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TITLE:

Real-time Flow Cytometry to Investigate Calcium Influx, Pore Formation, and Phagocytic Function of P2X7 Receptors in Live Adult Neural Progenitor Cells

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P2X7 receptors, flow cytometry, calcium, purinergic signaling, phagocytosis, pore formation, neural progenitor cells

SUMMARY:
Providing single-cell sensitivity, real-time flow cytometry is uniquely suited to quantify multimodal receptor functions of live cultures. Using adult neural progenitor cells, the P2X7 receptor function was assessed via calcium influx detected by calcium indicator dye, transmembrane pore formation by ethidium bromide uptake, and phagocytosis using fluorescent latex beads.

ABSTRACT:

Live-cell flow cytometry is increasingly used among cell biologists to quantify biological processes in a living cell culture. This protocol describes a method whereby live-cell flow cytometry is extended upon to analyze the multiple functions of P2X7 receptor activation in real-time. Using a time module installed on a flow cytometer, live-cell functionality can be assessed and plotted over a given time period to explore the kinetics of calcium influx, transmembrane pore formation, and phagocytosis. This simple method is advantageous as all three canonical functions of the P2X7 receptor can be assessed using one machine, and the gathered data plotted over time provides information on the entire live-cell population rather than single-cell recordings often obtained using technically challenging patch-clamp methods. Calcium influx experiments use a calcium indicator dye, while P2X7 pore formation assays rely on ethidium bromide being allowed to pass through the transmembrane pore formed upon high agonist concentrations. Yellow-green (YG) latex beads are utilized to measure phagocytosis. Specific agonists and antagonists are applied to investigate the effects of P2X7 receptor activity. Individually, these methods can be modified to provide quantitative data on any number of calcium channels and purinergic and scavenger receptors. Taken together, they highlight how the use of real-time live-cell flow cytometry is a rapid, cost-effective, reproducible, and quantifiable method to investigate P2X7 receptor function.

INTRODUCTION:

The study of purinergic signaling is broad and multifaceted, involving cellular physiology, biochemistry, and pharmacology. Purinergic signaling is involved in an infinite number of cellular and molecular processes, from cancer and cell cycle regulation to cell-cell communications and stem cell biology; as such, there often exists a potential to modulate purinergic signaling for a therapeutic benefit. Of the purinergic receptors, P2X7 receptor has received significant attention in recent years due to its potential as a therapeutic target for numerous inflammatory conditions[1]. Methods to study the receptor have evolved and been adapted over the years to facilitate this research[2–5]. Here, we describe a live-cell flow cytometry method to investigate the multiple functions of P2X7 receptors in adult neural progenitor cells derived from the subventricular zone (SVZ) and the hippocampal dentate gyrus.

The P2X7 receptor was first described as the P2Z receptor, or the 'cell death' receptor, as its activation with high concentrations of adenosine triphosphate (ATP) results in the formation of a large transmembrane pore permeable to molecules up to 900 Da[6]. This leads to cytoskeletal rearrangement, transmembrane pore formation, and, potentially, apoptosis and/or necrosis[7]. Traditionally, this function of P2X7 is quantified by the uptake of large molecular weight dyes such as YO-PRO-1 or ethidium bromide, which fluoresce when intercalated with DNA[3,8]. Plate reader methods, which are rapid and allow for upscaling, generally do not allow for the observation of kinetics. The method described here is based on ethidium uptake and allows the fluorescence increase to be observed over time, providing a greater depth of information with regard to the speed of pore formation. P2X7 receptors have since been shown to facilitate a number of nonimmune functions, with distinct responses depending on exposure time and agonist concentration[9,10]. Brief activation by lower concentrations of ATP results in cation influx for the purposes of neurotransmitter and signal transduction[11]. Using flow cytometry to measure calcium influx overcomes the problems associated with cumbersome and technically challenging methods—particularly, patch clamping to measure inward currents which provide invaluable details as to the change in

potential across a cell membrane but do not allow for population analysis[2]. The third function of P2X7 receptors occurs in the absence of ATP, where P2X7 receptors have been demonstrated to facilitate phagocytosis in both the immune system and the nervous system[9,12,13]. Advancements in microscopy techniques have allowed the visualization of cytoskeletal rearrangements during the uptake process, although quantification and population analysis can still present a challenge.

