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Fluorescence-Based Calcium Imaging in Primary Human Airway Epithelial Cultures Using Automated Cell Segmentation

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Abstract

Calcium signaling is critical in a multitude of biological processes, reinforcing the need to develop methods to study calcium flux in primary tissue. The study of calcium signaling at a single-cell resolution in complex primary epithelial cultures is challenging and therefore remains poorly studied. This study presents methods adapted for monitoring live calcium signaling in primary airway epithelial cultures and novel approaches for machine learning based analyses. Using patient-derived primary airway epithelial cultures differentiated at ALI (air-liquid interface) loaded with an extrinsic fluorescent indicator dye, calcium mobilization was measured at single-cell resolution using an epifluorescent microscope. We developed a novel software that utilizes machine learning-based cell segmentation to assign fluorescence intensities to distinct cells over time. Overall, the adapted imaging setup and software enable a rapid and unbiased approach to analyzing single cells within the primary epithelial cultures. The step-by-step protocol presented here will enable the future study of calcium in individual cells and cell types that make up the primary epithelial cultures.

Introduction

Changes in intracellular calcium mobilization dictate a wide variety of cellular processes, including changes in gene expression and modulation of innate immune responses[1,2]. Intracellular calcium levels can be altered through release from intracellular calcium stores, such as the endoplasmic reticulum and mitochondria[1,2,3]. Extracellular calcium influx into the intracellular space can also alter cytosolic calcium through both passive and active transport[2]. Live cell imaging of calcium mobilization is assayed primarily by intracellular dyes or genetically encoded dyes, GCaMP (Green Calcium-activated Multimer/Protein)[4]. Common in literature are AM (acetoxymethyl) conjugated dyes, which enable the dye to permeate the plasma membrane[5,6,7,8]. Upon entering the cell, the AM moiety is cleaved by endogenous intracellular esterases, and the dye gains a negative charge, which traps it in the cytosolic space[9]. Due to this gained charge, the dye can be pumped out by plasma membrane resident organic anion channels. Therefore, probenecid, an inhibitor of organic anion efflux transporters, is added to prevent cellular efflux of the dye and retain optimal cytosolic signal[10,11]. Studies commonly employ Fura-2 AM, a ratiometric dye, and Fluo-4AM, an intensitometric dye, to assay calcium signaling on stable cell lines cultured in a monolayer. This study employs a protocol adapted for epifluorescent imaging of multilayered cultures grown at the air-liquid interface using the recently developed intensitometric calcium dye, Cal-520 AM, which has enhanced sensitivity compared to Fluo-4 AM. Thus, it is suitable to robustly visualize subtle signals at a single cell resolution from complex primary epithelial cultures. Current literature is rich in live calcium imaging of bulk responses from stable cell lines. However, it is now commonly known that *in vivo*, tissues constitute a diverse cellular landscape supporting different cell type-specific roles in maintaining tissue homeostasis[12,13,14,15]. Despite this common knowledge, the mechanisms by which

DOI 10.3791/69470

distinct cell types coordinate calcium signals and regulate calcium ion homeostasis remain unknown. Therefore, the methods presented here will enable single-cell detection of calcium signaling and enhance the throughput of downstream analyses in primary airway tissues.

We are leveraging our previously published and rigorously validated primary epithelial culture models of the human nasal epithelium as a tool to image live cell, cytosolic calcium in a more biologically relevant model[16,17,18]. Briefly, epithelial cells are isolated from patient nasal brushes and are cultured on a semipermeable membrane on a ~~trans well,~~ which is suspended within a culture well in order to maintain an air-liquid interface (ALI). This interface, coupled with specialized media, has been rigorously tested using orthogonal techniques for the presence of different cell types[16,17,18]. Moreover, it has been shown that the current methods are translatable to primary bronchial cultures that are cultured in a similar way to our primary nasal cultures[19].

This article introduces an adapted method to measure and analyze live calcium signaling at a single-cell resolution in these primary airway epithelial cultures. These multilayered cultures are difficult to visualize using microscopy; however, the current setup enables us to focus reliably on single layers within the multilayered cultures, allowing us to detect calcium mobilization from these specific layers. This setup has several advantages, in that it does not require a perfusion system, which is often costly and difficult to adapt unless specialized microscopes are employed. Epifluorescence microscopy has been employed, which offers improved temporal resolution compared to confocal imaging. The use of light-emitting diodes (LEDs) minimizes phototoxicity relative to laser-based confocal systems. Extrinsic intensiometric dyes are used in the protocol presented here as they enable rapid, relative changes in calcium across the entire cell population and are compatible with most fluorescence microscopy setups. This is beneficial over common ratiometric dyes, which require deep ultraviolet (UV) excitation that is not available on many microscopes. Moreover, GCaMP requires transfection or transduction, which strengthens the technique for more targeted calcium imaging in specific cell types. Due to low transduction and transfection efficiencies in primary epithelial cultures, the technique is poorly suited for monitoring the entire cell population.

