STAR Protocols

Protocol
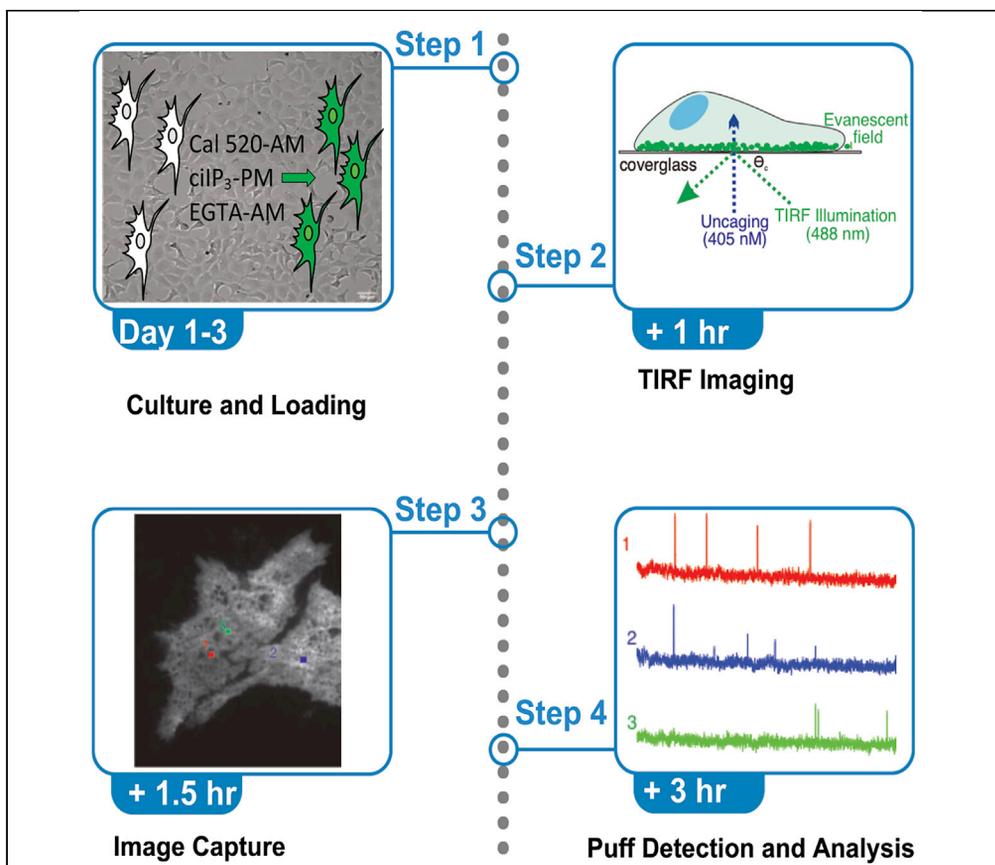
A protocol for detecting elemental calcium signals (Ca^{2+} puffs) in mammalian cells using total internal reflection fluorescence microscopy

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Highlights

Ca^{2+} puffs from cultured mammalian cells occur near the cell plasma membrane

Determine kinetics of active IP$_3$R clusters upon photo-liberation of caged IP$_3$

Solutions to common issues arising from detecting Ca^{2+} puffs using TIRF microscopy

This protocol outlines steps to visualize and detect Ca^{2+} puffs following photo-liberation of caged inositol-1,4,5-trisphosphate (IP$_3$) from HEK-293 cells expressing only the native IP$_3$R type 1 receptor using total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy offers high axial resolution and allows imaging at high speed, with a higher signal-to-background ratio. Additionally, we shed light on commonly encountered pitfalls, which should be considered while recording Ca^{2+} puffs using TIRF microscopy.

Protocol

A protocol for detecting elemental calcium signals (Ca^{2+} puffs) in mammalian cells using total internal reflection fluorescence microscopy

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SUMMARY

This protocol outlines steps to visualize and detect Ca^{2+} puffs following photo-liberation of caged inositol-1,4,5-trisphosphate (IP$_3$) from HEK-293 cells expressing only the native IP$_3$R type 1 receptor using total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy offers high axial resolution and allows imaging at high speed, with a higher signal-to-background ratio. Additionally, we shed light on commonly encountered pitfalls, which should be considered while recording Ca^{2+} puffs using TIRF microscopy.
For complete details on the use and execution of this protocol, please refer to Emrich et al. (2021) and Lock et al. (2015a).

BEFORE YOU BEGIN

The choice of probes and imaging conditions are critical to resolve elemental Ca^{2+} signaling events with high spatial and temporal resolution. We utilize Cal-520-AM, a membrane-permeable calcium-sensitive indicator for TIRF imaging as it offers enhanced quantum efficiency when excited using a 488nm laser, higher signal-tonoise ratio, and superior intracellular retention when compared to other green-emitting calcium indicators (Lock et al., 2015b). ci-IP$_3$-PM, a "caged" cell permeant derivative of IP$_3$ that is poorly metabolizable is employed to induce Ca^{2+} release upon photo-uncaging by near UV light. Additionally, a membrane-permeable ester of the slow Ca^{2+} buffer EGTA (EGTA-AM) is used to limit the recruitment of neighboring IP$_3$R clusters, increasing the spatial resolution and limiting the globalization of Ca^{2+} signals. We describe measuring Ca^{2+} puff activity in HEK-293 cells which have been genetically engineered by CRISPR/Cas9 to express endogenous levels of IP$_3$R type 1 in the absence of other subtypes. We term these cells endo hR1 cells. This protocol could be used with any adherent cell type.

