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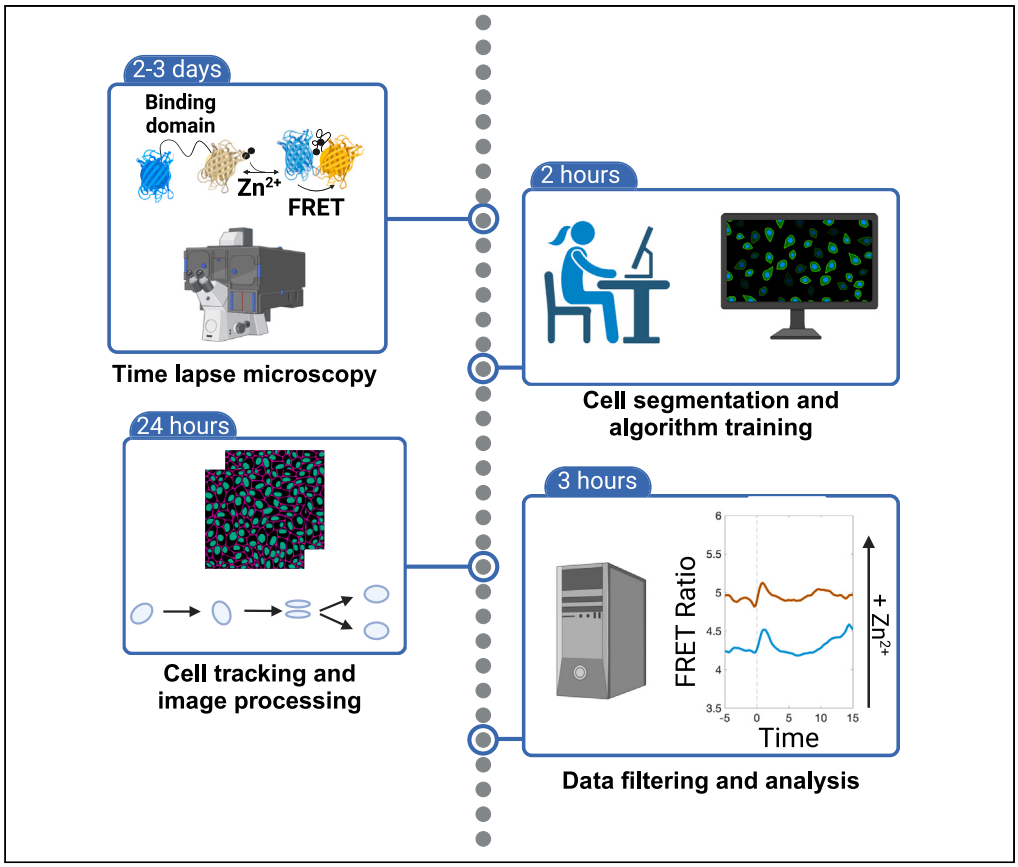
Protocol for measuring cell cycle Zn^{2+} dynamics using a FRET-based biosensor



The exchangeable Zn^{2+} pool in cells is not static but responds to perturbations as well as fluctuates naturally through the cell cycle. Here, we present a protocol to carry out long-term live-cell imaging of cells expressing a cytosolic Zn^{2+} sensor. We then describe how to track cells using the published pipeline EllipTrack and how to analyze the single-cell traces to determine changes in labile Zn^{2+} in response to perturbation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Culture and maintain MCF10a cells expressing NES-ZapCV2 and H2B-HaloTag

Monitor asynchronously cycling cells on a widefield microscope

Track and process asynchronously cycling cells with EllipTrack

Analyze single-cell FRET data to identify cell cycle changes in free Zn^{2+}

Holtzen et al., STAR Protocols 5, 103122
June 21, 2024
https://doi.org/10.1016/j.xpro.2024.103122

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Protocol for measuring cell cycle Zn^{2+} dynamics using a FRET-based biosensor

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https://doi.org/10.1016/j.xpro.2024.103122

SUMMARY

The exchangeable Zn^{2+} pool in cells is not static but responds to perturbations as well as fluctuates naturally through the cell cycle. Here, we present a protocol to carry out long-term live-cell imaging of cells expressing a cytosolic Zn^{2+} sensor. We then describe how to track cells using the published pipeline EllipTrack and how to analyze the single-cell traces to determine changes in labile Zn^{2+} in response to perturbation.

For complete details on the use and execution of this protocol, please refer to Rakshit and Holtzen et al.[1]

BEFORE YOU BEGIN

Zn^{2+} is an essential micronutrient that plays indispensable roles in catalysis, structure, and signaling.[2–4] Labile Zn^{2+}, which is the freely exchangeable pool of Zn^{2+} in cells, is highly regulated, and changes in response to perturbation.[1,5] To study Zn^{2+} at a single-cell level over time, scientists have developed genetically encoded Förster resonance energy transfer (FRET) sensors to quantify labile Zn^{2+} in cells.[5,6] ZapCV2, a FRET sensor specific for Zn^{2+}, is composed of two fluorescent proteins linked together with a Zn^{2+} binding domain. Upon Zn^{2+} binding, the two fluorescent proteins are brought together and can increase FRET efficiency. The FRET ratio (the ratio of FRET to CFP) is proportional to the free Zn^{2+} concentration in the cytosol.

In this protocol, we describe steps to monitor Zn^{2+} dynamics throughout the cell cycle using MCF10a cells expressing the FRET sensor ZapCV2 in the cytosol, as well as a nuclear marker H2B-HaloTag for cell tracking.[1] We include perturbations such as treatment with excess media Zn^{2+} and a Zn^{2+} specific chelator to influence Zn^{2+} dynamics throughout the cell cycle. While we apply this protocol to MCF10a cells, in principle it could be applied to most adherent cell types with subtle modifications. Similarly, we apply this protocol to cells expressing a nuclear excluded version of the FRET-based zinc sensor ZapCV2 (NES-ZapCV2) to measure free Zn^{2+} in the cytosol, but the protocol should also work with other FRET-based sensors. We do recommend making stable cell lines expressing the relevant fluorescent reporters to ensure adequate expression levels in imaging experiments lasting several days. We detail steps for tracking single-cells with an established pipeline,[7] as well as lay out guidelines for analyzing single-cell long-term FRET measurements. We advise that researchers have access to a widefield fluorescence microscope fitted with an environmental control chamber to maintain 37°C, 80% humidity, and 5% CO_2 to ensure optimal cell growth conditions. It is also critical that the widefield fluorescence microscope can capture FRET using a filter wheel or a two-camera system equipped with the proper emission filter sets.

Toy datasets and scripts used in this protocol are available at the link in the key resources table, and we strongly encourage researchers to run the pipeline with the toy data before generating and analyzing their own. This will help researchers develop an intuition for the tracking pipeline and analyzing data generated from a FRET-based biosensor.

Install MATLAB and EllipTrack

Timing: 1 h

1. Download MATLAB (version 2017a or later) and install.
2. Download EllipTrack (key resources table) and follow the instructions on the documentation page to install (https://elliptrack.readthedocs.io/en/latest/index.html#).
   a. Note that EllipTrack uses syntax from the old version of the BioFormats Image MATLAB package. To ensure the pipeline runs, update the pipeline by making the following edits:
      i. Open the read_image.m file in *functions* > *utils*.
      ii. Change line 44 to

```
I = bfReader.getPlane(image_info{3}, channel_id, image_info{5}-movie_definition.nd2_frame_range(reader_id,1)+1, 1);
```

      iii. Open the training_data_gui.m file in the *GUI* folder.
      iv. Change line 2529 to

```
I = all_bfReaders{row_id, col_id}{reader_id}.getPlane(site_id, channel_id, i-nd2_frame_range(reader_id,1)+1);
```

3. Install required toolboxes from MathWorks.
   a. Navigate to the Home tab of MATLAB and click "Add-Ons".
   b. Install the following Add-Ons:
      i. Computer Vision Toolbox.
      ii. Image Processing Toolbox.
      iii. Optimization Toolbox.
      iv. Parallel Computing Toolbox.
      v. Statistics and Machine Learning Toolbox.