Additionally, this study developed novel, specialized machine learning based software that allows single-cell segmentation based on live nuclear staining. The software is then able to record fluorescence intensity values for each cell over time. A stable epithelial cell line (Calu-3) was initially employed for method optimization. Then, these methods were translated to study single-cell calcium mobilization in primary epithelial cultures. Variables such as concentration of the calcium dye and probenecid, timing of incubation steps, as well as the imaging setup were optimized. Here, calcium signaling is recorded at a frame rate of 0.2 fps (frames per second), which is suitable for monitoring calcium signaling. The frame rate can be increased in order to measure more rapid calcium transients[20]. The optimal variables were then applied and further optimized in more complicated primary nasal cultures.

Protocol

The reagents and the equipment used are listed in the **Table of Materials**.

1. Calu-3 cell maintenance and culturing

1. Maintain Calu-3 cells in T-25 flasks in EMEM culture media supplemented with 20% FBS and 1% penicillin/streptomycin. Prewarm the media and change it on alternating days (5 mL/flask).
2. When cells are 70% confluent in the flask, aspirate the media in the flask.
3. Wash the flask with 5 mL 1x phosphate-buffered saline (PBS).
4. Add 1 mL of TrypLE directly to the cells for 10-15 min at 37 °C, 5% CO_2. Ensure that all cells have detached from the bottom of the flask and are dissociated into a single-cell suspension.
5. Neutralize the reaction with 4 mL of fresh culture media (step 1.1), then mix the cells well with a serological pipette to ensure even seeding.
6. Plate the cells onto a 96-well plate at 90%-100% confluence (approximately 40,000 cells/well).
    1. To seed wells, add 200 µL/well of well-mixed cell suspension.
    2. Allow the cells to adhere to the 96-well plate overnight.
7. Change media every day (100 µL/well) until ready for experiments.
8. Allow 2-5 days post confluency within wells to start experimentation prior to conducting experiments to allow for differentiation.

2. ~~Calu-3 Cal-520 AM, Fluo-4 AM protocol~~

CAUTION: Fluo-4 AM and Cal-520 AM are dissolved in DMSO, which is considered hazardous. Probenecid is listed as an acute hazard. Please wear gloves and a lab coat when handling chemicals to avoid contact with skin. Dispose of all chemicals into designated chemical waste bins.

1. On the day of the experiment, remove media from the wells by pipetting and wash cells twice (200 µL/well) in prewarmed calcium buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ 2 mM

DOI 10.3791/69470

$CaCl_2$, and 10 mM Hepes, adjusted to pH 7.38 (NaOH) 291 mOsm) to remove dead cells.

2. Incubate cells with 200 µL/well of a cocktail of 3 µM Cal-520 AM, 2.5 mM probenecid, and 1:5000 Hoechst nuclear stain in calcium buffer (step 2.1) for 1 h in an incubator set at 37°C and 5% CO_2.
    1. For Fluo-4 AM experiments, incubate cells with 200 µL/well of a cocktail of 3 µM Fluo-4 AM, 2.5 mM probenecid, and 1:5000 Hoechst nuclear stain in calcium buffer (step 2.1) for 1 h at 37 °C and 5% CO_2.

    NOTE: Keep the tubes of dye in the dark by covering them with aluminum foil.
3. During the incubation, prepare thapsigargin in calcium buffer (step 2.1). Adjust intermediate stock concentrations to ensure proper final concentrations within the well.

    NOTE: Here, 50 µL of drug is added to 100 µL of the existing calcium buffer. Therefore, a 3x intermediate stock of 6 µM was prepared.
4. After 1 h, aspirate the dye by pipetting and wash away extracellular dye with calcium buffer (step 2.1) twice (200 µL/well).
5. Following washes, add 100 µL/well of 2.5 mM probenecid diluted in calcium buffer (step 2.1) to maintain the dye in the cytoplasmic space.
6. Wrap the plate in aluminum foil to protect it from light. The cells are now ready to image.

3. Setting up microscopy for the Calu-3 stable cell line

1. Using the epifluorescence microscope equipped with a SuperFluor Objective, a stable, solid-state light source, and a CMOS fluorescence camera (6.5 µm pixel size). Illumination intensity is set at 500 mW.
2. Place the plate of cells on the stage using the 96-well plate adapter. Using Brightfield, focus on the cell layer at a low magnification to find the monolayer of cells (use 5-10x air objective).

    NOTE: This step is optional, but helpful to gain initial focus on the cell layer efficiently.
3. Once the cell layer has been identified, use Brightfield and switch to a higher magnification (20x air objective) and refocus on the cell layer.

