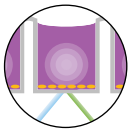

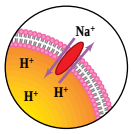

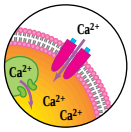

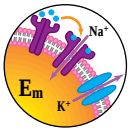

FLIPR
FLuorometric Imaging Plate Reader

Application Note

Measuring Intracellular pH With the FLIPR® I and FLIPR^{384} Fluorometric Imaging Plate Reader Systems

Molecular Devices

Section I: Introduction

The FLIPR® system is designed to perform functional cell-based assays. This application note provides a basic protocol for running an intracellular pH assay on the FLIPR system using adherent cells, as well as a discussion of some of the important parameters for optimization of the assay. Because each cell line has unique properties, the protocol will need to be optimized for your particular assay.

The "Principles" section of this application note outlines the purpose and the options for each step of the assay. The next section details the materials and methods used in the assay.

Section II: Principles of the intracellular pH assay

The Na^+/H^+ exchanger, or NHE, contributes to the pH stability of the cytoplasm by exporting protons out of the cells and importing sodium ions. BCECF is a pH sensitive dye. The extinction coefficient of BCECF is high in alkaline environment and low in acidic environment. It can be used to monitor the NHE activity by measuring changes in intracellular pH following an artificial acid-load of the cells. Cells are initially incubated in the presence of BCECF, then NH_4Cl, which causes the dye to enter the cell and the cytoplasm to alkalinize due to the formation of NH_3 (high fluorescence signal). When NH_4Cl is diluted by the addition of fluid on the FLIPR system, NH_3 diffuses out of the cell, leaving high concentration of H^+ inside the cytoplasm (resulting in rapid decrease of fluorescence signal). These protons are removed from the cells by the action of the NHE (demonstrated by a slow increase of fluorescence signal, proportional to the NHE activity). Figure 1 represents typical results obtained with the intracellular pH assay.

The activation of many G-protein coupled receptors causes acid excretion, mainly through the NHE activity. It is possible to measure the response of such receptors to ligands by monitoring the activation of the NHE in cells subjected to the BCECF and acid load treatment. The intracellular pH assay may provide an alternative to intracellular calcium assay for non-Gq-protein coupled receptors. This assay may also be used to assess inhibitors of the NHE function.

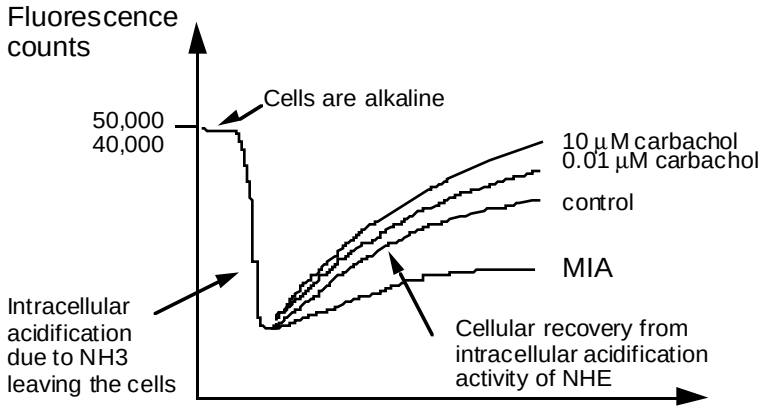

Figure 1: Drawing of intracellular pH results using M1-WT3 cells and carbachol agonist.

CELL PREPARATION

Adherent cells

Note: This application note provides a protocol for adherent cells only, since the volume added on the FLIPR system requires the cells to be firmly attached to the bottom of the plate.

Cells are seeded the day before the experiment and all steps are carried out in the same black wall 96- or 384-well plate. The basic steps of the assay are:

- Plate the cells in black-wall 96- or 384-well plate.
- Dye load the cells with BCECF for 45 minutes.
- Prepare the acid load of the cells by adding 20 mM NH_4Cl into the dye loading plate, incubate for 15 minutes.
- Wash the cells of extracellular dye with wash buffer containing 20 mM NH_4Cl.
- Assay the cells in the FLIPR system; the NH_4Cl buffer is diluted by the addition of a large volume of balanced salt solution and various ligands/controls.