⚠ CRITICAL: Ensure your computer meets the minimum requirements to run EllipTrack, which are listed on the documentation site.

⚠ CRITICAL: The original pipeline was written using a previous version of BioFormats Image which uses the function bfReader.getXYPlane. This function does not exist in the newer version, so the edits above are crucial to ensure the pipeline runs. Failure to edit the lines identified above will make the pipeline error when trying to extract the training data images, and when trying to run the pipeline.

Growth and maintenance of MCF10a NES-ZapCV2 H2B-HaloTag cells

Timing: 5 days

4. Thaw the frozen MCF10a NES-ZapCV2 H2B-HaloTag cell line quickly in a 37°C water bath.
5. Transfer to a 15 cm conical tube and add 8 mL of full growth media.
6. Centrifuge at 500 × *g* (RT) for 5 min.
7. Discard media and resuspend in new full growth media.

Note: MCF10a full-growth media is composed of 1:1 DMEM/F12 supplement, 5% horse serum, 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, and penicillin/streptomycin.

8. Plate in a 10 cm dish at 37°C incubator with 5% CO_2. Grow the cells to 80% confluence, then split cells 1:4.
9. Prepare MCF10a Zn^{2+} controlled minimal media (MM) using Chelex-100 treated horse serum and Chelex-100 treated insulin.
   a. Add 400 mg of Chelex-100 resin to 50 mL of horse serum.
   b. Incubate at 4°C for at least 12 h with slow rotation.
   c. Add 200 mg of Chelex-100 resin to 5 mL of recombinant human insulin.
   d. Incubate at 4°C for at least 12 h with slow rotation.

Note: MCF10a Zn^{2+} controlled minimal media is composed of 1:1 FluoroBrite DMEM/Phenol Red-free F12 supplement, 1.5% horse serum treated with Chelex-100 resin, 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin treated with Chelex-100 resin, and penicillin/streptomycin.

⚠ CRITICAL: All imaging experiments are conducted in the Zn^{2+} controlled MM. Full growth media contains 5% horse serum, which contains undetermined levels of metal ions, including Zn^{2+}. Treating the serum with Chelex-100 removes Zn^{2+} and other divalent metal cations, which are then replaced with the defined F12 media supplement. Perturbing Zn^{2+} in full growth media can lead to variable results, which is why all experiments outlined below are conducted in MM. In addition, the FluoroBrite and Phenol Red-free nature of the media for imaging experiments is important to reduce the background autofluorescence from typical media components.

Plate MCF10a NES-ZapCV2 H2B-HaloTag cells for imaging

Timing: 2 days

10. Digest cells using trypsin-EDTA to a single-cell suspension.
    a. Remove media and wash twice with phosphate-buffered saline (PBS).
    b. Add 1 mL of trypsin to cells and allow to incubate for 15–20 min at 37°C.
    c. Quench trypsin reaction with 4 mL of minimal media.
11. Centrifuge at 500 × *g* for 5 min and resuspend the pellet in 1 mL of minimal media.
12. Count cells and dilute the cell suspension into the required volume of minimal media for the number of wells to be plated.

Note: For a full 96-well imaging plate, we suggest plating 3,000 cells per well. Since each well of a 96-well plate can hold 200 μL of growth media, we suggest diluting cells to 300,000 cells per 20 mL of media.

13. Using a multi-channel pipette, add 200 μL of the cell suspension to each well of a 96-well glass-bottomed imaging plate.

Note: For ease of analysis using the data analysis pipeline, we recommend each column to be one treatment. Each well in that column should be one replicate of that treatment. For example, column 1 should be treatment A, column 2 should be treatment B, and column 3 should be untreated cells.

14. Leave the plate in a dark biosafety cabinet undisturbed for 30 min to allow for proper cell adhesion.

15. Allow to recover overnight at 37°C with 5% CO_2.

⚠ CRITICAL: MCF10a cells divide approximately once every 14 h, which means after 2 days of imaging, the cells will likely be crowded. Additionally, too few cells will lead to poor proliferation in very high or low Zn^{2+}. You will therefore need to optimize the initial plating density of the cells to allow for adequate proliferation, while reducing the chance of contact inhibition by the end of the time lapse. We have found that plating 3,000 cells/well is an adequate plating density for experiments up to 48 h.

⚠ CRITICAL: Leaving the plate in the dark biosafety cabinet allows cells the chance to adhere to the imaging plate. Moving the plate to the incubator too early will result in cell migration to the edges of the well and result in poor cellular imaging.

Acquire dark noise and illumination bias images

Timing: 30 min

16. Add 200 μL of the imaging media you are going to use for your experiment to one well of a 96-well plate.
17. Place the plate on the microscope.
18 Tune the illumination source and exposure times to emulate the ones you will be using in your long-term experiment.

Note: We acquire images at 30% illumination power and 300 ms exposure time.

19. Acquire five images from each channel and save them as .tif files with descriptive names for processing downstream.
20. Acquire five images with the light source turned off.
21. Save them as .tif files.
22. Follow instructions on the EllipTrack documentation website to create the bias and dark noise files.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM/F12, HEPES	Thermo Fisher Scientific	Cat# 11330057
Fluorobrite DMEM	Thermo Fisher Scientific	Cat# A1896701
F12 media supplement	Sigma-Aldrich	Cat# N6658-6X
Horse serum, New Zealand origin	Thermo Scientific	Cat# 16050122
Chelex-100, sodium form	Sigma-Aldrich	Cat# C7901
Gibco EGF recombinant human protein	Thermo Fisher Scientific	Cat# PHG0313
Cholera toxin	Sigma-Aldrich	Cat# C8052
0.05% tryspin-EDTA (1×)	Gibco	Cat# 25300-120
Pen/Strep	Gibco	Cat# 15140–122
Recombinant insulin	Life Technologies	Cat#12585-014
Janelia Fluor 669 HaloTag ligand	Luke Lavis, Janelia Research Campus	N/A
Tris(2-pyridylmethyl)amine	Sigma-Aldrich	Cat# 723134
Hydrocortisone	Sigma-Aldrich	Cat# H4001
0.1 M zinc chloride solution	Sigma-Aldrich	Cat# 39059

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
MCF10a cells transduced with NES-ZapCV2 and H2B-HaloTag	Rakshit and Holtzen et al., 2023	N/A
Software and algorithms		
MATLAB 2017a	MathWorks	N/A
Nikon Elements software	Nikon Inc.	N/A
EllipTrack	Tian et al.	32755578
Live cell FRET sensor analysis pipeline	Rakshit and Holtzen et al., 2023 (https://doi.org/10.5281/zenodo.7968726)	37330912
Other		
Nikon Ti-E widefield microscope	Nikon Inc.	N/A
Example data sets, including the EllipTrack pipeline, two time-lapse images of cells grown in minimal media and Zn^{2+} deficient media, and the post-processing data analysis pipeline.	https://doi.org/10.6084/m9.figshare.24970041	This work
Oko environmental cage incubator	Okolab	N/A
96-well glass bottom plate	Cellvis	Cat# P96-1.5H-N
All reagents and materials are US sourced. Catalog numbers may vary between regions.		