    NOTE: Ensure proper focus on the monolayer with Brightfield; cell boundaries should be clear.
4. Identify the calcium dye signal using the GFP (475 nm LED). Ensure that the LED power falls between 5%-10% to avoid photobleaching, and the exposure time is suitable to visualize baseline green signal without oversaturating the signal, typically between 300-500 ms.

    NOTE: To ensure reproducibility and even dye loading, qualitatively analyze the cell layer; most cells should exhibit the same brightness. Focus on fields of view with limited dead cells, which will fluoresce intensely before any stimulant is added. Relative baseline fluorescence intensity can be measured using Fiji (step 7.9). Baseline fluorescence variation between cells should be close to 10%-15% if dye has been loaded evenly (step 7.9).
5. Identify the Hoechst nuclear stain using the DAPI (405 nm) LED. LED power should be between 2%-5% with an exposure time between 50-100 ms.

    NOTE: If the cells are in focus, nuclei should appear with sharp outlines. Ensure that the nuclear signal does not reach saturation, as nuclei can interfere with the green channel.

4. Acquiring live calcium video

1. Start a live video using an interval of 1 frame every 5 s (0.2 fps) for a duration of 10 min.

    NOTE: Capture each fluorescent channel (DAPI 405 nm and GFP 475 nm) at each frame.
2. Once the video has started, allow a 5 min baseline reading prior to adding agonists.
3. Using a P200 pipette, add 50 µL of thapsigargin at a 3x intermediate stock to the well containing 100 µL of 2.5 mM probenecid in calcium buffer (step 2.1). Gently resuspend 1-2 times within the well to ensure the drug is evenly dispersed. Continue reading for 5 min following the addition of thapsigargin.

    NOTE: The drug addition step is critical; the pipette tip should not contact the plate to shift the plane of focus. Instead, float the pipette tip to only contact the buffer within the well. If drugs are mixed too vigorously, cells can detach from the plate and interfere with the signal.

    NOTE: Ensure to take note of the frame in which the drug is added for downstream analysis.

DOI 10.3791/69470

4. Save the video and the snapshot taken prior to the experiment. Proceed to data analysis.

5. Primary nasal and bronchial Cal-520 AM/Fluo-4 AM protocol

1. On the day of the experiment, remove basolateral media from the trans well and wash the apical and basolateral sides of the insert twice using 750 µL basolateral and 300 µL apical of the calcium buffer (step 2.1).
2. Incubate the cells with 600 µL basolateral and 200 µL apical of the Cal-520 AM or Fluo-4 AM dyes for 1 h at 37 °C and 5% CO_2. Prepare the dye in the same way as for the Calu-3 cells, for Cal-520 AM, 3 µM Cal-520 AM, 2.5 mM probenecid, and 1:5000 Hoechst in calcium buffer (step 2.1). For Fluo-4 AM, 3 µM Fluo-4 AM, 2.5 mM probenecid, and 1:5000 Hoechst in the same calcium buffer.
3. During the incubation period, prepare thapsigargin at a 3× intermediate stock for a final concentration of 2 µM (6 µM intermediate stock).
4. Following this, aspirate the dye and wash the cells twice with 300 µL apical and 750 µL basolateral buffer containing calcium (step 2.1).
5. Following washes, add 100 µL apical and 500 µL basolateral of 2.5 mM probenecid in calcium buffer (step 2.1).
6. Keeping the trans well within the plate, wrap the plate in foil. The cells are now ready for imaging.

6. Setting up microscopy for the primary nasal inserts

1. Acquire a glass-bottom Petri dish suitable for imaging, tweezers, and thin masking tape. Ensure that the Petri dish is clean by quickly wiping it with 70% ethanol and allowing it to dry completely.
2. Place the glass-bottom Petri dish within the small circular adaptor for the epifluorescence microscope.
3. Using tweezers, carefully place the insert into the center of the glass-bottom dish.

   NOTE: Be careful not to spill the buffer, which is placed in the apical compartment of the insert.
4. To ensure the insert doesn't move during drug additions, use masking tape to secure the top of the plastic of the insert onto the sides of the glass-bottom dish.
5. Place the adapter with the insert attached onto the stage of the microscope and begin by focusing on the cells using the 10x air brightfield objective.
6. Identify the calcium dye signal using the GFP (475nm LED). Ensure that the LED power falls between 5%-10% to avoid photobleaching, and the exposure time is suitable to visualize the baseline green signal without oversaturating it, typically between 300-500 ms.

   NOTE: Please see the NOTE under step 3.4 to ensure proper calcium dye loading.
7. Identify the Hoechst nuclear stain using the DAPI (405 nm) LED. LED power should be between 2%-5% with an exposure time between 50-100 ms.