It is necessary to optimize the cell seeding density so that a uniform, confluent monolayer is formed after an overnight incubation. Cells can be seeded more than one day in advance, as long as the seeding densities are adjusted to yield a confluent monolayer on the day of the assay. Cells that are weakly adherent or do not grow uniformly may need to be grown on plates coated with a matrix such as poly D-lysine, laminin or collagen (see Appendix for details). Cells that are normally maintained in culture at subconfluent levels should be seeded at lower densities.

DYE LOADING

In order to observe changes in intracellular pH, cells must be "loaded" with a pH-sensitive fluorescent dye. The dye loading protocol must be optimized for each cell type.

Fluorescent dye

To date, the most commonly used dye for intracellular pH assay has been BCECF.

Anion exchange protein inhibitor

Some cell types use mechanisms such as the anion exchange protein to export anionic molecules from the cells, including anionic forms of the fluorescent dyes. Not only will this result in poor dye loading, but it also may cause an artifact in the data; there will be a sharp decline in the measured fluorescence when the test compounds are added, due to dilution of the extracellular dye. (The test compounds are prepared in buffer without dye.) Therefore, it may be critical to the success of the FLIPR system intracellular pH assay to inhibit the action of the anion exchange protein.

Probenecid is an anion exchange protein inhibitor and when added to the loading medium may increase dye retention in the cells. An example of a cell type known to require probenecid is CHO. Although probenecid can be useful in slowing dye leakage from cells, it is toxic to the cells, and hence the duration of the dye loading should be kept to a minimum (see Loading duration).

Sulfinpyrazone is another anion exchange inhibitor. To date, little information about using sulfinpyrazone in the intracellular pH assay is available.

Dye loading media

Several types of loading media have been tested. The medium should be optimized for both satisfactory dye loading and good cellular responses. Possible choices include:

- Growth medium + 10% fetal bovine serum (FBS) + 20 mM HEPES
- Growth medium + 1% FBS + 20 mM HEPES
- Hank's BSS 1X (without phenol red) + 20 mM HEPES and 1% FBS
- Hank's BSS 1X (without phenol red) + 20 mM HEPES and 1% BSA

Loading duration and temperature

The optimal loading time will depend on the cell type. Because the fluorescent dye is toxic to cells, it is best not to exceed the optimal loading time. A 60-minute loading time at 37°C is usually effective for most cells and is the recommended starting point for assay development.

Note: If loading for 30 minutes yields an acceptable fluorescence signal, use the shorter loading time.

PREPARING FOR THE ACID LOAD OF THE CELLS

This step is actually not the acid load itself, but the addition of NH_4Cl to the cells. Once in the cells, NH_4Cl forms NH_3 and alkalinizes the cytoplasm. Subsequently, when NH_4Cl is diluted out by fluid addition on the FLIPR system, NH_3 diffuses out of the cells, leaving the protons H^+ in high concentration in the cytoplasm. This triggers the activity of the NHE and the export of protons out of the cell.

The solution made for this step is a 200 mM NH_4Cl solution. NH_4Cl remains in the wells throughout the wash step until diluted on the FLIPR system.

PREPARING THE COMPOUND PLATE

Depending on its complexity, preparing a compound plate can take a variable amount of time. Therefore, it is best to plan your experiment carefully to ensure that the compound plate will be ready for use as soon as the cells are ready. In this application note, it is assumed that the dye loading incubation is long enough to prepare the compound plate.

Preparing the compound diluent

In the intracellular pH assay, the buffer used to dilute the compounds and the wash buffer are different (in contrast to intracellular calcium and membrane potential assays). The wash buffer contains 20 mM NH_4Cl, whereas the compound diluent contains no NH_4Cl.

You can prepare a large volume of compound diluent and use it for all of the plates you will be assaying in a day. Using V-bottom plates to hold the compounds will minimize the dead volumes in the compound plate.

Preparing the compounds

The compounds should be prepared at 1.25X the final concentration in the cell plate during the assay. Adding a large volume of compound is essential for this assay since it allows the acidification of the cytoplasm, triggering the activation of the NHE.