The live-cell flow cytometry method detailed here allows for the investigation of all three main functions of P2X7 receptors in real-time. The inclusion of a time module device on the flow cytometer allows temperature control and continual stirring of cells in suspension. Agonist and antagonist stimuli can be delivered within a second, allowing the near uninterrupted measurement of the cellular response. This presents a rapid and simple method to reproducibly quantify function while avoiding the use of multiple assay systems. It is important to note that this protocol may easily be adapted to suit any cell type and could be used to examine other receptor subtypes given the inclusion of specific agonists or inhibitors, depending on their properties.

PROTOCOL:

Animals were treated in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved for use by the Griffith University Animal Ethics committee.

1. Neurosphere culture of neural progenitor cells from the adult SVZ and hippocampus

NOTE: The dissection protocol presented here is based on work by Walker and Kempermann, and a detailed protocol for the dissection of neural progenitor cells from adult mice is available elsewhere[14]. Culture conditions have been modified from Babu and colleagues[15]. Adult female C57BL/6 mice aged 8–12 weeks were used.

1.1. In a biological safety cabinet, prepare the culture medium (neural basal medium) supplemented with neural stem cell proliferation supplement, 2 mM glutamine, 20 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), and 2 µg/mL heparin.

1.2. Euthanize two mice by CO_2 inhalation. Alternatively, anesthetize the mice according to institutional guidelines and immediately euthanize them by cervical dislocation. Spray their heads with 70% ethanol and decapitate them. Transfer each head to a sterile tube containing Hank's balanced salt solution (HBSS) with 1x penicillin/streptomycin solution (P/S).

1.3. Perform dissections in a laminar flow hood under a dissection microscope.

1.4. Using forceps and dissection scissors, remove tissue and bone to expose the brain, and transfer it to a sterile dish containing HBSS with P/S.

1.5. Position the brain ventral side up and use a scalpel blade to make a complete coronal incision across the optic chiasm. Hold the brain steady with the forceps and use one swift,

clean, downward motion. Avoid sawing to minimize cell death and to help maintain tissue structure.

NOTE: If not held securely, the brain may tilt forward during the cut, compromising the number of progenitor cells obtained from the SVZ.

1.6. Isolate the SVZ from the rostral half of the brain.

1.6.1. Locate both ventricles, separated by the septum, with the corpus callosum forming a white bridge above them.

1.6.2. Use forceps to remove the septum separating the two ventricles and isolate the lateral walls of the anterior lateral ventricles. Do this by cutting away above, below, to the sides, and at the front with fine dissection scissors. There will remain just a small cup-like shape.

1.6.3. Prepare a clean Petri dish lid with a few drops of HBSS in the middle and transfer the SVZ to the liquid. Do not allow the tissue to dry or to touch a dry surface. Stand to the side while dissecting the hippocampi.

1.7. Isolate the hippocampi from the caudal half of the brain.

1.7.1. Make a midline cut between the hemispheres to sever the corpus callosum. Use the lateral ventricles as a guide and gently unfold the cortex to expose the hippocampus. Once the cortex has been unrolled, the hippocampus can be seen as a dense, white, curved structure.

1.7.2. Use fine dissection scissors or forceps to isolate the hippocampus from the neighboring tissue.

1.7.3. With forceps, remove excess white matter, blood vessels, and any membranous tissue covering the hippocampus.

1.7.4. Prepare a clean Petri dish lid prepared with a few drops of HBSS and transfer the hippocampus to the liquid. Repeat for the other hemisphere.

1.8. Once the hippocampus and the SVZ from two mice have been isolated and transferred to their respective Petri dish lids, mechanically dice the tissue using a scalpel blade. Chop the tissue in one Petri dish lid for approximately 1 min, rotating every 10 s until the tissue appears smooth and only fine pieces remain. Take a clean scalpel blade and repeat for the other dish, dicing the tissue for 1 min.