   NOTE: If the cells are in focus, nuclei should appear with clear, defined outlines. Focus on the most apical layer of cells. Adjust the microscope's z-axis to identify the highest focal plane where cells remain in sharp focus. Ensure that the nuclei do not reach saturation of signal, as the nuclear signal can interfere with the green channel.
8. To acquire the live calcium video, follow steps 5.1-5.4. During the drug addition step, add into the center of the insert and resuspend slowly using a P200 pipette.

   NOTE: Ensure to take note of the frame in which the drug is added for downstream analysis.

7. Analysis using the Fiji software

1. Open the Fiji application and **Select File # Open**, upload the video file, and on the resulting pop-up window, keep all default settings.
2. If desired, under **Image # Adjust # Brightness/Contrast**, alter the brightness and contrast of the separate fluorescence channels to have the most optimal view of the cells.
3. To ease cell tracing, merge the nuclear stain or brightfield channels using **Image # Colour # Merge Channels** and select the appropriate files for each individual channel in the pop-up.
4. To trace specific cells based on cell boundaries and nuclear stain, use **Analyze # Tools # ROI Manager** and check off both show all and labels in the resulting pop-up.
5. Carefully trace cells using the freehand selections tool and press t to add a cell.

   NOTE: Ensure that cells are traced in all 4 corners and the center of the video.
6. Once all cells have been traced accurately, within the open tab with all ROIs, click on **More # Multi Measure**, and press **OK** on the resulting pop-up.

DOI 10.3791/69470

7. Copy the entire sheet in the resulting pop-up entitled **Results** into an excel file.

   NOTE: The resulting sheet will have 6 subheadings (Area1, Mean1, Min1, Max1, IntDen1, RawIntDen1) for each ROI with a numerical value after each, which corresponds to the respective cell that was traced. The important value for downstream analysis will be the Mean of each ROI.

8. In order to monitor the variation between fluorescence intensity, calculate the average fluorescence during baseline for each cell, and calculate the percent variance. Experiments typically have between 20%-30% variance.

$$\frac{maximum - minimum}{average\ between\ maximum\ and\ minimum} \times 100$$

9. Normalize all readings by dividing the mean intensity values by the first reading of baseline for each of the cells traced.

10. Create an additional column for **time** in seconds, with the first frame corresponding to frame 0 and subsequent timepoints increasing by 5, which corresponds to the 0.2 fps interval.

11. Copy the data into an analysis and graphing software and generate a curve of change in fluorescence over baseline over time.

    NOTE: To generate single-cell maxima plots, use the =MAX() function in Excel to find the frame at which maximal fluorescence intensity is reached for each cell and plot using GrapPad Prism.

8. Analysis using the Calcium Suite

1. Using Fiji, open the video file and follow steps 8.1 and 8.2.

2. Once the nuclear and green channels are optimized for viewing, duplicate a single frame of the video by clicking on Image # **Duplicate**. In the resulting pop-up, deselect **Duplicate Hyper stack**. Within the same pop-up, under **channels**, type **1**. Save it as a PNG image file.

3. Repeat step 9.2 and under **channels**, type **2**.

   Merge the two images by following step 8.3. Upload the image into the Cell-Pose GUI (Graphical User Interface) and use a trained model for cell boundary tracing to generate a cell mask.

4. Upload the image into the Cell-pose GUI, and segment using the nuclear stain (Channel 1) and cytoplasmic stains (Channel 2).

5. Save the image as a PNG file, then open the PNG file in Fiji and convert it to a TIFF file.

   NOTE: Cell-pose GUI produces a black-and-white PNG file.

6. Open both the mask file and the video file in the Calcium Suite by dragging and dropping, and then click on **Analyze Data** to generate a downloadable plot, as well as a downloadable .csv file containing normalized and raw intensity values for all cells counted by the software.

Representative Results

To measure calcium mobilization in live cells, Calu-3 cells or differentiated primary nasal cultures are incubated with an intracellular dye. The relative change in calcium-dependent signal is measured as an increase in the mobilization of calcium either from intracellular calcium stores or the extracellular space into the cytosol. A sustained increase in calcium into the cytosol is represented by an increase in fluorescence after thapsigargin, a well-characterized irreversible inhibitor of the ER-resident SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase)protein, is added[21]. Here, it is shown that the calcium assay performs robustly in both the Calu-3 stable cell line and primary nasal cultures.

Utilizing microscopy-based assays permits one to focus on responses from individual cells. Cells were traced manually based on the cytoplasmic fluorescent calcium dye and confirmed by the presence of Hoechst live nuclear stain (**Figure 1A**, **Supplementary Figure 1**). Fluorescence intensity normalized to the first reading of baseline fluorescence plotted over time for single cells utilizing either Fluo-4 AM or Cal-520 AM displayed kinetic differences in the calcium response (**Figure 1B**,**C**). In that, distinct cells respond to thapsigargin at different times and with distinct profiles. Plotting the maximal intensities for traced cells across three biological replicates supports the increased sensitivity of the signal from Cal-520 AM compared to Fluo-4 AM (**Figure 1D**). Representative raw fluorescence traces and intensities are shown in **Supplementary Figure 2**.