Intracellular pH measurements are performed quickly in order to visualize rapid cellular kinetics. Therefore, it is necessary to pipet the compounds into the cell plate rapidly to effectively mix the fluids in the wells. The large volume added and the fast pipetting speed provide instantaneous mixing.

Because of the volume of compound added to the cell plate, strongly adherent cells are better candidates for this assay than weakly adherent ones.

WASHING THE CELLS

After dye loading and addition of NH_4Cl solution to the plate, several washes with the wash buffer are necessary to remove extracellular dye. The washes are performed in the presence of 20 mM NH_4Cl. An adequate automated cell washer should have adjustable dispensing and aspirating heights and speeds. We recommend Molecular Devices cell washer for 96- or 384-well plates. Alternative washers are BioTek or LabSytems cell washers for 96-well plates, and Skatron Embla340 for 384-well plates.

The quality of data obtained from the FLIPR system will depend partially on the cell washer's ability to leave a consistent residual volume in each well after the last wash. Results obtained after manual washing tend to be more variable. Also, if washing the cells manually, it is critical not to aspirate the cells dry between washes.

Note: The wash buffer is NOT the same as the buffer used to dilute the compounds.

FLIPR SETUP PARAMETERS FOR INTRACELLULAR PH ASSAYS

Checking the background and basal fluorescence signals

Start with the laser power, camera F-stop and exposure time set as shown in Table 1.

Settings	FLIPR I	FLIPR^{384}
Laser power	0.300 W - 0.400 W	0.600 W - 0.800 W
Camera F/stop	F/4-5.6	F/4-5.6
Exposure time	0.4 sec	0.4 sec

Table 1: FLIPR hardware settings for a cell plate basal fluorescence signal test.

At the beginning of a data run, perform a "signal test" to check the background fluorescence and the basal fluorescence signal (i.e., prior to compound addition) from the cells. For intracellular pH assays, it is best to work with a basal fluorescence signal of 40,000 to 50,000 counts above background (saturation of the camera occurs at 65,000 counts). The difference in laser power used on the FLIPR I system and on the FLIPR^{384} system is due to the difference in the way the excitation light scans the plate. The laser power used on the FLIPR^{384} sytem is typically twice as high as on FLIPR I to obtain the same basal fluorescence signal.

Basal fluorescence signal

The desirable basal fluorescence signal needs to be high enough to allow the drop of fluorescence after the fluid addition on the FLIPR system. However, it should not be so high that the camera reaches saturation.

Adjusting the basal fluorescence signal

Adjust the following instrument parameters to obtain an acceptable basal fluorescence signal:

Exposure time

If the basal fluorescence signal is too high, the exposure time can be decreased to a minimum of 0.1 second. If the basal fluorescence signal is too low the exposure time can be increased, but the sampling interval will have to be increased to at least the exposure time + 0.6 second (it takes 0.6 seconds for the camera to integrate data).

Camera F-stop

Increasing the camera F-stop number decreases the opening of the aperture. If the basal fluorescence signal is too high, increase the F-stop. If the basal fluorescence signal is too low, the laser power may be increased. F/1.4 is the largest aperture opening setting for the FLIPR system.

Laser power

Increase the laser power if the basal fluorescence signal is too low, or decrease it if the basal fluorescence signal is too high. The laser power during the assay should range between 0.1500 W and 0.800 W on FLIPR I, 0.300 W and 2.000 W on the FLIPR^{384} system.

PIPETTOR PARAMETERS FOR INTRACELLULAR PH ASSAYS

The FLIPR system software allows you to set the height of the pipet tips in the wells during fluid transfer, as well as the speed at which fluid is dispensed. These parameters are set independently for each pipetting sequence.