Preparation of cell culture media and other reagents

⏱ Timing: 5 h

1. Heat-inactivation of fetal bovine serum
   a. Heat-inactivate fetal bovine serum (FBS) by placing a 500 mL FBS bottle in a water bath set to 56°C. Swirl the bottle thoroughly every 8–10 min.
   b. Wait for 30 min.

c. Remove the bottle from water bath; wipe bottle externally with 70% ethanol using a paper towel.

d. In a laminar flow cabinet, dispense 50 mL FBS into 50 mL conical tubes and store the tubes at −20°C until the labeled expiration date.

2. Preparing aliquots of trypsin

a. Thaw a 100 mL bottle of trypsin (2.5%, 10✕) by incubating in a water bath at 37°C.

b. In a laminar flow cabinet, make 1✕ 10 mL (in 1✕ DPBS, Dulbecco's Phosphate Buffered Saline) aliquots in 15 mL conical tubes and store at −20°C until labeled expiration date.

3. Preparing cell culture media

a. In a laminar flow cabinet, to 450 mL of Dulbecco's Modified Eagle's Medium (high glucose, pyruvate), add 50 mL heat inactivated FBS and 5 mL Pen Strep Glutamine (100✕).

b. Store at 4°C and use within 8 weeks.

4. Preparing imaging buffer - Hanks' balance salts solution (HBSS)

△ CRITICAL: Ensure that the imaging buffer is freshly made on the same day.

Reagent	Final Concentration	Amount
NaCl	137 mM	7.95 g
1M MgCl$_2$	0.56 mM	560 μL
2M KCl	4.7 mM	2.35 mL
Na$_2$HPO$_4$	1 mM	9.1 mL
HEPES	10 mM	2.38 g
Glucose	5.5 mM	0.99 g
0.5M CaCl$_2$	1.26 mM	2.56 mL
pH to 7.4	n/a	n/a
Total	n/a	1 L

a. Add the appropriate amounts of the reagents to 500 mL de-ionized water in a beaker placed on a magnetic stirrer with gentle stirring.

b. Adjust the pH to 7.4 using 1M NaOH.

c. Using a 1 L measuring cylinder, makeup the volume to 1 L.

d. Store at 4°C until use.

5. Preparing Cal-520-AM indicator

a. Prepare 1 mM stock of Cal-520-AM by re-suspending 50 μg in 50 μL anhydrous DMSO.

b. Store at −20°C until use.

6. Preparing ci-IP$_3$-PM

a. Prepare 1 mM stock of ci-IP$_3$-PM by re-suspending 10 μg in 8.5 μL DMSO.

b. Store at −20°C until use.

7. Preparing EGTA-AM

a. Prepare 10 mM stock of EGTA-AM by dissolving 10 mg in 1.5 mL of DMSO.

b. Store at −20°C until labeled expiration date.

△ CRITICAL: Store all the reagents at appropriate temperatures and the reagents should be used before the labeled expiration date. Do not expose Cal-520-AM indicator to light. Protect the dye from light by wrapping tube in an aluminum foil to avoid photo-degradation.

Alternatives: PBS can be used as an alternative to DPBS. The calcium indicator dye and ci-IP$_3$-PM can be freshly prepared on the day of experiment and can be stored in dark at −20°C following re-suspension in DMSO for several weeks.
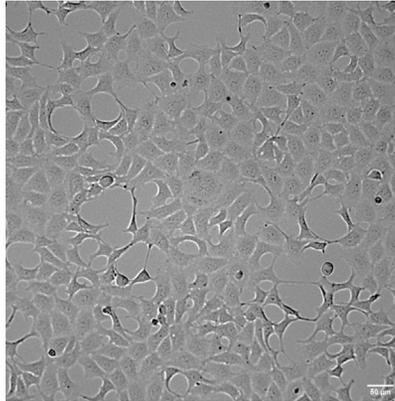
Figure 1. Bright field image of hR1 endo cells at ~80% confluency prior to passage
Scale bar: 50 μm.