1.9. Using a 1 mL pipette, transfer all the tissue from each Petri dish lid to separate tubes containing 1 mL of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA). Use one tube for the SVZ and one tube for the hippocampus.

1.9.1. Do this by first pipetting about half of the trypsin-EDTA to the tissue in the dish to minimize air bubbles and to prevent the tissue coming into contact with the dry pipette tip as

it is transferred to the tube. Rinse the dish and the scalpel blade with the rest of the trypsin-EDTA to collect as much tissue as possible and add it to the tube.

1.10. Incubate the tissue with the trypsin in a 37 °C water bath for 30 min, agitating the tube every 10 min to properly dissociate the tissue.

1.11. Triturate the tissue using a fire-polished glass Pasteur pipette to create a single cell suspension. Take care not to overtriturate as this will cause excessive cell lysis. This is a critical step for optimal tissue dissociation.

1.12. Observe the tube contents during the trituration process and stop at the earliest signs that the majority of tissue clumps have been removed. The trypsin solution should become slightly cloudy when the cells have gone into single-cell suspension, although some clumps may remain.

NOTE: As an indication, passing the suspension up and down 10x–15x is adequate.

1.13. Add culture medium up to 5 mL to neutralize the trypsin, and spin at 300 x *g* for 3 min. Wash a further 2x with HBSS and pass the medium through a 70 µm cell strainer to remove any tissue clumps.

1.14. Transfer all the cells in 15 mL of medium to a T75 cell culture flask.

NOTE: Spheres should form in the passage zero (P0) SVZ culture after 7–10 days and after 15–20 days in the hippocampal culture. Refrain from washing or feeding the cells during this time to maximize the number of neural progenitor cells in culture. If more progenitors are required, or to decrease the P0 incubation time, it is possible to increase the number of mice sacrificed.

1.15. Maintain cultures at 37 °C with 5% CO_2 and, following the initial P0 culture phase, passage every 7–10 days as necessary, using a biological safety cabinet and standard tissue culture practices.

NOTE: The P0 hippocampal culture should generate enough spheres to passage into a T25 flask; the SVZ culture will generate many more spheres and can be passaged into a T75. If the P0 hippocampal spheres have adhered, use a 200 µL pipette to gently lift the sphere.

1.16. Passage the spheres when they reach 150 to 200 µm in diameter. Collect the spheres and medium in a 15 mL tube and allow the spheres to settle by gravity for approximately 5 min. Alternatively, spin at a low speed (100 x *g*) for 2 min.

1.17. Remove the medium and dissociate the cells with dissociation reagent for 7–10 min, depending on the size of the spheres.

NOTE: The dissociation reagent used will have significant effects on the outcome of the culture, so please refer to the **Table of Materials** for specifics.

1.18. Wash the cells by adding 5 mL of HBSS to the cell solution and centrifuge at 300 x *g* for 3 min. Remove the supernatant, resuspend the cells in culture medium, and seed at approximately 150,000 cells in 5 mL of medium in a T25 cell culture flask or equivalent. Maintain the cultures at 37 °C and 5% CO_2.

1.19. Confirm neural progenitor cell status by identifying the expression of stem cell markers such as Sox2 and ASCL1 before proceeding to any downstream protocol[9].

NOTE: This can be done by the researcher's preferred method (e.g., immunochemistry by microscopy, flow cytometry or western blot, or by using quantitative polymerase chain reaction (qPCR)).

2. Preparation of a single-cell suspension for analysis by flow cytometry

2.1. Prepare the required media. These include the following.

2.1.1. Prepare **Na^+ medium** containing 145 mM NaCl, 5 mM KOH, 10 mM HEPES, 5 mM D-glucose, 0.1% bovine serum albumin (BSA), and 0.1 mM $CaCl_2$. This medium is also used in a calcium-free form, with 0.1 mM $CaCl_2$ omitted.