Pipettor height

After the pipettor picks up fluid from the compound plate, it draws a small bubble of air into the bottom of each pipet tip to ensure that fluid doesn't leak out. The bubble will be the first thing out of the pipet tip when fluid is dispensed. To avoid blowing bubbles in the wells (which can cause random light reflections and spurious signals), it is best to start dispensing with the tips above the fluid level in the wells. It is also preferable to have the pipettor tips submerged after the addition has been completed to ensure that all the sample is dispensed. If the pipet tips are in the air at the end of the fluid delivery, a drop can form on the end of the tip due to surface tension. Therefore, the pipettor height before fluid addition should be somewhere above the starting level of fluid in the wells but below the final volume after the addition. For example, if the wells contain 100 µL and the sample volume to be added is 50 µL, the pipettor can dispense the compounds from a height of 120–140 µL.

Fluid dispensing speed

The default pipettor dispense speed is 50 µL/sec for 96-well plates and 20 µL/sec for 384-well plates. Table 2 provides the limits for the fluid dispense speed for both 96- and 384-well plate formats.

	96-well plate	384-well plate
Slow dispense speed (weakly adherent cells)	10 µL/sec - 40 µL/sec	5µL/sec - 10 µL/sec
Fast dispense speed (strongly adherent cells)	50 µL/sec - 80 µL/sec	15 µL/sec - 20 µL/sec

Table 2: Fluid dispense speeds.

These values must be determined experimentally for each cell type, but generally it is preferable to dispense as fast as possible to enhance mixing of the compounds in the wells. The trade-off is that the pipetting speed must not be so forceful that it dislodges cells from the well.

Section III: Method for adherent cells

PREPARING THE CELLS

Adherent cells are typically plated the day prior to the experiment.

Materials

1 Clear, flat-bottom, black-wall 384- or 96-well plates (see consumables list in Appendix B). The flat bottom ensures that the cellular fluorescence is localized to a single horizontal plane, while the black walls prevent well-to-well crosstalk.

2 Cells ready to be transferred into 96- or 384-well plates (see Table 3).

3 Incubator (5% CO_2, 37 °C).

4 Pipettor and sterile tips suitable for use with microplates.

Plating the cells

	96-well plate	384-well plate
Cell density in growth medium	$2x10^4$ - $1x10^5$ cells/well	$1x10^4$ - $5x10^4$ cells/well
Total cell number	$2x10^6$ - $1x10^7$ cells	$4x10^6$ - $2x10^7$ cells
Growth medium volume	150 µL/well, (15 mL/plate)	30 - 80 µL/well, (12 - 32 mL/plate)

Table 3: Recommended cell seeding densities for adherent cells.

Step 1 Prepare a cell suspension in growth medium (see cell densities in Table 3).

Step 2 Pipette the cell suspension in plate (see volume per well in Table 3).

Step 3 Incubate the cell plate overnight in a 5% CO_2, 37°C incubator.

Note: To minimize edge effects, avoid stacking cell plates in the incubator.

DYE LOADING

Preparing the dye

The 1 mg/mL stock of BCECF dye is mixed with an equal volume of 20% (w/v) pluronic acid immediately before use.

*Note: Some cell lines generate better results when pluronic acid is **not** used in the loading medium.*

1 mg/mL (= 1.6 mM) BCECF stock. (See Appendix B for consumables details.)

1 Solubilize 1 mg BCECF in 1 mL low-water DMSO.

2 Store 25 µL aliquots at -20°C.

20% pluronic acid solution:

Note: A ready-to-use 20% pluronic acid solution is available from Molecular Probes.

1 Weigh out 400 mg pluronic acid into a tube, then solubilize in 2 mL low-water DMSO. Warming the solution to 37 °C will increase the acid solubility. Mix the solution gently to avoid forming excess bubbles.

2 Allow the solution to cool to room temperature before aliquoting or using, and store 25 µL aliquots at room temperature.

Dye/pluronic acid mixture:

1 Immediately before use, combine equal volumes of the dye stock and 20% pluronic acid. The dye and pluronic acid will be at concentrations of 0.8 mM and 10%, respectively.

Preparing the anion exchange protein inhibitor

Note: probenecid is only required for some cell types, e.g., CHO.

Probenecid should be prepared fresh every day at a stock concentration of 250 mM, and used at a working concentration of 2.5 mM.