MATERIALS AND EQUIPMENT

MCF10a full growth media (500 mL)	
Component	Concentration
1:1 DMEM/F12 Media	468 mL
Horse serum	25 mL (5% v/v)
Recombinant EGF	20 ng/mL
Hydrocortisone	0.5 μg/mL
Cholera toxin	100 ng/mL
Insulin	10 μg/mL
Penicillin/streptomycin	1×
Once sterile filtered, store at 4°C for 6 months.	

MCF10a controlled minimal media (500 mL)	
Component	Concentration
Fluorobrite DMEM	243 mL
Ham's F12 Supplement	243 mL
Chelex-100 treated horse serum	7.5 mL (1.5% v/v)
Recombinant EGF	20 ng/mL
Hydrocortisone	0.5 μg/mL
Cholera toxin	100 ng/mL
Chelex-100 treated insulin	10 μg/mL
Penicillin/streptomycin	1×
Once sterile filtered, store at 4°C for 6 months.	

⚠ CRITICAL: To acquire FRET images, the microscope must be able to acquire images for the donor fluorescent protein and the FRET channel nearly simultaneously. This requires the use of an emission filter wheel to swap between the donor emission wavelength and the acceptor emission wavelength (Figure 1B). Ensure your microscope setup is capable of this acquisition scheme.

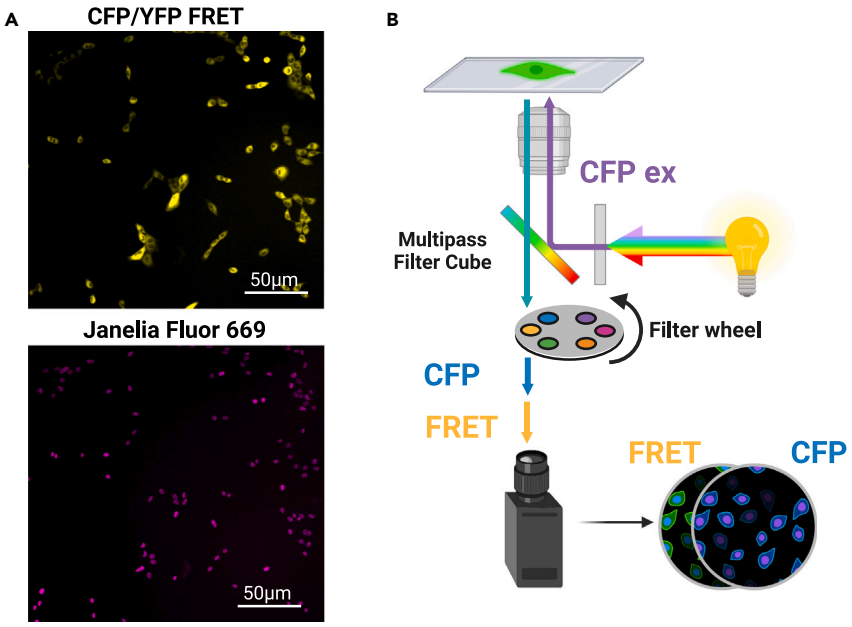

Figure 1. Cellular seeding density and fluorescence microscopy optical setup.

(A) Representative images of cells plated at 4,000 cells per well at the first frame of a time lapse. Density and cell distribution will change depending on the cell type. CFP excitation/YFP emission is the FRET channel, and Janelia Fluor 669 refers to the nuclear marker H2B-HaloTag labeled with JF669.

(B) Diagram of fluorescence microscopy acquisition setup. The FRET sensor is excited by the CFP excitation wavelength from the illumination source. The emission is composed of CFP emission and sensitized emission from CFP/YFP FRET. Both wavelengths are allowed to pass through a multi-pass filter cube to the filter wheel. A filter wheel is used to quickly swap between the CFP emission wavelength (CFP) and the CFP/YFP FRET emission wavelength (FRET).

STEP-BY-STEP METHOD DETAILS

Time-lapse microscopy of Zn^{2+} dynamics

Timing: 2–3 days

The time-lapse microscopy step acquires long-term live-cell images of cells to monitor their Zn^{2+} dynamics. This step includes treatment of cells with a membrane permeable Zn^{2+} chelator tris(2-pyridylmethyl)amine (TPA) and excess Zn^{2+}. To reduce background, cells are grown in controlled minimal media, which does not contain phenol red, and contains lower concentrations of horse serum. Serum and insulin contain high amounts of Zn^{2+}, so they are both treated with Chelex-100 to reduce the amount of Zn^{2+} to approximately 3 μM in the minimal media. The cells described in this protocol are either untreated or treated with 2 μM TPA to reduce free Zn^{2+} in cells.

1. Start the Nikon Ti-E widefield microscope and open the Nikon Elements software.
2. Start the environmental chamber and set the temperature to 37°C, CO_2 to 5%, and the relative humidity to 90%.
3. While the environmental chamber reaches the set point, prepare cells for imaging.
   a. Remove media from cells and replace with fresh, pre-warmed minimal media containing 5 nM Janelia Fluor 669 ligand.

Note: You may also use a different HaloTag ligand for nuclear tracking as long as it has minimal spectral overlap with ECFP and mVenus (such as JF646, JF635, and SiR700).[8] Ensure adequate signal-to-noise ratio before beginning acquisition. In addition, any H2B-basic

red (or far red) fluorescent protein fusion can be used as the nuclear marker (for example H2B-mCherry) if it has minimal spectral overlap with the ZapCV2 sensor. In this case, staining with HaloTag ligand can be omitted.

b. Return to the incubator for 30 min.
c. Remove cells from the incubator, remove media, and wash once with 1× PBS.
d. Add pre-warmed minimal media to cells.
e. Add any other treatments you want to each column. Leave one column untreated.

Note: For MCF10a cells, various concentrations from 15–50 μM excess Zn^{2+} with no issues in proliferation or cell survival.[1,9,10] In addition, the Zn^{2+} chelator TPA has been used at concentrations between 2–3 μM; however, it is well documented that this concentration causes changes in cell proliferation.[1,9] Other cell types may require optimization of Zn^{2+} supplementation or TPA treatment.

4. When the environmental chamber reaches the set point, place the 96-well plate into the sample holder.
5. Select a 10× air objective for acquisition.

Note: A 20× air objective can be used as well.

6. Focus on the cells in one well of the 96-well plate.
   a. Use Perfect Focus or another autofocus feature to find the focal plane of the bottom of the dish.
   b. Manually offset the focus until the cells are in the focal plane.
7. Set up acquisition to measure fluorescence with the following optical configurations.
   a. Set exposure times and illumination power to yield at least 2:1 signal to noise ratio (Figure 1A).

Note: Researchers may test signal to noise ratio by determining the intensity of the nucleus or cytosol divided by the background. For example, determine the nuclear intensity of the JF669 H2B-HaloTag construct and divide it by the intensity in an area not in the nucleus. To ensure at least a 2:1 signal to noise ratio, adjust illumination power and exposure times. To decrease phototoxicity, ensure exposure time is under 500 ms and illumination power is kept under 50%. In addition, researchers must be careful not to saturate the detector by increasing exposure time or laser power. If pixels are saturated, reduce exposure time or illumination power until the pixel intensity is within the range of the detector.

   b. Add the following optical configurations to your acquisition.

Note: Below are the excitation and emission properties of the fluorophores used in the sample data, as well as the filter properties of our optical configurations:

H2B-HaloTag conjugated to Janelia Fluor 669, $\lambda_{max,}$ ex: 674 nm, $\lambda_{max,}$ em: 685 nm, Excitation filter: 640/30, emission filter: 705/72, Dichroic mirror: 640 nm.