Preparation of cell culture

⏱ **Timing: 1 week**

8. Thawing and culturing hR1 endo cells
   a. Pre-warm cell culture media (prepared in step 3) by incubating in a water bath set to 37°C.
   b. Remove a vial of hR1 endo cells (~1 × 10^6 cells/vial) stored in freezing media (70% DMEM, 20% FBS, 10% DMSO) from liquid nitrogen and thaw gently in a laminar flow cabinet.
   c. Aseptically, transfer the contents of vial into a 15 mL conical tube containing 9 mL sterile 1× DPBS.
   d. Centrifuge the conical tube at 200 × g for 5 min using a centrifuge.
   e. In the laminar flow cabinet, carefully discard the supernatant and re-suspend the pellet in 8 mL cell culture media.
   f. Transfer the media with cells into a 10 cm^2 cell culture dish, cell density ~30%.
   g. For cell adhesion, incubate the cells in an incubator set to 37°C with humidified atmosphere of 5% CO$_2$ for 24 h.
   h. Following 24 h, view the cells using an optical microscope to confirm cell adhesion.
9. Passaging cells
   a. Passage cells when grown to 80%–90% confluent (Figure 1).
   b. Pre-warm cell culture medium, trypsin, and DPBS by incubating in a water bath set to 37°C.
   c. In the laminar flow cabinet, carefully discard the spent cell culture media.
   d. Gently rinse the cells by adding 2 mL DPBS to the culture dish. Discard DPBS.
   e. Add 2 mL trypsin to the dish and place it back in the incubator for 2 min.
   f. Following 2 min, under aseptic conditions in a laminar flow cabinet, add 4 mL cell culture media to inactivate trypsin.
   g. Transfer the cells to a 15 mL conical tube and centrifuge at 200 × g for 5 min.
   h. Discard the supernatant and re-suspend the cells in 1 mL cell culture media.
   i. Transfer 10 μL of the re-suspended cells to a 0.5 mL tube containing 90 μL 0.4% trypan blue solution.
   j. Count the number of cells using a cell-counting chamber. Calculate the number of cells and seed ~ 2 × 10^6 in 8 mL cell culture media in a 10 cm^2 cell culture dish.
   k. Leave the cells in the incubator and passage the cells when 80%–90% confluent (~48 h). Passage the cells at least twice after thawing before starting the experiment to ensure that the cells have recovered from the stress caused by freeze-thawing. After thawing a vial, the cells can be used for imaging up to 20 passages.

⚠ CRITICAL: Take appropriate precautions to avoid contamination while culturing cells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	VWR	7647-14-5
Glucose	Fischer Chemical	D16-500
$MgCl_2.6H_2O$	Sigma-Aldrich	M9272-500G
KCl	AMRESCO	7447-40-7
$CaCl_2.2H_2O$	Sigma-Aldrich	223506-500G
HEPES	Fischer Bioreagents	BP310-500
Na_2HPO_4	Sigma	S5136-500G
NaOH	J.T. Baker Inc.	5635-02
DMSO	Sigma-Aldrich	34869
ci-IP_3-PM	Tocris	6210
EGTA-AM	Invitrogen	E1219
Cal-520-AM	AAT Bioquest	21130
Fetal Bovine Serum	Gibco	10437-028
Trypsin (2.5%, 10×)	Gibco	15090-046
Dulbecco's phosphate-buffered saline	Gibco	14190-144
Dulbecco's modified Eagle's medium (DMEM)	Gibco	11995-065
Pen Strep Glutamine (100×)	Gibco	10378-016
Bovine Serum Albumin	Fisher Bioreagents	BP1600-100
Trypan blue solution	Gibco	15250-061
Poly-D-lysine	Sigma	A-003-E
Experimental models: cell lines		
HEK-293 cells engineered using CRISPR-Cas9 technology to only express IP_3R1	Yule Lab	Dr. David Yule
Software and algorithms		
Fiji	Schneider et al., 2012	https://fiji.sc/
FLIKA	Ellefsen et al., 2014 (Dr. Ian Parker)	https://flika-org.github.io/
GraphPad Prism	Prism	https://www.graphpad.com/scientific-software/prism/
CellSens Dimensions 2.3 (Build 189987)	Olympus	Olympus
Other		
10 cm^2 Cell culture dish	Thermo Scientific	130182
Cell culture incubator	VWR	Symphony
Laminar flow cabinet	Labconco	Class II Biohazard
Centrifuge	Beckman Coulter	Allegra X-22R centrifuge
Optical microscope	Nikon TMS	211821
pH meter	VWR	Symphony SB70P
Precision scale	Mettler	PB1502
50 mL Conical tubes	TruLine	TR2003
15 mL Conical tubes	TruLine	TR2000
Ethanol	Koptec	64-17-5
Water bath	Precision	180 Series
Cell-counting chamber	Propper	090001
15 mm Coverslips	Warner Instruments	64-0703
12-Well plates	TruLine	TR5001
35-mm Dish	NEST	706001
Vacuum grease	Dow Corning	1597418
Perfusion chambers	Warner Instruments	64-0233
Perfusion system	Warner Instruments	VC-6 six channel valve controller
Kimwipes	Kimtech	34155
Olympus IX81 inverted microscope	Olympus	IX81
PlanApo 60x/1.45 Oil TIRFM Microscope Objective	Olympus	17763

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Low Autofluorescence Immersion Oil, n=1.518, Olympus Type F	Olympus	MOIL-30
488 nm Laser (60 mW)	Olympus	CMR-LAS-488-60-D
405 nm Laser (100 mW)	Olympus	CMR-LAS-405-100-D
Hamamatsu ORCA-fusion digital camera C14440	Hamamatsu	C14440

STEP-BY-STEP METHOD DETAILS
Preparing cells for imaging

⏲ Timing: 2 days

1. Coating coverslips with poly-D-lysine
   Coating the coverslips promotes cell adhesion and ensures that the cells are intact (not washed off) during the subsequent washing steps with imaging buffer.
   a. Pre-warm cell culture media, DPBS, trypsin, poly-D-lysine (0.1 mg/mL) by placing in a water bath set to 37°C.
   b. Using tweezers gently place pre-autoclaved 15 mm No1 coverslips in each well of a 12-well cell culture plate.
   c. Coat the coverslips by adding 0.1–0.2 mL poly-D-lysine/well of the 12-well plate to sufficiently cover the coverslips in each well.
   d. Incubate for 1 h at room temperature (25°C) in the laminar flow cabinet.
   e. Remove poly-D-lysine and rinse the wells 3 times with DPBS.
   f. Leave the plates uncovered for at least 1 h in the laminar flow cabinet for drying.