250 mM probenecid (100X stock, enough for 1L of buffer):

1 Solubilize 710 mg probenecid in 5 mL 1.0 N NaOH.

2 Mix in 5 mL Hank's BSS 1X without phenol red with 20 mM HEPES.

Preparing the loading medium

Possible choices of loading medium include:

- Growth medium + 20 mM HEPES + 10% fetal bovine serum (FBS)
- Growth medium + 20 mM HEPES + 1% FBS
- Hank's BSS 1X (without phenol red) + 20 mM HEPES and 1% FBS
- Hank's BSS 1X (without phenol red) + 20 mM HEPES and 1% BSA

Note: HEPES is typically added to Hank's buffer and /or any loading medium where probenecid is present in order to maintain constant pH.

Dye loading the cells

Using a cell washer instrument (for 96- or 384-well plates)

Constituents	96-well plate (90 µL/well)	384-well plate (35 µL/well)
Loading medium	11 mL/plate	13 mL/plate
BCECF/pluronic acid mixture (2X for cell washer method)	88 µL (6-10 µM dye final concentration)	104 µL (6-10 µM dye final concentration)
Probenecid stock (if necessary)	110 µL	130 µL

Table 4: Preparation of loading medium for one plate of adherent cells, using the cell washer.

Step 1 Wash off the growth medium using a cell washer. For most cell lines, one to two washes is sufficient. Make sure the residual volume after the last wash is defined and consistent across the plate. The recommended

residual volumes are 45 µL for 96-well plate and 17.5 µL for 384-well plate.

Step 2 Add an equal volume of a 2X concentration of loading buffer using a multi-channel pipettor. The recommended final volumes in the wells are 90 µL for 96-well plate and 35 µL for 384-well plate.

Step 3 Cover the plate, then incubate in a 5% CO_2, 37°C incubator for 45 minutes.

The cell washer method is effective at not perturbing the cell layer. It is strongly recommended to use the cell washer method only for 384-well plates, since the entire well area is read by the FLIPR^{384} system. For 96-well plates, you can use either a cell washer or the manual method.

Using the manual method (for 96-well plates only):

Constituents	96-well plate (90 µL/well)	384-well plate (35 µL/well)
Loading medium	11 mL	13 mL
BCECF/pluronic acid mixture (1X for manual wash method)	44 µL (3-5 µM dye final concentration)	52 µL (3-5 µM dye final concentration)
Probenecid stock (if necessary)	110 µL	130 µL

Table 5: Preparation of loading medium for one plate of adherent cells, using the manual method.

Step 1 Aspirate all of the growth medium out of the wells, being careful to avoid disturbing the cells in the area read by the FLIPR system.

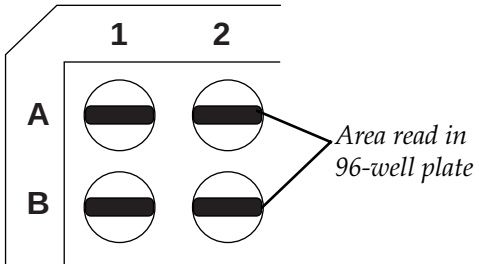

Figure 2: The region in a microplate well read for data in a 96-well plate.

Step 2 Dispense the loading medium into each well. The recommended volumes are 90 µL for a 96-well plate and 35 µL for a 384-well plate.

Step 3 Cover the plate, then incubate in a 5% CO_2, 37°C incubator for 45 minutes.

PREPARING FOR THE ACID LOAD OF THE CELLS

The acid load solution is a 200 mM NH_4Cl solution (10X concentration).

Preparing the acid load solution (buffer/200 mM NH_4Cl)

Step 1 Prepare a stock in H_2O: 535 mg NH_4Cl + 10 mL H_2O = 1 M stock

Step 2 Dilute this stock in buffer to make the 200 mM NH_4Cl acid load solution:

1 40 mL Hank's BSS 1X

2 800 µL HEPES (1 M)

3 10 mL (1 M) NH_4Cl stock. New stock concentration is 200 mM.

Optional, if required by your cells:

4 *400 µL (250 mM) probenecid.*

Preparing for the acid loading of the cells

	96-well plate	384-well plate
Volume during dye loading	90 µL/well	35 µL/well
NH_4Cl 200 mM added	10 µL/well	5 µL/well

Table 6: Recommended volumes during dye loading and acid load.