2.1.2. Prepare **K^+ medium** containing 145 mM KCl, 5 mM KOH, 10 mM HEPES, 5 mM D-glucose, and 0.1% BSA.

NOTE: These media were extensively optimized and are detailed in previous publications[4,5].

2.1.3. Prepare **Stock ATP** by weighing enough ATP powder for around 20 mL of a 100 mM stock. Dissolve the powder in 17 mL of KCl buffer (145 mM KCl, 5 mM KOH, and 10 mM HEPES, pH 7.5) and slowly, while stirring, add 2 mL of 18% (w/v) tetramethylammonium hydroxide (TMA) to the solution to bring the pH to 6.8–7.0.

2.1.4. Adjust the final volume to 20 mL. Do not overshoot the pH past 7.0. Store the stock at -80 °C; note that the ATP aliquots are stable for at least 6 months.

NOTE: The free molecular weight of anhydrous ATP is 551.14 g/mol, and this does not include the molecular weight of the water and disodium molecules, which may vary from batch to batch and should be taken into consideration for the calculations. TMA is toxic, and care must be taken when handling. Read the safety data sheet and prepare the stocks in a fume cabinet.

2.1.5. Prepare **stock BzATP** by dissolving the BzATP in ultrapure H_2O for a final stock concentration of 10 mM.

2.2. Create a single-cell suspension as described in steps 1.16 and 1.17. Count the cells using a hemocytometer or automatic cell counter. Resuspend the cells in the required medium (e.g., Na^+ medium, culture medium) for the experiment being conducted (as described below) and place the cells on ice in the meantime.

2.3. For each experiment, ensure there is enough sample to include controls for forward and side scatter, as well as for the calibration of voltage and compensation settings. Note, as an indication, that spheres grown in a T75 flask typically yield around 8 x 10^6 cells per flask.

2.4. Set the flow cytometer settings using control samples.

2.4.1. Use forward and side scatter to selectively gate the living cells.

NOTE: Forward scatter provides information regarding cell size based on light diffraction, while side scatter provides a measure of internal complexity or granularity. Flow events with small forward and side scatter may be considered as dead cells.

2.4.2. Adjust the voltage and gain of the flow cytometer according to the manufacturer's instructions. Run a trial sample to ensure the capture of data at the maximum fluorescence intensity. No compensation is required for single channel acquisitions.

3. Measuring calcium influx by live-cell flow cytometry

3.1. Following the preparation of a single-cell suspension, resuspend the cells in 1 mL of calcium-free Na^+ medium and load them with 2 ng/mL of calcium indicator dye according to the manufacturer's protocol (refer to the **Table of Materials**) with 10 µL of 5% pluronic acid. Incubate the cells for 30 min at 37 °C.

3.2. Wash the cells by adding 3–5 mL of calcium-free Na^+ medium and centrifuging gently (200 x *g* for 4 min). Remove the supernatant and resuspend the cells in calcium-free Na^+ medium, washing a second time.

3.3. Resuspend the cells in 1 mL of calcium-free Na^+ medium, place them on ice, and allow them to de-esterify for 30 min.

3.4. Wash 1x more by adding 3–5 mL of K^+ medium and centrifuging (200 x *g* for 4 min); then, resuspend the cells in K^+ medium and aliquot them into fluorescence assisted cell sorting (FACS) tubes at a concentration of 1 x 10^6 cells per 500 µL per FACS tube.

NOTE: The number of FACS tubes per sample will depend on the number of treatments and repeats that are included.

3.5. Place the FACS tubes on ice until the cells are ready to be analyzed. Do not leave the cells on ice for an extended period but begin the assay as soon as possible.

3.6. For some samples, preincubate the cells with P2X7 receptor-specific inhibitor AZ10606120 (1 µM for 10–15 min) or A438097 (10 µM for 30 min) at 37 °C prior to analysis.

3.7. A few minutes prior to running the first sample, add $CaCl_2$ (to a final concentration of 1 mM in the FACS tube) and place the tube in a 37 °C water bath to recover.