Next, differentiated primary nasal and bronchial cultures seeded on trans wells and differentiated at ALI were utilized. Trans-well inserts were placed on a glass-bottom Petri dish fitted to the specialized stage adaptor of the epifluorescence microscope (**Figure 2A**). In a similar way to the Calu-3 cells, individual nasal cells were manually traced based on the cytoplasmic calcium dye and the presence of Hoechst nuclear signal (**Figure 2B**). The change in normalized fluorescence intensity over time, normalized to baseline, is plotted for randomly selected cells using Cal-520AM and Fluo-4AM (**Figure 2C**,**D**). Primary nasal and bronchial cells exhibit cell-specific fluorescence response profiles upon stimulation with thapsigargin. Plotting the maximal intensity of cells across three biological replicates (**Figure 2C**) supports the increased sensitivity of Cal-520 AM relative to Fluo-4 AM in primary cells (**Figure 2E**).

DOI 10.3791/69470

It is noted that there is variability in the response sizes in both the Calu-3 stable cell line and the primary airway cultures in response to thapsigargin. Heterogeneity likely reflects both technical and biological variation. Technical variation may arise due to uneven dye loading or, in the case of the primary cultures, layers of cells that are out of focus. Biological variation may arise from the presence of different cell types, which may have altered calcium levels as well as variable expression of the target of thapsigargin, the SERCA2 protein.

Utilizing manual analysis methods can be time-consuming and may introduce unintentional subjectivity. To improve the throughput of analysis, machine learning-based software was developed to trace all cells within a specified field of view, by segmenting them based on live nuclear stains (Hoechst). Therefore, the software provides a robust and non-biased approach to monitor cell-specific changes in fluorescence over time. The software uses two inputs of identical pixel size, a video of the experiment using the green channel over time and a mask generated by the cellpose GUI software. The software will count all cells in the view and normalize each individual cell's fluorescence over time relative to the initial baseline reading. The novel machine learning software described here drastically increases the number of cells that can be processed, allowing over 1000 cells to be analyzed in a similar time frame compared to 12 cells analyzed manually. A screenshot of the calcium software applied to the same replicate analyzed manually in **Figure 2D** is presented (**Figure 3A**,**B**). The software enables exporting a CSV-format file of normalized intensity values for all cells specified by the software at each frame of the video. This data was exported and plotted on the graphing software to represent individual traces for all 1441 cells identified by the software in **Figure 3A** (**Figure 3C**).

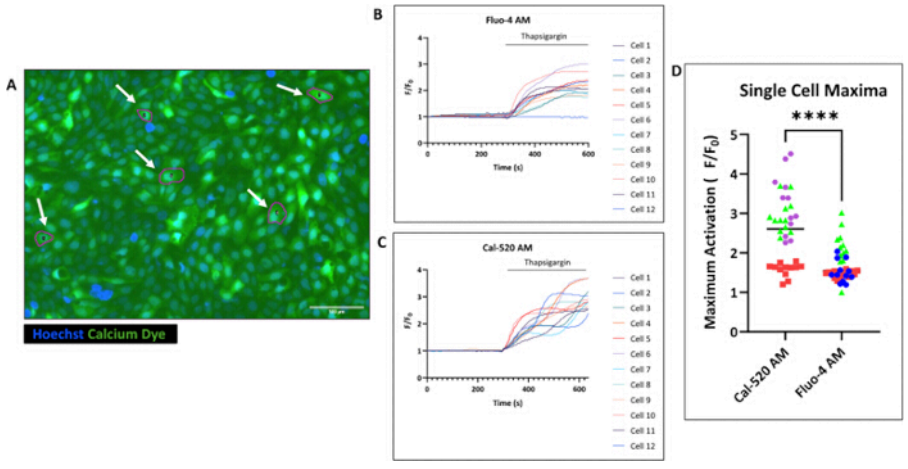

Figure 1: Live Calcium Assay performed in the Calu-3 cell line. (**A**) Zoomed in representative cell tracing of a 20X magnification image of microscopy-based assays, denoted by white arrows and a magenta outline using the freehand tool in Fiji. The scale bar in the bottom right corner represents 100 µm. (**B**) Representative traces for fluorescence of cells measured by the Fluo-4 AM dye stimulated by thapsigargin. Normalized fluorescence over baseline (F/F_0) plotted on the Y-axis over time on the X-axis. Each trace represents a different cell defined by Fiji tracing. (**C**) Identical to (**B**); however, the Cal-520 AM dye is employed here to report calcium mobilization. (**D**) Maximal activations to thapsigargin (n = 3) after 5 min of the 12 cells traced in different cell passages, denoted with different shapes and colors ($p < 0.0001$ (Welch's unpaired t-test) mean ± SEM of -0.8791±0.1647). Please click here to view a larger version of this figure.