Step 1 Approximately 45 minutes after the beginning of the dye loading incubation, add the 200 mM NH_4Cl solution to all wells: 10 µL for a 96-well plate and 5 µL for a 384-well plate. The final concentration of NH_4Cl is 20 mM.

Step 2 Transfer the plate to the 37°C incubator for an additional 15-20 minutes.

PREPARE THE COMPOUND PLATE

Preparing the compound diluent

The compound plate should be prepared during the dye loading incubation. The compound diluent **does not** contain NH_4Cl.

For 100 mL compound diluent:

1 100 mL Hank's BSS 1X (without phenol red).

2 2 mL (1 M) HEPES.

Optional, if required by your cells:

3 *1 mL (250 mM) probenecid.*

Note: If compounds are proteins or peptides, it may be necessary to add 0.1%BSA to the wash and the compound diluent buffer to prevent the compounds from sticking to the plastic of the plate.

Preparing the compounds

The compound concentration is determined by the necessity to dilute out the NH_4Cl in the wells. Therefore, compounds are prepared at a 1.25X concentration.

WASHING THE CELLS

Preparing the wash buffer (buffer/20 mM NH_4Cl)

For 500 mL wash buffer containing 20 mM NH_4Cl (1X concentration), enough for two to three 96- or 384-well plates:

1 500 mL Hank's BSS 1X (without phenol red).

2 10 mL (1 M) HEPES

3 50 mL NH_4Cl 200 mM (final concentration 20 mM)

Optional, if required by your cells:

4 *5 mL (250 mM) probenecid.*

Washing the cells

Step 1 You will need approximately 150 mL/96-well plate or 200 mL/384-well plate of the wash buffer. Dead volumes for cell washers vary and must be determined by the user for their cell washer.

Step 2 Wash the cells three to four times with the cell washer. Use a "gentle" setting if the cells are weakly adherent. Leave a defined, consistent volume of fluid covering the cells after the last wash. For the intracellular pH assay, the residual volume should be as small as possible to allow a large volume to be added on the FLIPR system to dilute out NH_4Cl. The recommended volume ratio is 4:1 (4 parts of volume added on FLIPR to 1 part of volume in cell plate). Some washers do not permit such a small volume; you may also leave a large volume after the wash, and manually pipette out of the wells a defined volume to reduce the final volume after the wash.

	96-well plate	384-well plate
Final volume after wash	50 µL/well	15 µL/well
Volume added on the FLIPR	200 µL/well	60 µL/well

Table 7: Recommended volumes in assay.

RUNNING THE ASSAY

Instrument settings

Start with the laser power set at 0.300 Watts for the FLIPR I system, 0.600 Watts for the FLIPR^{384} system, the exposure time set to 0.4 seconds, and a camera F-stop of F/4 or 5.6. At the beginning of a data run, perform a "signal test" to check the basal fluorescence signal from the dye-loaded cells. If it is not between 40,000 to 50,000 counts above background, adjust it by modifying the exposure time, the camera F-stop, and the laser power.

EXAMPLE OF INTRACELLULAR PH ASSAY SETUP PARAMETERS

The following FLIPR system setup parameters are for an assay using CHO cells and BCECF:

Experiment Setup Dialog Box	Settings for 96-well Plates	Settings for 384-well Plates
General Tab		
Residual Volume in Cell Plate	50 µL	15 µL
Compound Concentration	1.25X	1.25X
Exposure Length	0.4 sec	0.4 sec
Presoak Tips	None	None
Multiple Sequences	Neither activated	Neither activated
Filter #	1	1
Automation	All n/a	All n/a
First Sequence	First interval: Sample Interval = 1.0 sec Sample Count = 60	First interval: Sample Interval = 1.0 sec Sample Count = 60
	Second interval: Sample Interval = 3.0 sec Sample Count = 20	Second interval: Sample Interval = 3.0 sec Sample Count = 20
Fluid Addition Tab		
Active	Yes	Yes
Fluid Volume	200 µL	60 µL
After Sample	10	10
Pipettor Height	170 µL	40 µL
Dispense Speed	60 µL/sec	20 µL/sec
Mix After Addition	No	No
Add From	Right Tray	Position 2
Pipetting Tab		
Mixing Volume	n/a	n/a
Number of Mix Cycles	n/a	n/a
Tip Positioning	Leave in well	Leave in well
Remove Fluid After Addition	No	No