3.8. Drop a clean, small magnetic stirrer into the FACS tube and position the tube in the time module linked to a circulating 37 °C water bath to control the sample temperature. Select a low stirring speed to ensure movement of the sample without introducing a vortex effect. Place the water jacket tube adapter onto the sample platform and close the lever arm of the FACS machine.

3.9. Initiate sample acquisition and run the sample for 3 min at around 1,000 events per second.

3.10. At the 40 s mark, quickly remove the tube and add the P2X7 agonist, either 1 mM ATP or 300 µM BzATP, and replace the tube to continue the acquisition.

3.11. While the first sample is recording, prepare the second sample with $CaCl_2$ and place it in 37 °C to allow sufficient time for the cells to warm up prior to analysis. Once the first sample has finished, clean the intake by running a water sample, and then the acquisition of the second sample can begin as described in steps 3.8 and 3.9. Always clean the intake between samples.

4. Measuring pore formation by live-cell flow cytometry

4.1. Create a single-cell suspension as described in steps 1.16 and 1.17. Save a few milliliters of the old medium, use it to resuspend the cells at a concentration of 1 x 10^6 cells per 100 µL per FACS tube, and place it on ice until ready.

4.2. Prior to running the assay, add 900 µL of K^+ medium for a final volume of 1 mL and place the tubes in a 37 °C water bath for 10 min to recover.

4.3. If applicable, preincubate the cells with treatments, including the P2X7-specific inhibitors AZ10606120 (1 µM for 10–15 min) or A438097 (10 µM for 30 min).

4.4. Immediately prior to running the assay, add 25 µM ethidium bromide to the FACS tube; then, add the magnetic stirrer, place it on the FACS machine according to step 3.7, and begin the acquisition.

NOTE: Ethidium bromide is toxic, and care must be taken when handling it. Dispose of used FACS tubes appropriately.

4.5. To induce the formation of pores in the cell membrane, add 1 mM ATP or 100 µM BzATP 40 s after the start of the acquisition.

4.6. Run the samples at around 1,000 events per second for 6 min.

4.7. While the first sample is running, take the second sample from the ice and place it in the 37 °C water bath to allow sufficient time for the cells to recover prior to the analysis. Once the first sample has finished with the acquisition, clean the intake by running a water sample; then, the second sample can be placed on the machine to begin recording as described in steps 3.8 and 3.9.

5. Measuring phagocytosis by live-cell flow cytometry

5.1. Create a single-cell suspension as described in steps 1.16 and 1.17. Resuspend the cells in conditioned medium and aliquot them into FACS tubes at a concentration of at least 1 x 10^6 cells per 100 µL per FACS tube. Dilute the cells to a final concentration of 1 x 10^6 cells/mL with Na^+ medium (e.g., add 900 µL of Na^+ medium) and place the cells on ice until the analysis is performed.

5.2. Use 1 µm of YG latex beads (microspheres) as phagocytic targets for real-time phagocytosis assays.

NOTE: Other colors may also be substituted, but differently sized beads were found to be inadequate targets of phagocytosis[4].

5.3. Prior to running the first sample, transfer the cells to a 37 °C water bath and incubate them for approximately 7–10 min to allow the cells to recover.

5.4. Add any treatments requiring preincubation to their respective tubes, including 1 mM ATP for 15 min, 300 µM oxidized ATP (oxATP) for 40 min, 20 µM cytochalasin D for 20 min, and 4% paraformaldehyde (PFA) for 20 min.

5.4.1. No preincubation is required for 5% human serum. If treatments are added at approximately the same time, the samples can be run in reverse order while the others continue to incubate. For example, run the controls and serum first, then the ATP-treated sample, followed by cytochalasin D and PFA, and oxATP last.

5.5. Place the sample on the cytometer with the magnetic stirrer as described in steps 3.8 and 3.9 and, then, initiate sample acquisition.

5.6. Remove the sample tube from the machine 15–20 s after the start of the acquisition, and add 5 µL of undiluted YG beads. Return the sample FACS tube and continue the acquisition. Run the samples for 7–8 min at around 1,000 events per second.