2. Seeding cells for imaging
   a. Seed 0.5×10^6 cells in each well of a 12-well plate with poly-D-lysine coated coverslip (prepared in the previous step) in 1 mL cell culture media.
   b. Leave the plates in an incubator for 48 h.

Detecting calcium signals using TIRF microscopy

⏲ Timing: 5 h

3. Loading cells with Cal-520-AM (Ca^{2+} indicator), ci-IP_3-PM (cell permeable caged IP_3 analog), and EGTA-AM (cell permeable Ca^{2+} chelator).
   a. After 48 h, view the cells under a microscope using brightfield illumination and ensure that the cells have adhered and attained flattened morphology.
   b. Prepare 5 μM Cal-520-AM and 1 μM ci-IP_3-PM by dissolving 5μL 1 mM Cal-520-AM and 1 μL 1 mM ci-IP_3-PM in 1 mL imaging buffer supplemented with 1% BSA.
   c. Prepare 5 μM EGTA-AM by dissolving 5 μL 10 mM EGTA in 10 mL imaging buffer.
   d. Using fine-tip forceps, transfer the cells on coverslip from step (a) into a 35-mm dish in the laminar flow cabinet.
   e. Wash the coverslip gently three times with 750 μL imaging buffer.
   f. Discard the imaging buffer and incubate the coverslip in 750 μL Cal-520-AM and ci-IP_3-PM supplemented imaging buffer (prepared in step b).
   g. Incubate at 25°C for an hour. Following 1 h incubation, rinse the coverslip gently with imaging buffer three times.
   h. Discard the imaging buffer and gently add 750 μL of EGTA-AM supplemented imaging buffer (prepared in step c).
   i. Incubate at 25°C for 45 min. Following 45 min incubation, rinse the coverslip gently with imaging buffer three times.
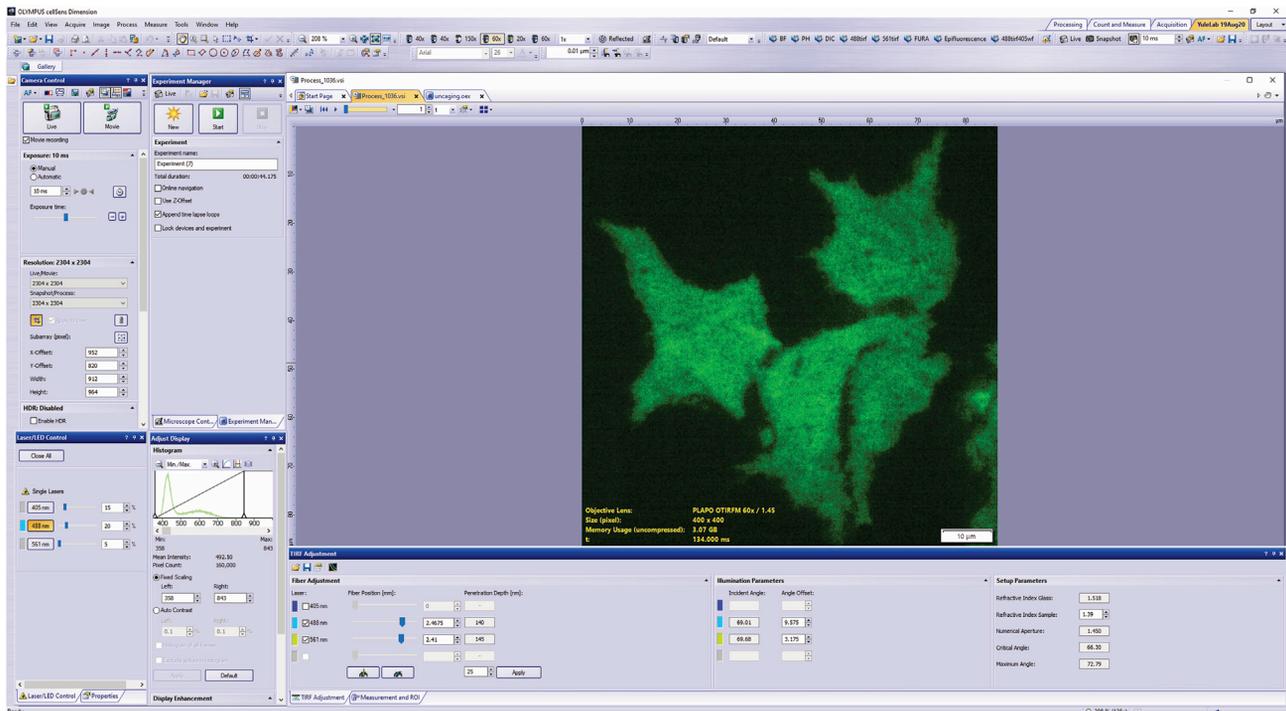
Figure 2. Graphical user interface of cellSens Dimension imaging software used to acquire Ca²⁺ puffs
A layout of the acquisition software which enables selection of appropriate objective, laser, TIRF settings, camera control, field of view, monitoring progress of experiment, and acquisition/display settings.

    j. Discard the imaging buffer. Add 750 μL fresh imaging buffer and incubate at 25°C for another 45 min to facilitate complete de-esterification of the loaded reagents.