Appendix A: Improving confluence and/or adherence of weakly adherent cells

If you are working with a cell line that doesn't grow uniformly on microplates, or that adheres weakly, coating the bottom of the cell plate with a matrix (i.e, poly D-lysine, laminin, fibronectin, gelatin, etc.) may improve the homogeneity of the monolayer and may help weakly adherent cells to remain attached to the plate through the washing process. Sample procedures for poly D-lysine, laminin, and collagen follow.

a) Poly D-lysine Coating (or Becton Dickinson part # 354640)

Step 1 Prepare a sterile, 100 µg/mL solution of poly D-lysine (Sigma Chemical Co., catalog # P7280) in tissue culture grade water. You will need approximately 8 mL per 384-well plate or 5 mL per 96-well plate to be coated.

Step 2 Working in a tissue culture hood, aliquot approximately 20 µL into a 384-well plate or 50 µL into a 96-well plate, then leave the plate(s) in the hood for 30 minutes.

Step 3 Aspirate off the poly D-lysine with a Pasteur pipet, then rinse the plate once with sterile water: 100 µL/well for a 384-well plate or 200 µL /well for a 96-well plate. Aspirate off the water.

Step 4 Allow the plates to dry in the hood before use.

b) Laminin Coating

Step 1 Prepare a sterile, 66.7 µg/mL (500 µg/7.5 mL) solution of laminin (Sigma Chemical Co., catalog # L-6274) in Hank's BSS. You will need approximately 8 mL per 384-well plate or 5 mL per 96-well plate to be coated.

Step 2 Working in a tissue culture hood, aliquot approximately 20 µL/well for a 384-well plate or 50 µL /well for a 96-well plate, then leave the plate(s) in the hood for 30 minutes.

Step 3 Aspirate off the laminin with a Pasteur pipet.

Step 4 Allow the plates to dry in the hood before use.

c) Collagen Coating (or Becton Dickinson part # 354649)

Step 1 Prepare a sterile, 0.3 mg/mL solution of Collagen (Vitrogen 100, from Collagen Biomedical, Palo Alto, California) in sterile 0.01 N HCl. You will need approximately 8 mL per 384-well plat or 5 mL per 96-well plate to be coated.

Step 2 Working in a tissue culture hood, aliquot approximately 20 µL /well for a 384-well plate or 50 µL for a 96-well plate, then leave the plate(s) in the hood overnight.

Step 3 Aspirate off the collagen with a Pasteur pipet.

Step 4 The next day, rinse once with sterile PBS 1X to neutralize the pH.

Appendix B: List of consumables

Description	Suggested Supplier	Item number
Black wall plates, clear bottom, tissue culture treated, sterile, 96-well	Corning/Costar	3603
	Packard Instrument	6005182
Black wall plates, clear bottom, tissue culture treated, sterile, 384-well	Corning/Costar	3712
	Greiner/distributor E&K	781092
Nunc V-bottom 96 well plate	Fisher	12-565-216 Nunc part #249128
Non-sterile lids for Nunc plates	PGC Scientific	5-6112-21
Clear plate, 384 well, for compounds, with lid	Corning/Costar	3702
	Greiner/distributor E& K	781186
FLIPR pipet tips, black, non-sterile (for experiments), 96-well	Molecular Devices	9000-0240
FLIPR pipet tips, black, non-sterile (for experiments), 384 well	Molecular Devices	9000-0258
Aspirator manifold (12 pin)	Wheaton Science Products	851388
Aspirator manifold (8 pin)	Wheaton Science Products	851381
Hank's Balanced Salt Solution (10X solution)	Gibco	14065-056
HEPES buffer solution 1 M	Irvine Scientific	9319
Probenecid, crystalline	Sigma	P8761
Pluronic acid 20% solution	Molecular Probes	P-3000
BCECF AM ester for intracellular pH assay	Molecular Probes	B-1150
DMSO, low water content	Sigma	D2650