5.7. While the first sample is running, take the second sample from the ice and place it in a 37 °C water bath to allow sufficient time for the cells to recover prior to analysis. Once the first sample is finished, clean the intake by running a water sample and, then, begin acquisition on the second sample as described in steps 3.8 and 3.9.

6. Data analysis

6.1. Export the data to a spreadsheet. The data analysis will depend on the experimental question.

NOTE: Be aware that different runs may have different baseline intensities, so it is important to run the assay for a designated time at the start (around 40 s) prior to adding any agonists

and to normalize the data by calculating the change in fluorescence (the fluorescence at any given time point [F] divided by the fluorescence at time point zero [F0], or F/F0).

6.2. To quantify the rate or kinetics of the P2X7 function in question, calculate the area under the curve or the sum of the trapezoids created under the curve for each 10 s time period[16].

6.3. To determine the effects of treatments, average the fluorescence intensity over the final 10–20 s of recording and compare the treatments. Determine the significance by *t*-test or analysis of variance.

REPRESENTATIVE RESULTS:

Neural progenitor cell cultures

Neural progenitor sphere cultures derived using this method should be phase bright and have a smooth round edge (**Figure 1A,B**). In healthy cultures, small microspikes can be observed on the edges (**Figure 1C**). At late passages, or if fed inadequately, spheres can form a hollow cup shape (**Figure 1D**) or large oblong shapes (**Figure 1E**, indicated by arrow). These cultures should not be used for flow cytometry or any other downstream applications, as these features it may be indicative of differentiation. To confirm the neural progenitor status, the cells were plated on glass coverslips coated with poly-L-ornithine and laminin for immunocytochemistry (**Figure 1F** and, at higher confluency, **Figure 1G**). Cells were stained for GFAP, nestin, Sox2, vimentin, ASCL1, BLBP, Prox1, and DCX to identify the cells as Type 2 progenitor cells (hippocampus) or Type C progenitor cells (SVZ)[9]. Cells should have a well-defined nucleus and extended processes.

[Place **Figure 1** here]

Calcium influx by live-cell flow cytometry

This protocol allows for the analysis of P2X7 receptor function as a calcium channel in real-time. The kinetics of receptor function, as well as the effects of different agonists and antagonists, can also be assessed. When plotted over time, calcium influx in the hippocampal and SVZ neural progenitor cells was generally similar (**Figure 2A** and **Figure 2B**, respectively). Agonists (either ATP or BzATP) were added at the 40 s mark, as indicated by the red arrow. For a brief moment, the tube is removed from the recording point to add the agonist, resulting in data points of zero. This will allow for the identification of the time when the agonist was added. BzATP rapidly activates P2X7 receptors, opening the ion channel and allowing calcium influx, which binds to Fluo-8 and fluoresces. ATP application generally results in a more gradual calcium influx. It has a lower affinity to P2X7 when compared to BzATP and will also result in G-protein-coupled receptor activation, a slower signaling pathway which releases calcium from the endoplasmic reticulum. The inclusion of P2X7 antagonists A438079 and AZ10606120 (data not shown) reduced the calcium influx in response to agonist application.

[Place **Figure 2** here]

Pore formation by live-cell flow cytometry

Transmembrane pore formation is a canonical feature of P2X7 receptors, results in macromolecule exchange, and can lead to cell death. Ethidium$^+$ is a large molecule (314 Da)

excluded from healthy cells; its uptake and subsequent intercalation with DNA results in fluorescent emissions and can be used to assess the ability of P2X7 receptors to form transmembrane pores. Following the application of the agonists ATP (1 mM ATP) and BzATP (100 µM) at the 40 s (indicated by the arrow), time-resolved flow cytometry captures the ethidium bromide entering the cells in real-time (**Figure 3A**). This effect was attenuated by the P2X7-specific inhibitor AZ10606120. The ethidium bromide uptake assay demonstrates a functional P2X7 receptor C-terminus[17] and is good evidence for full-length P2X7 receptor expression. ATP concentration-response assays illustrate the effects of agonist concentration on P2X7 pore formation, using change in ethidium bromide fluorescence over time (**Figure 3B**). Agonist dose concentration curves together with receptor-specific inhibitors provide strong evidence for receptor activation.