ECFP, $\lambda_{max,}$ ex: 435 nm, $\lambda_{max,}$ em: 475 nm, Excitation filter: 440/20, emission filter: 480/20, Dichroic mirror: 455 nm.

ECFP/mVenus FRET, $\lambda_{max,}$ ex: 435 nm, $\lambda_{max,}$ em: 530 nm, Excitation filter: 440/20, emission filter: 540/21, Dichroic mirror: 455 nm

⚠ CRITICAL: Since NES-ZapCV2 is a FRET sensor, it is imperative that the exposure time for each channel is the same. For example, if the donor exposure is 200 ms, the FRET channel must also be exposed for 200 ms.

8. Take one time-lapse image for each channel every 12 min for a total of 24 h or longer.

Note: Most commercial systems will have an autofocus feature (e.g. Nikon's Perfect Focus or Zeiss's Definite Focus) to ensure cells are kept in focus through the acquisition. At 10× magnification, focus issues are rare, but it is suggested that researchers use an autofocus feature regardless of magnification.

Note: Because of the long-term nature of the imaging, ensure that any treatments you add to your cells are gentle enough as to minimize apoptosis. If apoptosis is common, consider reducing the strength of your treatment.

Note: Over time, the dye-labeled H2B-HaloTag will be diluted by consecutive cell divisions, and it is possible that the nuclear fluorescence will be diluted as well. With an extremely bright dye such as Janelia Fluor 669, we have tracked cells for at least 72 h with no issues; however, it may be necessary to optimize the HaloTag ligand for adequate cell tracking. For longer time lapse experiments, it may be necessary to take advantage of a constitutively fluorescent H2B-FP fusion.

Note: Each file can be upwards of 10 GB in size. Be aware of space considerations and storage infrastructure before beginning the experiment.

Training data set construction

Timing: 2 h

Since perfect segmentation and tracking are difficult to achieve, EllipTrack uses a machine learning algorithm to identify and correct errors in initial segmentation and tracking. To do this, the pipeline will measure features of each ellipse and identify whether it is accurately segmented, poorly segmented, or undergoing mitosis. It will then apply corrections to each ellipse and subsequently each track. This section will guide you through segmenting cells based on their nuclear marker and training a machine learning algorithm on cellular phenotypes and cell movement tracking (Figure 2). This is required for accurate cellular tracking with EllipTrack. Because different H2B fusion proteins have slightly different appearances in the nucleus, a new training dataset is required for each new H2B fusion protein. The following steps will be for processing time-lapse images of .nd2 files using a multi-well plate format. Attached will be the time-lapse files, the parameters file, and the training data file to use as a starting point for your own analysis.

9. Transfer files to the computer where analysis will take place.
10. In MATLAB, Open the EllipTrack pipeline folder and select *GUI > parameter_generator_gui.m* and run the file.
11. Select "New" then click "Start".
12. Fill in the fields with the information and directories of your imaging and illumination bias files.
    a. For each channel except the nuclear marker channel, ensure you select "Calc Cytoring" to measure the cytoplasmic ring of each cell.
    b. Ensure you put the name of each channel exactly as it appears in the file metadata.
    c. Assign a name to the signal which represents the signal that channel is tracking.
13. Click "Next".
14. Input your plate information.
    a. Input the well you would like to use for training.

Note: For training the machine learning algorithm, we suggest using one well of your untreated control cells.

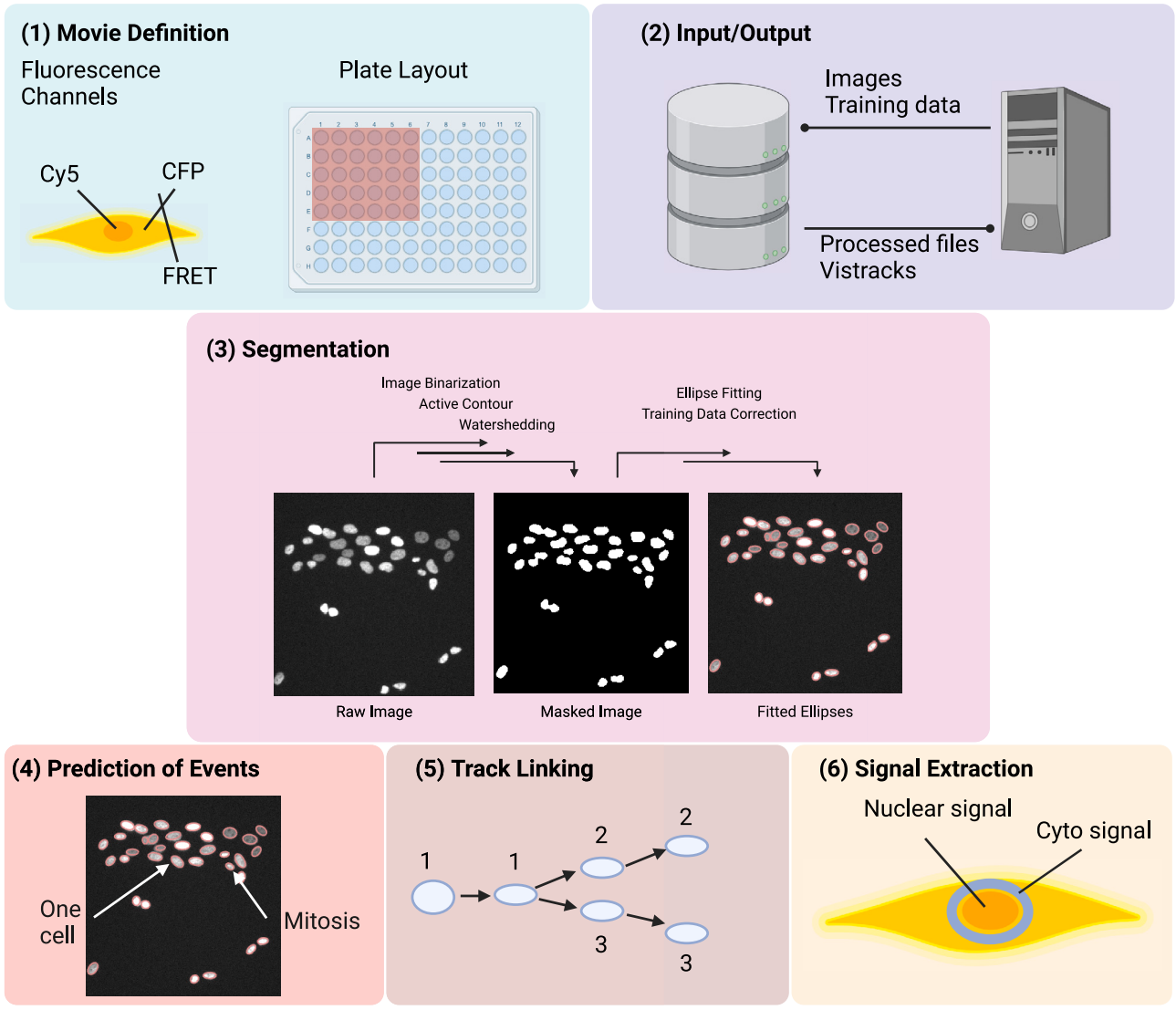

Figure 2. Summary of the EllipTrack pipeline

Users first input the names and locations of their movies along with the fluorescent channels they want to monitor. Users will then identify where to input training data and image locations along with the output directories. Users will then define segmentation parameters for their cell nuclei. Users will then change any parameters necessary in the Prediction of Events, Track Linking, and Signal Extraction steps of the pipeline.

b. Input the range of frames you would like to analyze.
c. Check the box indicating which jitter correction algorithm you will use.

