of transient or quickly changing events. Whereas the point and slit scanners tend to cause photobleaching, the spinning disk minimizes this effect (9).

Whereas confocal microscopes collect optical slices by rejecting light above and below the focal plane, another imaging modality termed two photon microscopy works by only exciting fluorophores within the focal plane. Thus, this technology is considered "inherently confocal." Furthermore, because two photon imaging functions at near infrared wavelengths it can penetrate samples several fold more deeply than confocal imaging (16).

2. The purpose of the acid wash is to provide a

clean surface as well as electrostatic interactions that will allow maximum adherence of acini. Cell Tak (BD Bioscience #354240) can be used in lieu of or in addition to this method.

3. We generally custom-make this syringe setup and use gravity to perfuse our perfusion chamber. However, automated flow systems are available through Harvard Apparatus (Holliston MA).
4. We use a custom-made perfusion chamber that uses a rubber gasket (Figure 1). However, commercial chambers are available through Warner Instruments (Hamden CT).
5. A properly regulated vacuum is a critical factor in preventing acini from being suctioned off the coverslip.
6. We use a Zeiss LSM 510 laser scanning confocal microscope which has an Argon laser. The imaging software is LSM Imager, provided by Zeiss. Analysis of data can be performed using Image J software from the

National Institutes of Health.

7. Spatial and temporal changes in Ca^{2+} signaling patterns (such as waves and oscillations, respectively) can be reliably measured using the F/F_0 calculation, but this is not a true indicator of $[\text{Ca}^{2+}]$. Quantitative measurements of $[\text{Ca}^{2+}]$ can be calculated using single wavelength excitation and emission Ca^{2+} dyes such as Fluo-4, although errors can occur in these measurements due to photobleaching and dye loss (5, 8). $[\text{Ca}^{2+}]$ can be measured more accurately by using ratiometric dyes, such as Fura2 or Indo1 (10), but this is impractical on most confocal microscopes because of the requirement of a UV laser for excitation. On the other hand, concomitant use of Fluo3 or Fluo4 and Fura-Red can allow ratio imaging using the 488 nm excitation line that is present on most confocal microscopes (13).

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