DOI 10.3791/69470

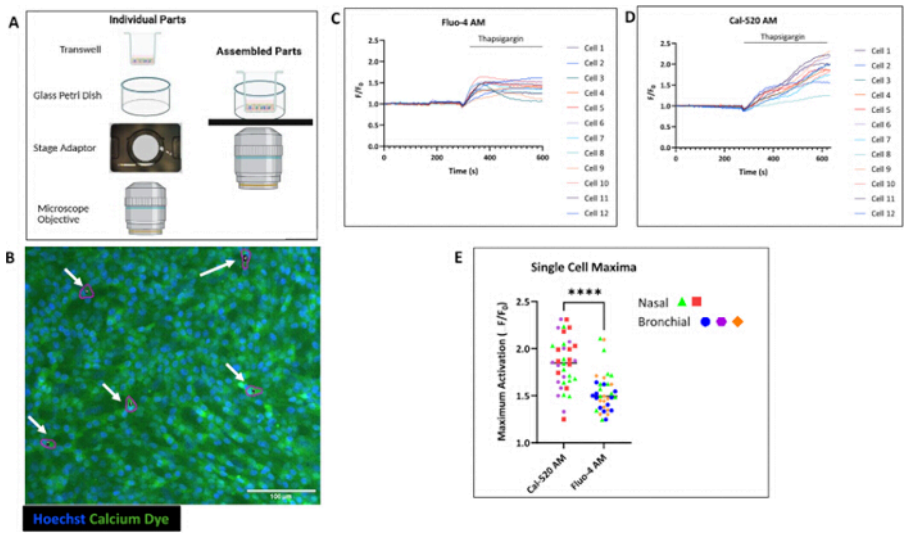

Figure 2: Live Calcium Assay performed in primary nasal and bronchial cultures. (**A**) Cartoon schematic of the microscopy setup for both primary nasal and bronchial cultures. (**B**) Zoomed in cell tracing of a 10x magnification image of individual cells in nasal cultures, denoted by white arrows and magenta outlines using the freehand tool in Fiji. The figure scale bar in the bottom right corner represents 100 µm. (**C**) Representative traces for fluorescence of cells measured by the Fluo-4 AM dye stimulated by thapsigargin. Normalized fluorescence over baseline (F/F_0) plotted on the Y-axis over time on the X-axis. Each trace represents a different cell defined by Fiji tracing. (**D**) Identical to (**B**), except the Cal-520 AM dye is employed to readout calcium mobilization. (**E**) Maximal activations to thapsigargin (n = 3) after 5 min of the 12 cells traced in different biological replicates, denoted as different shapes and colors. Nasal cultures are denoted with a green triangle or red square, and bronchial cultures are denoted with a blue circle, purple hexagon, or orange rhombus ($p < 0.0001$ (Welch's unpaired t-test), mean ± SEM of -0.3079 ±0.05689). Please click here to view a larger version of this figure.