    ⚠ CRITICAL: All the incubations steps are at room temperature (25°C) to avoid indicator sequestration into organelles and in the dark to prevent photo-degradation of the loaded indicator. Do not freeze/re-use the diluted Cal-520-AM/ci-IP$_3$-PM mixture.

4. Imaging Ca²⁺ puffs using TIRF microscopy
    a. Using fine forceps, attach the coverslip to a Warner perfusion chamber using vacuum grease. For each experimental set, image at least 5 coverslips.
    b. Add 1 mL imaging buffer to the perfusion chamber to ensure that the cells are properly immersed.
    c. Clean the bottom of the cover slip using a kimwipe to avoid artifacts while focusing/imaging.
    d. On an Olympus IX81 inverted microscope equipped with PlanApo 60x/1.45 Oil TIRFM Microscope Objective, place a drop of low autofluorescence immersion oil.
    e. Next, fasten the Warner perfusion chamber on to the microscope stage.
    f. We use Olympus CellSens Dimensions 2.3 (Build 189987) software for imaging (Figures 2 and 3).
    g. Excite Cal-520 indicator using a 488 nm laser (set the intensity to a minimum such that the indicator fluorescence does not photobleach; see solution in "problems 2 and 3"); ensure that the cells are in focus and in the incident angle of the laser achieves TIRF (Figures 2, 3A, 3C, and 3E). Fluorescence in TIRF mode characteristically reveals the interface between the plasma membrane and the coverslip (Figure 4A). See solution in "problem 1" if this does not occur.
    h. Set the protocol in the software to record baseline (during which the cells are quiescent) for at least 3/5 s; deliver a UV-flash using a 405 nm laser uniformly to uncage ci-IP$_3$ for 1 s
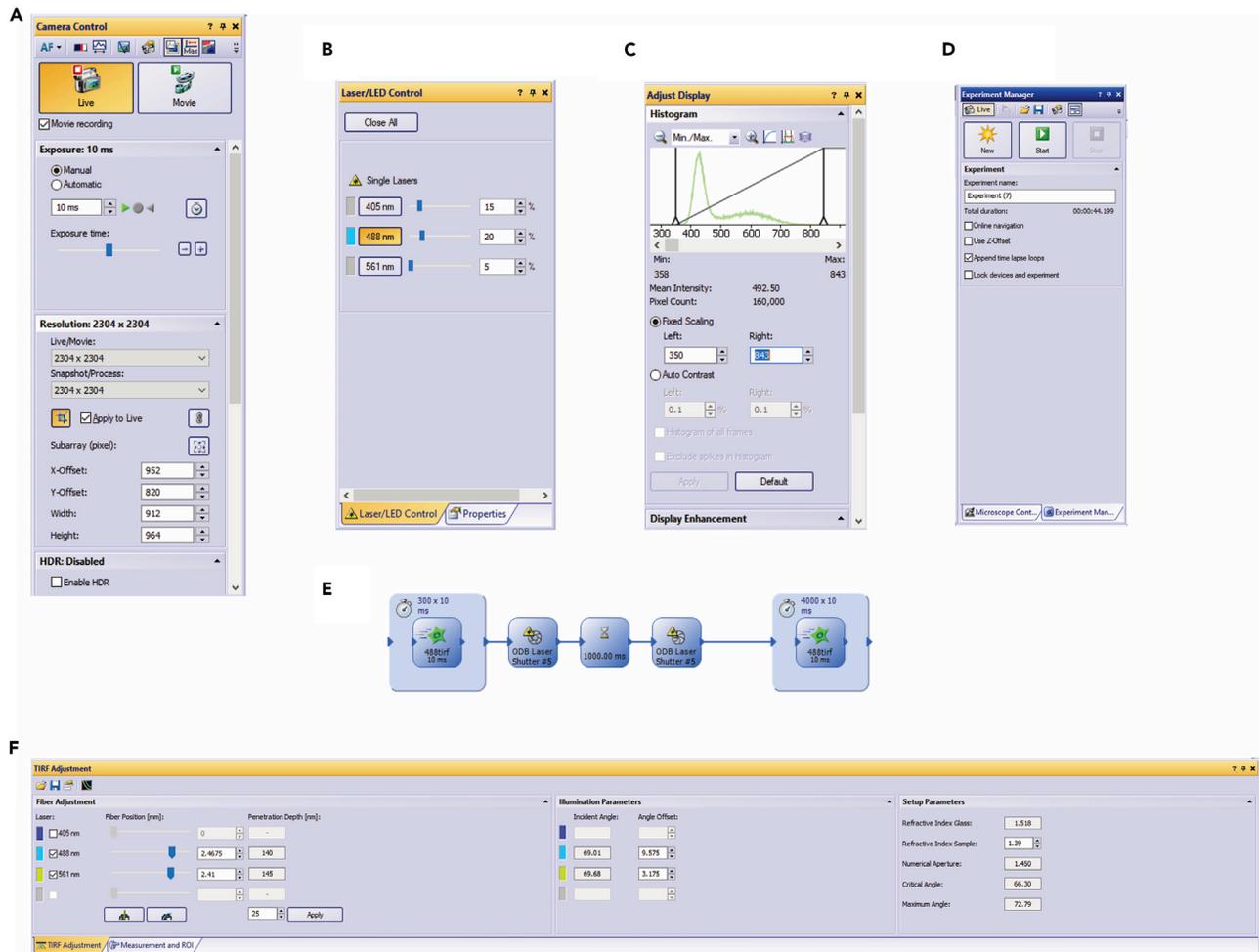
Figure 3. Parameters that can be adjusted in the cellSens Dimension imaging software to acquire Ca^{2+} puffs

(A) Camera control window allows acquiring movies/snapshots, adjusting the exposure time (gain), resolution, and field of view/region of interest for recording Ca^{2+} puffs.