Note: "Global" considers all wells joined together and will use the same registration offset for all of them. "Local" will analyze the registration offset separately for all wells. If using a 96-well plate, choose "Global."

d. Input the number of cores you would like to use to analyze the movies.

Note: This requires availability of more than one core on your computer. If your computer only has one core, just leave it at 1.

15. Extract your training data images.

a. Input the well location of the untreated control cells you specified in Step 14a.
b. Select a range of frames to save as .tif files.

Note: We suggest selecting a range of 10–15 frames from at least two segments of the time lapse.

c. Assign your output directory and click "extract".

16. Click "Next".
17. Assign your output directories.
    a. Leave the training data field blank, since you have not created any.
    b. Select the output folder to store the "MAT" files, as well as the "Segmentation" files.
    c. If desired, select one or all of the other fields and assign an output directory.
18. Click "Next".
19. Identify optimal segmentation parameters.
    a. Examine frame 50 of one well by inputting the "coordinates" of this frame in the Row/Column/Site/Frame selection box.
    b. Adjust the Intensity boxes to the minimum and maximum intensities that make the cells visible.
    c. Adjust the parameters in all subsequent segmentation steps and inspect the segmentation after each step.
    d. Optimize the parameters so that most cells are accurately segmented, and the fitted ellipse tightly surrounds each nucleus.
20. Click "Save". A parameters.m file will populate in the *GUI* folder.
21. Copy the parameters.m file to the same folder where mainfile.m is located.
22. Open mainfile.m and highlight lines 1–14.
23. Right click and select "Evaluate Selection."

Note: Segmentation will take between 30–40 min, depending on the length of the time-lapse and the cell density.

24. When the segmentation is done, navigate to the *GUI* > *training_data_gui.m* and run the file.
25. Input the locations of each file, as well as the frames to analyze, and the output the location, then click "Import Data".
26. Label ellipses by clicking and assigning a morphology to it.

Note: It is easy to miss mitosis events when labeling the morphologies of cells. Therefore, we suggest starting by labeling all mitoses in the movie segment, then returning to fill in the other morphologies listed below. Be sure to label cells from a wide range of brightnesses and behaviors. For more information on the execution of this training algorithm, refer to the original EllipTrack publication.[7] We have also included examples of each cell morphology in Figure 3, and a brief explanation of each morphology below:

No cells: The nucleus is over segmented, and one nucleus is segmented into two or more ellipses. Label 50 instances of these ellipses. You may label fewer if over segmentation is rare.

One cell: The nucleus is adequately segmented and only one nucleus is present inside of the ellipse. Label 100 instances of these ellipses.

Two cells: The ellipse contains two nuclei and is under segmented and two or more nuclei are present inside of this ellipse. Label 50 instances of these ellipses. You may label fewer if under segmentation is rare.

Before mitosis: Label an ellipse that divides in the next frame. Identifying these events will require scanning back and forth between two frames of the time lapse and locating cells that split from one ellipse to two ellipses. Label all mitoses in the segment of the movie you are analyzing.

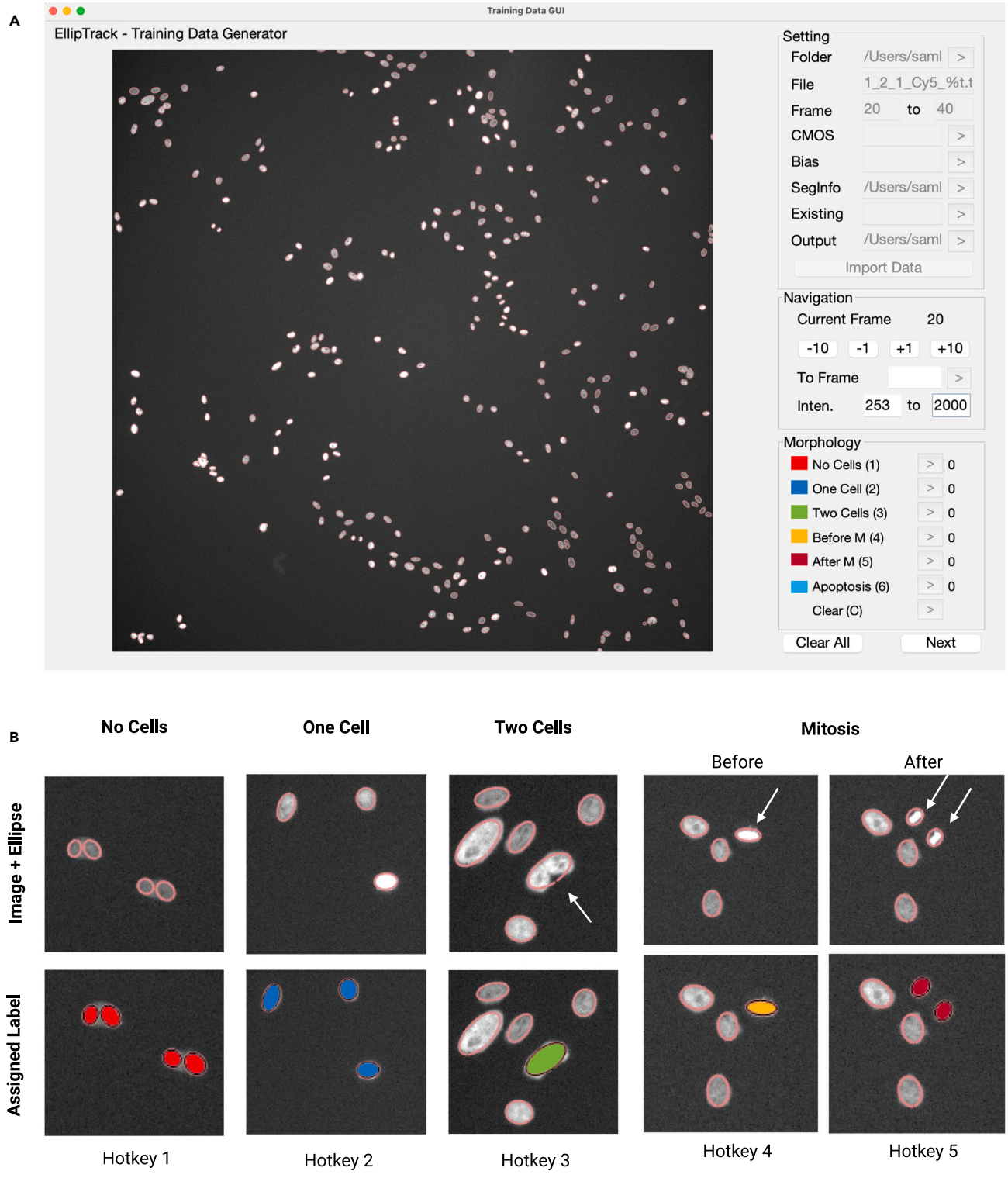

Figure 3. Representative images of EllipTrack training

(A) A screenshot of the EllipTrack training data GUI showing settings, navigation GUI, and morphology assignment GUI.

(B) Representative images of each type of morphology. Apoptosis is not shown because it is rare in this cell type and was not represented in the dataset.

After mitosis: Label both cells that are born after cell division. This can be done concurrently with step 24d, since identifying a pre-division ellipse will allow you to mark the post-division ellipse in the subsequent frame.

Apoptosis: Do not label unless apoptosis is common in your treatment conditions.

27. Click "Next".
28. Label ellipses and track across the movie segment. Be sure to label cells from a wide range of brightnesses and behaviors.
    a. Assign a number to the cell you are currently tracking by filling in the "To Cell" field.
    b. Select a cell and record it by using the hotkey "R".
    c. Follow the cell to the next frame, select the cell and record it using the hotkey "R".
    d. Repeat this for at least 10 cells in the field of view.
29. Click "Finish".
30. Repeat steps 24–29 for the remaining movie segments you would like to use for training.