[Place **Figure 3** here]

Phagocytosis by live-cell flow cytometry

Our group has previously demonstrated that extracellular ATP inhibits P2X7-mediated phagocytosis by dissociating the P2X7 C-terminus from the cytoskeleton, specifically, nonmuscle myosin IIA[18,19]. This method expands on these findings to demonstrate P2X7 receptor involvement in phagocytosis by hippocampal and SVZ neural progenitor cells in real-time (**Figure 4**, an example of hippocampal phagocytosis). Uninhibited phagocytosis (control) levels of 1 µm YG latex beads were established as the positive control. ATP inhibited the phagocytosis of YG beads to the same extent as the nonspecific inhibitors, namely PFA fixation and the actin polymerization inhibitor cytochalasin D, while 5% serum abolished all innate phagocytosis[20].

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Representative hippocampal neural progenitor cell culture. (**A**) Hippocampal neural progenitor cells are isolated from adult mice and cultured as neurospheres until approximately 100 to 150 µm in diameter. (**B**) Neurospheres should have a smooth periphery, (**C**) and small microspikes may be observed on their surface. When spheres are too long in culture, they can form (**D**) cup or (**E**) oblong shapes. These cultures should not be used for experiments. To confirm the neural progenitor status of the cells, seed them as a single-cell suspension on poly-L-ornithine (PLO) and laminin-coated glass coverslips for immunochemistry. Cells should have a small soma and branching processes, (**F**) at low confluency and (**G**) ready for immunochemistry. The scale bars represent 100 µm.

Figure 2: Live-cell calcium influx in neural progenitor cells from the hippocampus and SVZ. P2X7 receptor calcium channel function was demonstrated in (**A**) hippocampal and (**B**) SVZ-derived progenitor cells by changes in Fluo-8 fluorescence. Application of the general P2X agonist ATP and the P2X7 agonist BzATP result in P2X7 ion channels opening, allowing calcium influx. The influx was blocked with the P2X7-specific inhibitors A438079 or AZ10606120 (data not shown). F = fluorescence; F0 = fluorescence at time point zero.

Figure 3: P2X7 transmembrane pore formation measured by ethidium uptake. The addition of ethidium bromide moments before the start of acquisition is used to measure the formation of P2X7 transmembrane pores. High concentrations of ATP and BzATP result in (**A**) P2X7 receptor pore formation, allowing ethidium bromide to enter the cell. The P2X7 inhibitor AZ10606120 attenuates this phenomenon and provides evidence for functional P2X7 receptors. (**B**) ATP concentration-response assays demonstrated significant pore formation at 500 µM and 1 mM but not at lower concentrations.

Figure 4: YG bead uptake demonstrating the phagocytic capacity of neural progenitors via P2X7 receptors. YG bead uptake by neural progenitor cells is observable using live-cell flow cytometry in real-time. Control levels of phagocytosis are established initially, and if the number of cells allows, reconfirmed at the end of the run. Involvement of P2X7 receptors is indicated by the inhibition of phagocytosis in the presence of ATP, as this dissociates the C-terminus from the membrane cytoskeleton, preventing P2X7-mediated cytoskeletal rearrangements. The application of ATP blocked phagocytosis to the same extent as the use of nonspecific inhibitors of phagocytosis, including paraformaldehyde (PFA) and cytochalasin D (CytD).