(B) The laser/LED control tab permits selection of appropriate laser and laser intensity to illuminate the dye loaded cells.

(C) Adjust display facilitates optimizing the appearance of dye loaded cells.

(D) Experiment manager window enables creating, saving, and executing a protocol for image acquisition. In this window, a series of commands including the laser type, exposure time, controlling UV laser shutters, and recording time for running an experiment can be defined.

(E) A protocol used for acquiring a three second baseline (prior to photolysis), delivering UV flash from a 405 nm laser for 1 s (photolysis of ci-IP$_3$), followed by recording Ca^{2+} puffs for 40 s using the 488 nm laser is presented.

(F) The angle of incidence of laser to achieve total internal reflection can be optimized by adjusting the angle offset in the TIRF adjustment window. The penetration depth which varies with incident angle and other fixed parameters such as refractive indexes of glass, sample, numerical aperture, critical angle, maximal angle is displayed in this tab.

(Figures 3B and 3D; see solution in "problems 4 and 5"). Alternatively, the UV laser light may be focused to a spot to illuminate a region of a cell.

i. Following uncaging, record the Ca^{2+} puffs for a user-defined time period (for example 1 min or until the calcium signals globalize).

j. Capture the emitted fluorescence using a Hamamatsu ORCA-fusion CMOS digital camera C14440. Images were acquired from a final field of (86.7 × 86.7 μm) 400 × 400 pixels (one pixel=216 nm), penetration depth of ~140 nm at a rate of ~97 frames/s (binning 2 × 2), and at an exposure of 10 msec (Methods video S1).

⚠ CRITICAL: All the necessary precautions must be considered before operating the lasers. Lasers can cause irreparable damage to the eye and skin burns. Although the Olympus
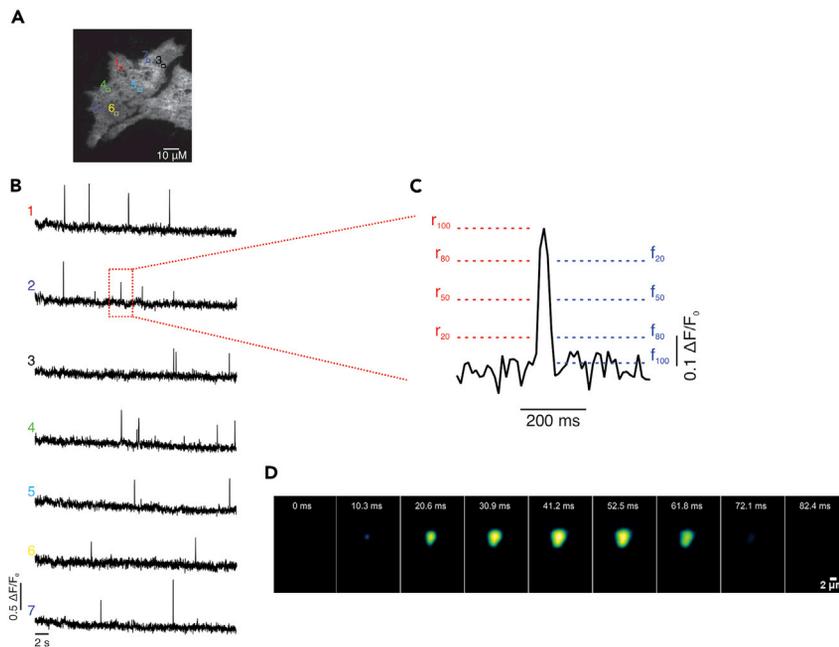
Figure 4. TIRF imaging using hR1 endo cells

(A) A hR1 endo cell illustrating the sites at which puffs were observed upon uncaging ci-IP$_3$. Scale bar: 10 μm.

(B) Traces showing the changes in fluorescence from the depicted regions of interest (1 μm by 1 μm) in A.

(C) Time course of a Ca^{2+} puff is shown on an expanded time scale.

(D) Gaussian blurred images of a representative Ca^{2+} puff over the entire duration of the puff obtained from a region of a cell. Scale bar: 2 μm.

TIRF condenser employs a laser interrupt when laser light is directed to the eyepieces it is recommended that appropriate goggles to protect eyes and long-sleeve gloves to protect skin from laser burns are worn.