Note: We recommend training time lapse segments from the beginning, middle, and end of the time lapse.

Cell tracking and image processing

Timing: 24 h

This step will guide you in setting up the parameters for predicting events of each ellipse, tracking the cell migration, and signal analysis. This step will also guide you through assessing the quality of the tracking using the vistrack movies. Keep in mind, we do not expect perfect tracking, since the "cell tracking problem" is still an active area of research and no algorithm tracks cells with complete accuracy. This pipeline will take a few hours, depending on the number of wells, channels, and frames each time lapse has. Parallel processing using multiple cores on a single CPU is encouraged.

31. Run the *GUI > parameter_generator_gui.m*.
32. Select "Load Existing" and select the parameters.m file you generated before running the segmentation and training.
33. Navigate to the "Section 1. Movie Definition (2/2)" page and ensure the "Wells" are filled in with the wells you would like to analyze.
34. Click "Next".
35. Load the training data files that you created and press the "+" button to add them to the pipeline.
36. Add the directory where you want the vistrack movies to be saved.
37. Click "Next" twice.
38. Select which method estimates your cell line's migration speed the best.

Note: For MCF10a cells, cell density strongly impacts the migration speed, so we suggest using this method. For other cell lines or treatments that inhibit migration speed, you may need to select a different method.

    a. Select "Cell Density" and then click "Calculate".
    b. Determine if there is a trend between "Cell Density" on the horizontal axis and "Migration speed" on the vertical axis. If migration speed changes based on cell density, select "Cell Density."
    c. If migration speed does not change based on cell density, repeat steps 38a-b on the "Time" method. If migration speed changes throughout the movie, select this method.
    d. If there is no correlation in either of the previous methods, select "Global".

39. Click "Next".
40. Select "Both mitotic and newly born cells". Keep all other parameters the same.
41. Click "Next". Keep all parameters the same.
42. Click "Next". Keep all parameters the same.
43. Click "Save" and transfer the parameters.m file to the directory that holds the mainfile.m file.
44. Open mainfile.m and click "Run".
45. When the pipeline is finished, navigate to the output folder and inspect the vistrack files, which are saved following the scheme "[Row]_[Column]_[Site]_[Nuclear Channel]_[Frame].tif".
    a. You can load these files into ImageJ as an image sequence, or manually inspect each.
    b. Pick a handful of cells and watch throughout the time lapse and inspect for track swapping (i.e., two nuclei swapping track IDs) or premature termination (i.e., track number turning red and then disappearing in the next frame).
    c. If most cells are adequately tracked, the tracking is satisfactory, and you may continue with the analysis.

Note: Vistrack movies will be the same size as the original time lapse images. Be aware of the space each will take up. We suggest that users select one or two images and analyze the vistrack movies. Then, if the tracking is satisfactory, remove the output directory for the vistrack movies from the parameters file and run the pipeline to conduct tracking without exporting the vistrack movies. See Methods video S1 for examples of acceptable and unacceptable tracking.

Data filtering and analysis

Timing: Variable

Once the individual tracks and relevant information have been extracted from the images, the tracks can be further analyzed to identify changes in Zn^{2+} concentration using the ZapCV2 biosensor. Since the biosensor is ratiometric, the pipeline will calculate the FRET ratio to display relative changes in Zn^{2+} concentration. The script will calculate the FRET ratio based on the equation:

$$FRET\ Ratio = \frac{CFP_{ex}YFP_{em}}{CFP_{ex}CFP_{em}}$$

where $CFP_{ex}YFP_{em}$ is the background-subtracted FRET channel intensity, and $CFP_{ex}CFP_{em}$ is the background-subtracted CFP channel intensity. Subtle changes in expression of the sensor and therefore intensities will change the FRET ratio, so there is a filter step to ensure that low-expressing cells are removed from the analysis.

The output file from EllipTrack is large and contains information that is not necessary to the downstream analysis of the ZapCV2 sensor. We offer a bespoke pipeline to extract the relevant information from the native EllipTrack output, as well as link each mother-daughter lineage together into their own lineage traces. Finally, we outline steps to run the analysis pipeline to display single-cell and mean FRET ratio information from the time-lapse image. This pipeline is modular and can be adapted to any required downstream signal extraction and data presentation.

46. In MATLAB, navigate to *live-cell-zinc-sensor* > *preprocessing* and open the export_lineage_struct.m file.
47. Click Run and select the signals.m file that was created by the EllipTrack pipeline.
    a. In the command window, enter a string that describes the cell line you are analyzing.
    b. In the command window, enter the date of the experiment as an integer in the format YYYYMMDD.
48. Navigate to the main_file.m and change necessary information.
    a. Change the variable colors_cell to incorporate any additional colors you want. Ensure this is the same length as the variable condition_cell.

b. Inside of the switch/case statement, you can have the program set different parameters depending on the cell type used in your analysis.
   i. You may adjust the strings inside of condition_cell, as well as change which conditions you would like to plot in conditions_to_plot.
   ii. You may change the minimum and maximum y-values each graph can take on, since different cells will have higher or lower sensor expressions and therefore FRET ratio.
c. Set the FRET_min and FRET_max variables.

Note: The FRET_min and FRET_max variables correspond to the minimum and maximum values the ZapCV2 sensor can take on. Any cells outside of this will be discarded. These values will depend on the optical setup of your fluorescence microscope. You should therefore determine this experimentally for your own imaging system by treating cells with either strong chelation or high media Zn^{2+} to get a sense of the FRET ratio range of your system. Afterward you may change these variables to better reflect your system.

49. Click "Run" and select the "XXX_struct_cell.mat" file that was exported by the export_lineage_struct.m file.
50. Tune the CFP channel threshold to remove low-expressing cell traces.
    a. Open set_sensor_threshold.m and run the script.
    b. Iteratively change the CFP_thresh parameter until plots in Figure 4 (After Threshold) do not show a visible correlation between the CFP intensity and FRET ratio.

    Note: Setting the CFP threshold higher will yield fewer but higher quality traces.

    c. When you are satisfied with this threshold, navigate to main_file.m and change the CFP_thresh parameter to the threshold you just determined.
51. Run main_file.m once more.
52. Select your "XXX_struct_cell.mat" file to import it.
53. Open up any file other than "main_file.m" and execute it to generate graphs of single-cell FRET ratio, or grouped FRET ratio and save your data.

    Note: Examples of graphs are shown in Figure 5. A brief description of the files is shown below.

    a. cellproliferation.m: This script will plot the number of cells at each frame, normalized by the number of cells at the initial frame. This is a proxy for cellular proliferation (Figure 5A).
    b. resting_FRET_asynchronous.m: This script will plot the average FRET ratio of every cell across the time lapse. This is a proxy for relative FRET ratio across the time lapse that will vary between different Zn^{2+} treatments (Figure 5B).
    c. mitosis_red_dots.m: This script will plot the single-cell FRET ratio of all cells in the time lapse as dark gray lines. It will also plot each cell division as a red dot (Figure 5C).
    d. mitosis_FRET_align.m: This script will align all FRET ratio traces to mitosis and average them. This yields the population average of the Zn^{2+} pulse after mitosis, as described in Rakshit and Holtzen et al. (Figure 5D).
    e. mitosis_FRET_align_alltraces.m: This is similar to the script in 53d, except it plots every cell trace.
54. You can also modify the files to suit your analysis needs as well.
    a. Copy the text of one of the scripts to a new script editor window and change accordingly.