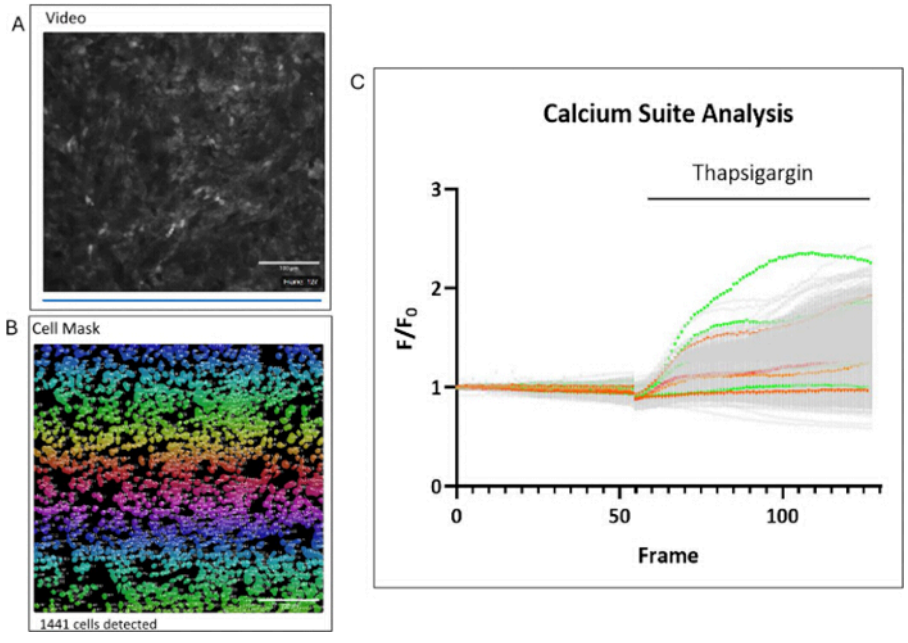

Figure 3: Analysis using the machine learning based software. (**A**) Screenshot of the calcium suite software with the video of the green fluorescence channel, representing calcium signal intensity change, reported by the Cal520AM indicator (n = 1). Scale bars in the bottom right corner represent 100 µm. (**B**) The cell mask generated from the identical region of interest in panel A by the cellpose GUI and respective cell count of 1441 cells, colored randomly by the Calcium Suite software. (**C**) Resulting curve of all 1441 cells exported from the Calcium Suite software in grey, select traces of cells are shown using different colors for readability. Please click here to view a larger version of this figure.

Supplementary Figure 1: Accuracy of the cell segmentation program. (**A**) A zoomed-in image taken using a 10x objective of the primary airway cultures stained using the live nuclear stain, Hoescht (blue). The scale bar is shown in the bottom right corner

DOI 10.3791/69470

of 50 pixels. (**B**) A further zoomed-in image of (**A**) to show nuclei more clearly for comparison with the mask, which falls in the dotted box emphasized in (**A**). (**C**) Mask generated by cellpose based on nuclear stain of the image in (**B**), distinct nuclei are white against the black background. Please click here to download this File.

Supplementary Figure 2: Raw baseline fluorescence measurements. (**A**) Raw fluorescence intensity of identical 12 cells in the Calu-3 cell line replicate is presented in **Figure 1C**. (**B**) Normalized Fluorescence intensity (F/F_0) of all 12 cells plotted in (**A**), identical to the plot in **Figure 1C**. (**C**) Plotted first baseline readings and maximal activations without normalization from each of the 12 cells plotted in (**A**). (**D**) Plotted normalized first baseline readings and normalized maximal activation readings (F/F_0) for each of the 12 cells plotted in (**B**). In (**C,D**), orange dots correspond to baseline, and green dots correspond to readings after the addition of thapsigargin. Please click here to download this File.

Discussion

This article has presented methods for analyzing single-cell calcium signaling in live primary nasal and bronchial cultures as well as a stable epithelial cell line. Both the Cal-520 AM and Fluo-4 AM calcium reporters are useful calcium indicators in both setups. Cal-520 AM is more sensitive as it has a higher quantum yield of 0.75 compared to Fluo-4 AM of 0.16 and slightly higher affinity for calcium (Cal-520 AM is 320 nM and Fluo-4 AM is 345 nM). Moreover, Cal-520 AM is reported as having improved cellular retention compared to Fluo-4 AM[22]. However, both dyes performed well in the current studies of thapsigargin-mediated calcium release[22,23].

This biological model is unique in that it utilizes primary epithelial cells cultured from human tissue. The differentiation process in the present culturing methods results in a multilayered tissue that consists of multiple distinct cell types. Here, methods to measure live calcium signaling *in vitro* are presented. The lack of enzymatic dissociation or opening of tight junctions maintains the tissue in its native state.

These studies are impactful as calcium is a crucial regulator of many biological processes, such as host innate immune responses to invasion from bacteria, and can be useful in the comparison of calcium mobilization in healthy and diseased states. Methods presented here have the potential to study these phenomena in live cells. Moreover, the ability to focus on calcium mobilization at a single cell resolution is exciting, as it is well known that differentiated tissue is composed of a diverse cellular landscape. Therefore, these methods provide the framework for cell-type-specific calcium mobilization in response to various agonists *in vitro*.

The protocol presented here has many advantages. In addition to the preservation of the tissue, it is easily adaptable to most laboratories as it does not require the use of a perfusion system, which is often costly and difficult to set up. Moreover, the Cal-520 AM and Fluo-4 AM dyes are commercially available and accessible. Furthermore, utilizing microscopy-based assays greatly improves the spatial and temporal resolution of the assay compared to plate reader assays, which only report the mean fluorescence in an entire well of seeded cells. Thus, the methods presented here have the potential to track cell-type-specific regulation of calcium mobilization. The machine learning software offers the ability to analyze over 1000 cells in roughly the same amount of time it takes to analyze 12 cells manually, which drastically decreases the time spent on analyses.

The success of the assay depends on a few critical steps. Namely, it is important that the trans well insert remains immobile throughout the imaging analysis, including the drug addition step. Additionally, remaining in the exact field of view enables the accurate tracking of a fluorescence change in a single cell over time. Furthermore, it is crucial that nuclei are stained with stains such as Hoechst. This live nuclear stain greatly improves confidence in cell tracing using the cell-pose GUI for the use of the machine learning software, as well as manual tracing using Fiji.