⚠ CRITICAL: Ensure that the lights are off during the entire process of imaging. Set the 488 nm laser intensity to a minimum to avoid photobleaching/spontaneous puff activity. The optimum laser intensity for imaging can be confirmed by using HEK-293 cells which lack all the 3 endogenous IP$_3$Rs (HEK-3KO). It is also critical to optimize the intensity of the UV flash (typically in the range of 0.1–2 mW) and duration (100 ms–1000 ms) for uncaging ci-IP$_3$-PM. The speed of recording can be enhanced by increasing the binning to 4 × 4. Following dye loading, cells appear healthy and can be used for acquiring images for at least ~45 min. Each coverslip can be used to acquire multiple recordings since the focused UV-laser used to uncage ci-IP$_3$ triggers uncaging in the field of view and only a small fraction of cells on the coverslips are exposed to laser light. Nevertheless, avoid recording puffs from the same field of view more than once.

Alternatives: Cells can also be superfused with a G$_{\alpha q}$-coupled agonist using a perfusion system to elevate intracellular levels of IP$_3$ to evoke Ca^{2+} puffs (Emrich et al., 2021; Lock et al., 2017). Cal-590-AM indicator can be used in place of Cal-520-AM which differ in their excitation/emission wavelengths (558/584 nm and 492/514 nm for Cal-590 and Cal-520, respectively) and also their affinities for Ca^{2+} (k$_d$=561 nM and 320 nM for Cal-590 and Cal-520, respectively). The long excitation/emission wavelength of Cal-590 makes it compatible for use with multicolor detection, for example, with GFP tagged proteins (Thillaiappan et al., 2017).

Analyzing Ca^{2+} puffs using FLIKA software

⏱ Timing: 3 h

5. Convert the .vsi files obtained using CellSens Dimensions 2.3 software into .tif format using Image J (Fiji). Further analysis to determine various spatio-temporal and biophysical parameters such as number of puffs/cell, number of puff sites/cell, rise and fall times are obtained using FLIKA software developed by Dr. Ian Parker's group (Ellefsen et al., 2019; Ellefsen et al., 2014). The steps involved in analyzing Ca^{2+} puffs using FLIKA software are outlined below:

   a. Load .tif file (Crop to accommodate a single cell, trim frames to be analyzed).

   b. On graphical user interface, select Process – Math – Subtract (for example 450, black levels).

   c. Select the Subtracted window - Process – Math – Ratio by Baseline [First frame – 0, Number of frames – 290 (before uncaging ci-IP₃), Ratio Type – Average, Black Level – 0.00] – **Data window containing F/Fo (A)** (Figure 4).

   d. Select the Ratioed by Average window – Process – filter – Butterworth filter – Filter order 3 – Low cutoff Frequency (e.g.: 2) High cutoff Frequency (e.g.: 10), Frame rate (Hz) (e.g.: 97 fps).

   e. Select the Ratioed by Average – Butter Filtered window – Process – Math – Ratio by Baseline [First frame – 0, Number of frames – 290 (before uncaging ci-IP₃), Ratio Type – standard deviation, Black Level – 0.00] – **Normalized window containing data with baseline at 0 (B).**

   f. Select the Ratioed by average – butter filtered – Ratioed by standard deviation window –Process – Filter – Gaussian Blur – Sigma (pixels) (e.g.: 3), normalized edges, preview (true); adjust the histogram in a manner to detect puffs (e.g.: 1). – **Gaussian Blurred normalized window (C)** (Figure 4D).

   g. Plugins – Detect Puffs – Threshold cluster – select the appropriate windows **(A, B, C)**. Enter the values pertaining to Roi_width (e.g.: 5), paddingXY (e.g.: 20), padding_pre (e.g.: 20), padding_post (e.g.: 20), maxSigma For GaussianFit (e.g.: 90), radius (e.g.: 5), maxPuffDiameter (e.g.: 30), blur_thresh (e.g.: 1), time_factor (e.g.: 2). Load_flika_file (false). Rotatedfit (true).

   h. Blur Threshold (enter the desired value (e.g.: 1.00). Hit set threshold.

   i. Right click and circle the clusters.

   j. Hit set threshold.

   k. Fit Gaussian.

   l. In control panel, monitor the individual puffs adjust start and end of individual puff.

   m. Export to excel which has details about events assigned to clusters (Figures 4B and 4C), the amplitudes (Figure 5A), number of puff sites/number of puffs (Figure 5B), rise and fall times (Figure 5C).

   n. Save (.flika) for future references.

Note: The parameters mentioned in parenthesis as examples work best for hR1 endo cells and with our microscope settings, however, these parameters vary based on the cell types/speed of recording/number of puffs/frequency of puffs/duration of puff/radius of puffs and other factors. Hence, they need to be optimized accordingly. All the puffs identified automatically by the algorithm were manually confirmed before analysis. For a detailed guide on all the parameters for detect puff plugin, please refer to the help section in FLIKAGUI (https://github.com/kyleellefsen/detect_puffs/blob/master/docs/detect_puffs_parameters.md) (Ellefsen *et al.,* 2019).

EXPECTED OUTCOMES

Cells successfully loaded with the fluorescent indicator should emit fluorescence at the appropriate emission wavelength in the TIRF field. Multiple Ca^{2+} puffs in several regions of the cell should be detectable following uncaging ci-IP₃, as shown in Methods video S1.