EXPECTED OUTCOMES

In this protocol, we describe steps to conduct a long-term live-cell imaging experiment with cells expressing a nuclear marker and a FRET-based Zn^{2+} sensor. We then describe steps to process the data using a machine learning cell tracking pipeline. Finally, we describe how to analyze single-cell FRET using a previously published pipeline, including data quality control, filtering steps

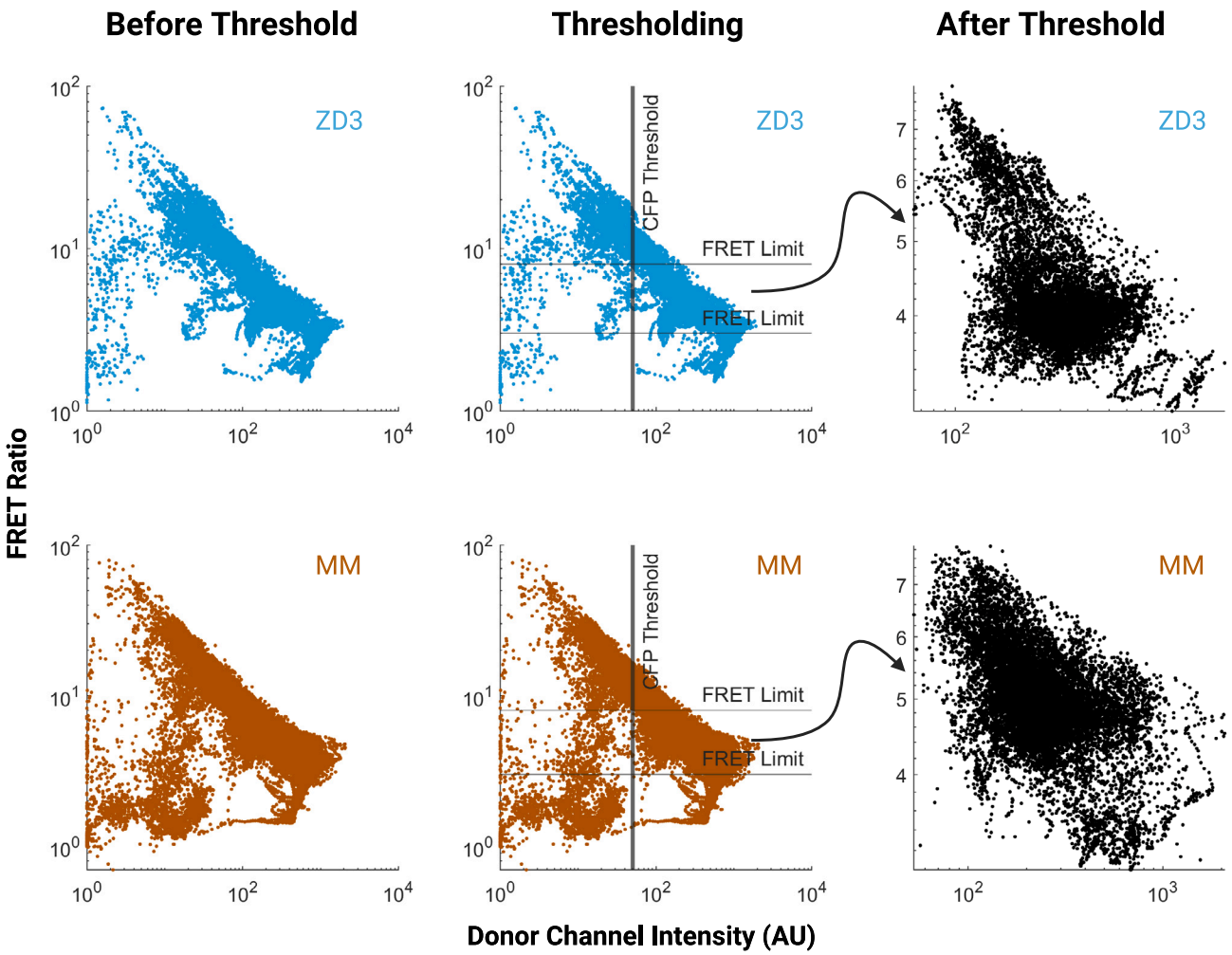

Figure 4. Donor channel and FRET ratio thresholding scheme

(Left column) All measurements of CFP intensity on the horizontal axis and the corresponding FRET ratio on the vertical axis. Cells treated with minimal media (bottom) and mild Zn^{2+} chelation using 3 μM TPA (top). All points are plotted on a log-log scale. Each point represents the FRET ratio and corresponding donor channel intensity of one cell at each time point of the time lapse. (Middle column) Plots from the left column showing FRET limits (FRET Limit) as well as the donor channel intensity limits (CFP Threshold) imposed on the single cell traces. All points are plotted on a log-log scale. The thresholds will depend on your imaging system, the expression level of the sensor in your cells, as well as the optics you use for your experiments. (Right column) Plots of CFP and FRET intensity of cells that fall within the constraints set in the middle column. The yield of tracks within this column will depend on the expression level and signal to noise ratio of your specific cell lines. Typically, a yield of greater than 50% is expected. All points are plotted on a log-log scale.

and estimation of labile Zn^{2+}. Depending on the plating density, duration of the time lapse, and stringency of filtering, you should expect to yield at least 300 single-cell tracks per time lapse image.

QUANTIFICATION AND STATISTICAL ANALYSIS

Refer to the "resource availability" section for a link to the toy data and code presented in this protocol. Follow the steps and use the data to get accustomed to the pipeline.

LIMITATIONS

Microscope limitations

Imaging FRET sensors comes with its own challenges. First, the most important part of the microscope setup is the ability to acquire the donor channel and the FRET channel almost simultaneously.

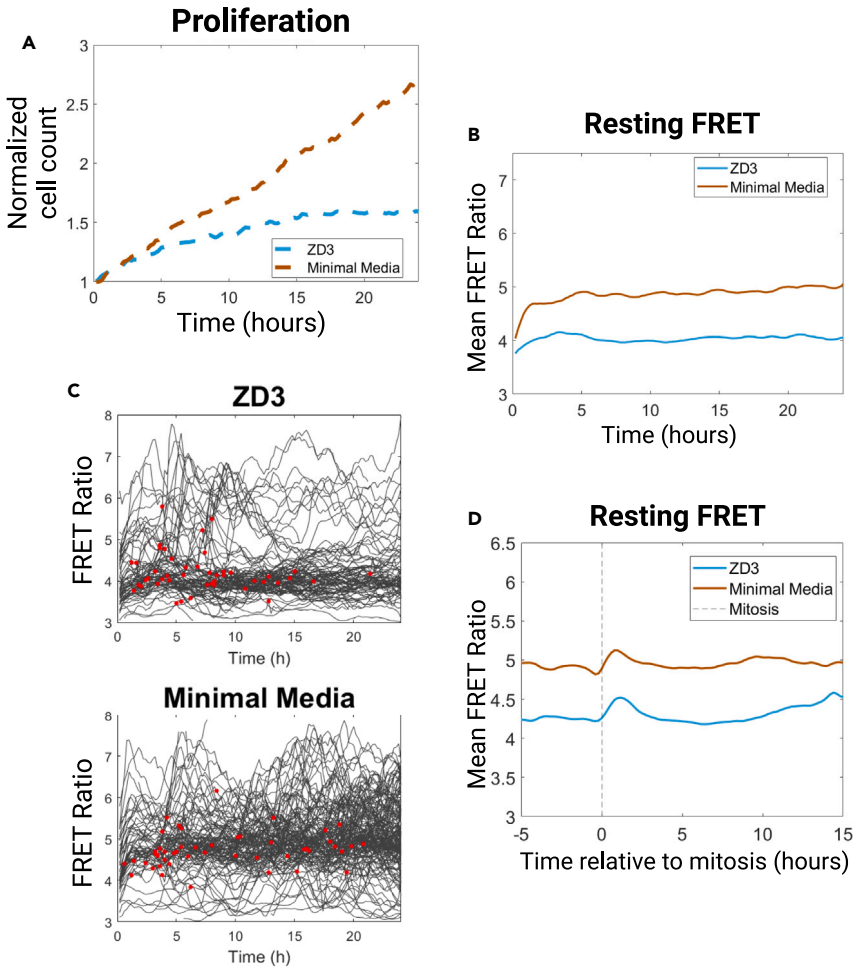

Figure 5. Graphs of single-cell and population averaged FRET ratios

MCF10a cells were treated with minimal media (MM) and 3 μM TPA in MM (ZD3) and imaged for 24 h. More than 300 tracks were analyzed in (A, B, and D). 100 random tracks were plotted in (C).