Researchers may encounter common issues when completing the protocol, which require troubleshooting. In order to troubleshoot uneven dye loading, Plurionic F-127 may be used to improve the solubility of the dye and aid in its even dispersal. Ensure that the dye is freshly prepared or stored in aliquots of small volumes to minimize freeze-thaw cycles. If photobleaching occurs, lower the LED intensity and exposure times while finding proper focus, and ensure that the dye and cell plate are protected from light during incubation.

Moreover, we acknowledge that different groups may have access to different materials. Therefore, a few troubleshooting clues specific to imaging setups are presented. It is recommended that groups use imaging plates and Petri dishes of thickness suited for the objective correction on the specific microscopes available to optimize focusing ability. Additionally, a short baseline reading is recommended to optimize exposure time for cell culture models, as thicker tissues are well-suited for longer exposure times. Short baseline reads will enable groups to monitor whether specific exposure and intensity settings will photo-bleach samples, and therefore specific intensity and exposure times can be optimized until the baseline fluorescence intensity remains stable. Multiple orientations of the insert were tested, including carefully cutting the insert on which cells are seeded and placing the insert in a specialized spaceship imaging adapter. However, the setup presented here gives robust calcium signal results and greatly minimizes artifacts associated with solution additions. Notably, novel research from a few groups has used a flipped orientation,

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where cells are seeded upside down on the trans well filter. These groups have been successful in monitoring cilia beating using the flipped approach[24,25]. In this setup, the flipped orientation can be considered to study agonists that need to interact with the basolateral membrane.

In experiments presented here, there is notable variation in fluorescence between individual cells in both the Calu-3 stable cell lines and primary cultures. A potential explanation is the different cell types in the differentiated primary cultures, as well as the potential for the Calu-3 epithelial stable cell line to be at different stages of differentiation, which may affect the expression of the SERCA pump, the target of thapsigargin. Moreover, it is well known that extrinsic calcium dyes have limitations, including uneven dye loading, which may affect intensity readings, as some cells may report a higher intensity value in response to thapsigargin simply because there is more dye contained within the cell[26]. Additionally, the primary cultures are well differentiated and form multiple layers. The use of the epifluorescent microscope here is beneficial, as it allows one to focus on additional cells in multiple layers. However, a drawback is that other layers remain slightly out of focus, which may diminish the relative fluorescence intensity of these out-of-focus cells after thapsigargin stimulation. Moreover, primary airway cultures are composed of cells with heterogeneous shapes and depths, and dye loading may be uneven across the different layers of the culture. Here, the fluorescence intensities of specific cells were analyzed over their respective baseline intensities ($\frac{F}{F_0}$). Background subtraction is not performed, but it can be employed in future studies to correct for out-of-cell areas that are out of focus or have heterogeneous sizes.

The machine learning cell-pose segmentation tool was used to identify single cells. Qualitatively inspecting the segmentation in comparison to nuclear stain demonstrates that the algorithm is able to generate masks for the majority of nuclei. However, there are occasional nuclei that are missed, and merged nuclei were observed (**Supplementary Figure 2**). The accuracy of the segmentation tool can be improved by focusing on smaller regions of interest and by adjusting the brightness and contrast using Fiji to ensure all nuclei are of the desired brightness.

Additionally, we acknowledge that the temporal resolution used here is low, 0.2 fps, which is suitable for measuring the gradual mobilization of calcium into the cytosol induced by thapsigargin. However, the frame rate can be adjusted on the pifluorescent microscope. Therefore, methods have the potential for scalability in response to different agonists that would trigger more intricate and rapid calcium fluxes.

As mentioned, the lack of a perfusion system eases setup; however, this setup is more biologically relevant in comparison to the simplified format in which thapsigargin is added stagnantly to the cultures. Moreover, perfusion systems often employ temperature and CO_2-controlled caged environments, which the current setup lacks.

Overall, the methods presented here are widely applicable and offer the potential to monitor live biological phenomena in complex primary cultures seeded on trans well inserts. For instance, the protocol can be utilized to study calcium signaling in specific cell types within the heterogeneous landscape using immunofluorescence of cell-type-specific proteins. These methods can also be adapted to monitor calcium signaling in response to more biologically relevant agonists, including ATP, succinate, and LPS (lipopolysaccharide). Moreover, the protocol can be utilized in disease modelling, wherein calcium signaling can be compared in healthy and affected individuals in response to different stimuli or pharmacological compounds.

Compared to current published protocols, this adapted imaging setup enables reliable imaging of multilayered primary airway cultures, and novel machine learning segmentation analyses offer the potential to analyze thousands of cells simultaneously.

Disclosures

The authors declare no competing interests.

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