LIMITATIONS

A major consideration for the TIRF methodology is that imaging is restricted to a region close to the coverslip/sample interface (typically less than 200 nm). However, imaging using this technique minimizes out-of-focus intracellular fluorescence which facilitates detecting various processes that occur near the plasma membrane.
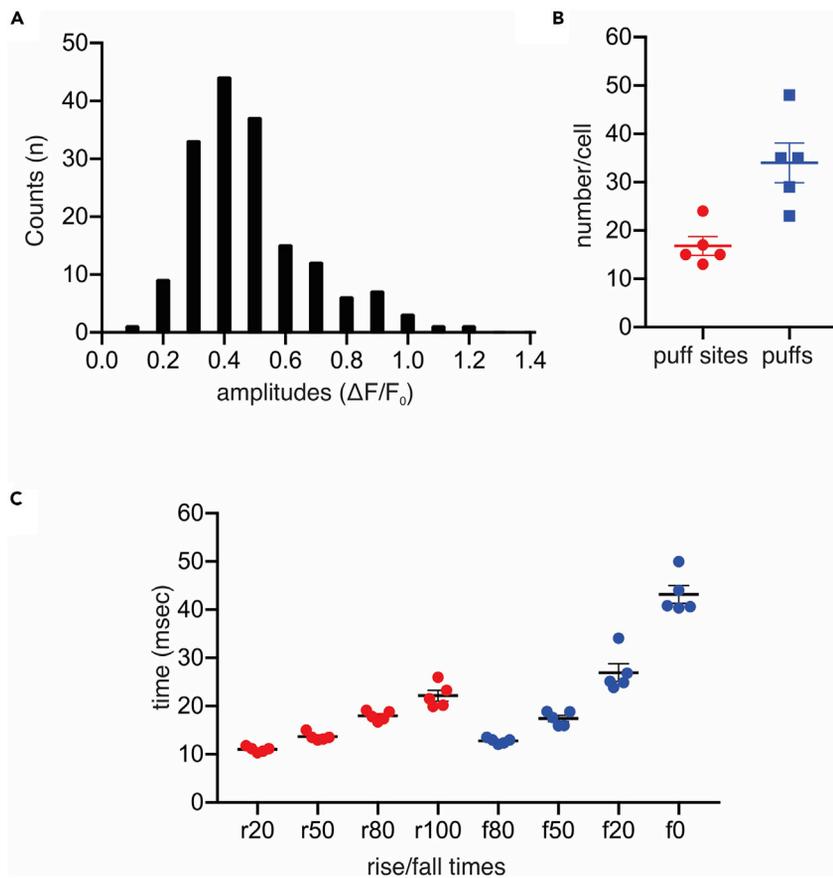
Figure 5. Ca²⁺ puffs evoked following photolysis of ci-IP₃ in hR1 endo cells (n=5)
(A) Graph showing amplitude distributions of puffs in hR1 endo cells.
(B) Scatter plot depicting the number of puff sites/cell and puffs/cell.
(C) Mean-rise (r) and -fall (f) times for the fluorescence to increase/decrease to various levels (0%–100%) are obtained using FLIKA.

TROUBLESHOOTING

Problem 1
There is no difference in the way the cells appear upon switching from epifluorescence mode to TIRF mode (step 4).

Potential solution
This could arise because the incident angle may not have surpassed the critical angle or the penetration depth exceeds that required to facilitate optimal total internal reflection. TetraSpeck™ Microspheres, 0.1 μm, fluorescent blue/green/orange/dark red (Catalog #: T7279, Invitrogen) can be used to confirm if the microscope is in TIRF mode. In TIRF mode, at the coverslip some of the beads will be immobile with a high signal to background ratio while the other beads keep rapidly appearing and disappearing. In epifluorescence mode, a significant amount of out-of-focus fluorescence will appear from the beads. In TIRF mode, adjust the incidence angle to ensure only the fluorescent microspheres which are close to the membrane are excited by the evanescent field. These parameters along with refractive index of glass/sample (which are fixed) can be adjusted in the software (Figure 2E).

Problem 2
Cells loaded with fluorescent indicator are not visible upon excitation with appropriate wave length (step 4).

Potential solution

Varying the exposure time/gain, binning, and/or laser intensity can help in determining the best setting for recording Ca^{2+} puffs

Problem 3

Cells are photobleaching over-time during recording in TIRF mode or spontaneous puffs appear before uncaging ci-IP$_3$ (step 4).

Potential solution

This problem usually arises due to the heat/free radicals generated by the excitation laser to illuminate the indicator loaded cells. This can be avoided by decreasing the intensity of laser used to excite the fluorescent indicator loaded cells.

Problem 4

How to determine the laser power at the objective (step 4)?

Potential solution

The laser power at the objective can be determined using a Hand-held Optical Meter (Newport, Model 1918-C).

Problem 5

Ensuring the field is evenly illuminated by the 405 nm laser for uncaging ci-IP$_3$-PM (step 4).

Potential solution

In order to test this, place a drop (2–5 μL) of Fura-2-AM (or other indicator which is excited by UV light) on a cover slip attached to a perfusion chamber on the TIRF objective. Excite Fura-2-AM by switching on the 405 nm laser. In the software, measure the decay in fluorescence intensity starting from the center of the illuminated spot to the periphery by placing a region of interest. Ensure that the fluorescence intensity remains uniform throughout the field of view.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vikas Arige (vikas_arige@urmc.rochester.edu)

Materials availability

This study does not generate any novel reagents.

Data and code availability

This study does not generate any novel dataset/code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100618.

AUTHOR CONTRIBUTIONS

V.A. drafted the manuscript and performed the example experiments. D.I.Y., M.T., S.M.E., and R.E.Y. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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