(A) Graphs of cellular proliferation. Lines represent the normalized cell count in each condition over time.

(B) Graphs of resting cellular FRET over the time lapse. Lines represent the population average FRET ratio in each condition.

(C) Graphs of single-cell FRET ratio as a function of time, plotted with each mitosis detected by the tracking software.

(D) Graphs of the average FRET ratio after being aligned to anaphase.

The best way to accomplish this is to use an emission filter wheel in which the donor emission filter and the acceptor emission filter are in adjacent positions. This enables one filter cube containing the donor excitation filter and dichroic to be used for excitation, and rapid changing of emission filters via the filter wheel. A two-camera system that can acquire both the donor channel and FRET channel simultaneously is also acceptable only if each camera is equipped with the proper filters (CFP emission and CFP/YFP FRET emission filters specifically).

If a filter wheel is not available, FRET ratio images can be collected with two cubes: one containing the donor excitation filter, dichroic, and donor emission filter and the other containing another donor excitation filter, dichroic, and acceptor emission filter. The limitation of the 2-dichroic cube set-up is that it takes more time to switch the cube than to switch an emission filter, so there is a greater time delay between the donor and FRET images. Ensuring the capability of your microscope to capture FRET is critical for the success of this protocol. Any perturbation in temperature can influence the experiment, so it is important that cells are equilibrated in the environmental chamber before the acquisition.

Pipeline limitations

Cell tracking is a continually evolving field, and although cell tracking algorithms have advanced significantly, there is no "one size fits all" algorithm that tracks cells perfectly. Although EllipTrack does exceptionally well at tracking cells in 2D space, it is not amenable for 3D tracking, nor is it amenable for multinucleated cells or cells that undergo multipolar mitosis. For these applications, researchers should use alternative tracking algorithms specifically designed for these analyses.[11]

Sensor limitations

FRET-based biosensors are inherently ratiometric and hence ideally suited to experiments like this one in which the cells undergo significant changes in volume during mitosis. However, FRET-based biosensors generally suffer from low dynamic range, which can limit sensitivity to small changes in the ligand concentration. In addition, the FRET ratio is sensitive to the brightness of the sensor. In an ideal system, one can increase the exposure time or the illumination intensity to increase signal to noise of the sensor; however, this becomes problematic when one is imaging cells for an extended period of time. Researchers must therefore balance the signal to noise of the sensor and the health of the sample.

TROUBLESHOOTING

Problem 1

Cells are inadequately tracked.

Potential solution

Inaccurate cell tracking is an issue with any cell tracking pipeline, and one should always expect some imperfections in tracking (Methods video S1); however, if cell tracks are consistently incorrect, or a pattern in cell track loss is apparent, there are steps that can be taken to identify issues and increase the cell tracking efficacy.

First, ensure cell segmentation is correct. Oftentimes, when cell nuclei are under-segmented or over-segmented, the algorithm stops tracking these cells. To remedy this, optimize nuclear segmentation by tuning parameters in the cell segmentation step. This will allow the algorithm to more accurately link tracks together by reducing the error associated with poorly segmented nuclei.

If cell segmentation is adequate and over- and under-segmentation is rare, there may be an issue with the training data set. Since EllipTrack uses a linear discrimination model trained on nuclear parameters to identify cell morphologies, it is imperative that the training data set is representative of a wide variety of cell behaviors and brightnesses. Therefore, improper training can cause downstream issues with tracking cells that are present in the time lapse, but not represented in the training data. To remedy this, export the vistrack movies and identify when the pipeline stops tracking cells, indicated by a cell ID turning red and disappearing. Determine whether dim cells, bright cells, oddly shaped cells, or cells entering or exiting mitosis are triggers for premature termination. Then, return to the training data GUI and ensure that the training data set covers these cellular morphologies. If it does not, label bright cells, dim cells, oddly shaped cells, or mitotic cells and rerun the pipeline with this updated training data set.

Problem 2

The FRET ratio of cells in a subset of cells is not between the predefined FRET ratio window.

Potential solution

In the vast majority of cells, the NES-ZapCV2 will respond to free Zn^{2+} and have a FRET ratio between a well-defined minimum and maximum. As discussed previously, the minimum and maximum FRET ratio will depend on the specific optics of each individual imaging system. With our optical configuration and illumination conditions, the FRET ratio should be between three and eight. In a small percentage of cells, the ZapCV2 sensor is either lowly expressed, highly overexpressed, or the

ZapCV2 sensor is somehow malfunctioning, which can lead to issues in quantifying the FRET ratio. In a perfect FRET sensor, the FRET ratio is independent of the brightness of the sensor, and thus plotting the FRET ratio against the donor channel intensity will yield a plot of pure noise. This is not always the case with sensors that are extremely dim or extremely bright, since FRET and brightness become nonlinear. The lower the intensity of the signal, the lower the signal-to-noise, and therefore the more susceptible that cell is to changes in sensor brightness. This will show a characteristic scatter plot as shown in Figure 4. The quality of the FRET ratio measurements is particularly sensitive to CFP intensity since CFP is less bright than the acceptor mVenus and the CFP intensity is in the denominator of the FRET ratio measurement. Tune the "CFP_thresh" parameter to yield a population whose FRET ratio is not correlated with the donor channel intensity. This can be done using the set_sensor_threshold.m script.

If a large percentage of cells are still too dim and their FRET ratio and donor channel intensity show a correlation, it may be necessary to repeat the experiment with modified acquisition parameters. When modifying acquisition parameters, try to optimize the donor channel intensity while keeping the exposure times exactly the same between the donor and FRET channels.

Problem 3

Running the analysis software returns an error.

Potential solution

If any part of the analysis code returns an error, check all previous steps and research the error to pinpoint the issue. Since errors can be due to any number of syntax or logical issues with the code, it is impossible to give a precise fix for the error.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Amy Palmer (amy.palmer@colorado.edu).

Technical contact

Requests for further technical information should be directed to the lead contact, Amy Palmer (amy.palmer@colorado.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

A repository of all scripts and pipelines used in this protocol are uploaded to Figshare (https://doi.org/10.6084/m9.figshare.24970041). We have also shared the time lapse images generated in this publication to this repository.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103122.

ACKNOWLEDGMENTS

We thank the University of Colorado Biochemistry Cell Culture Core (specifically Theresa Nahreini) for assistance with cell culture. We would like to acknowledge NIH Director's Pioneer Award DP1-GM114863 (A.P.) and NIGMS MIRA R35 GM139644 (A.P.) for generous financial support.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, A.E.P., A.R., S.E.H.; investigation, S.E.H., A.R.; writing, S.E.H., A.R., A.E.P.; funding acquisition, supervision, and project administration, A.E.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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