DISCUSSION:

This paper presents a detailed protocol for the analysis of P2X7 receptor function in neural progenitor cell cultures derived from the adult neurogenic niches. The potential applications for adult neural progenitor cells range from research to therapeutic purposes, and so the method of culture must be robust and reproducible. There are a number of key aspects to this protocol that may impact the quality of the endpoint culture. Once removed from the skull, the brain should not be allowed to dry and the dissection should be performed as quickly as possible. Particularly with the hippocampus, extra care taken to remove any blood vessels or membranous tissue will result in superior progenitor cell yields. The dissociation and trituration process can heavily impact the number of spheres obtained in a culture; agitating the tissue during the incubation with trypsin-EDTA will result in a more homogeneous solution. The use of a fire-polished glass pasture pipette over a P1000 plastic pipette tip is highly recommended to reduce cell death and improve the resulting culture. Avoid overtriturating. Despite these precautions, the procedure can create a lot of debris in the P0 culture, and to avoid losing progenitor cells, washing or feeding the culture should be avoided until spheres have formed.

A number of differences between the hippocampal and SVZ cultures will be obvious at P0. Hippocampal cultures yield fewer spheres, and these generally adhere. Use a pipette tip to gently lift off the spheres for the initial passage. Adherent spheres were not observed in subsequent passages. Different brands of tissue culture flasks may cause the spheres, particularly hippocampal spheres, to adhere and grow as colonies on the bottom of the dish. This was not found to alter any downstream results for this protocol but should be monitored, and consistency should be maintained where possible.

Previous methods used to measure P2X7 receptor function, such as patch clamping to record calcium influx, are time-consuming and laborious and may only provide information on a single cell. This protocol presents a rapid and reproducible method to analyze all three main functions of P2X7 receptors using one machine. Time-resolved live-cell flow cytometry allows

for whole population analysis and provides the researcher with information regarding the kinetics of calcium influx, pore formation, and/or phagocytic function. In addition to this, flow cytometry can be easily used as a method for assessing marker expression patterns and population analysis based on cell size or protein expression levels.

When conducting these experiments, differences in maximal calcium influx, ethidium uptake, or phagocytosis rates may be observed between repeats. To minimize this, the sphere size, culture conditions, and feeding regime must be consistent as the health of the cells will have a significant impact on the results obtained. The time on ice can also influence the data, so ensure everything is prepared ahead of time so that the time on ice is minimal. Ensure that the calcium indicator dye loading time is consistent. Another factor that may lead to large inconsistencies in the maximal calcium recordings is the variation between ATP batches. The preparation of ATP stocks is crucial, and the use of different batches for the same experiments should be avoided. Comparing old and new batches to ensure the ATP is consistent is also recommended. The effectiveness of P2X7 antagonists may also be cell-line- and batch-dependent, so optimization of incubation times and concentrations may be required.

It is worth noting that calcium influx/efflux is one of the most fundamental and complex cellular functions and can be mediated by many receptors. The ATP-induced calcium influx, as a classic measurement for P2X7 channel/pore function, may not accurately reflect the true function of P2X7 receptors, as ATP may also activate P2Y receptors to release intracellular calcium. In this case, barium may be a better cation to use instead of calcium as its influx is unidirectional[16]. To differentiate the contribution from P2Y receptors in calcium influx, conditions where 1 mM EDTA or ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) is added to the K^+ medium instead of $CaCl_2$ may be used in this assay.

This protocol may also be easily adapted to suit other cell types and can be useful for investigating the functionality of alternate ion channel receptors or receptors that participate in phagocytosis. This method may also be adapted to a flow cytometry machine without a time module. As an example, phagocytosis assays may be performed where YG beads are added 7–8 min prior to the analysis by conventional flow cytometry. Keep the cells at 37 °C and continuously swirl them. This will not provide real-time information but differences in the mean final fluorescence will still inform the researcher regarding the functionality of P2X7 receptors.

Interest in P2X7 receptors as a drug target[21,22] or even as a drug delivery route[23,24] is growing rapidly, and so methods to study this enigmatic receptor must be continuously adapted and improved upon to facilitate these studies. This protocol outlines methodologies that may be used to explore P2X7 function in adult neural progenitor cells, and it is hoped that achieving a greater understanding of P2X7 receptors in the neurogenic niches may lead to advancements in the treatment of stroke and other ischemic injuries.

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DISCLOSURES:

The authors have nothing to disclose.

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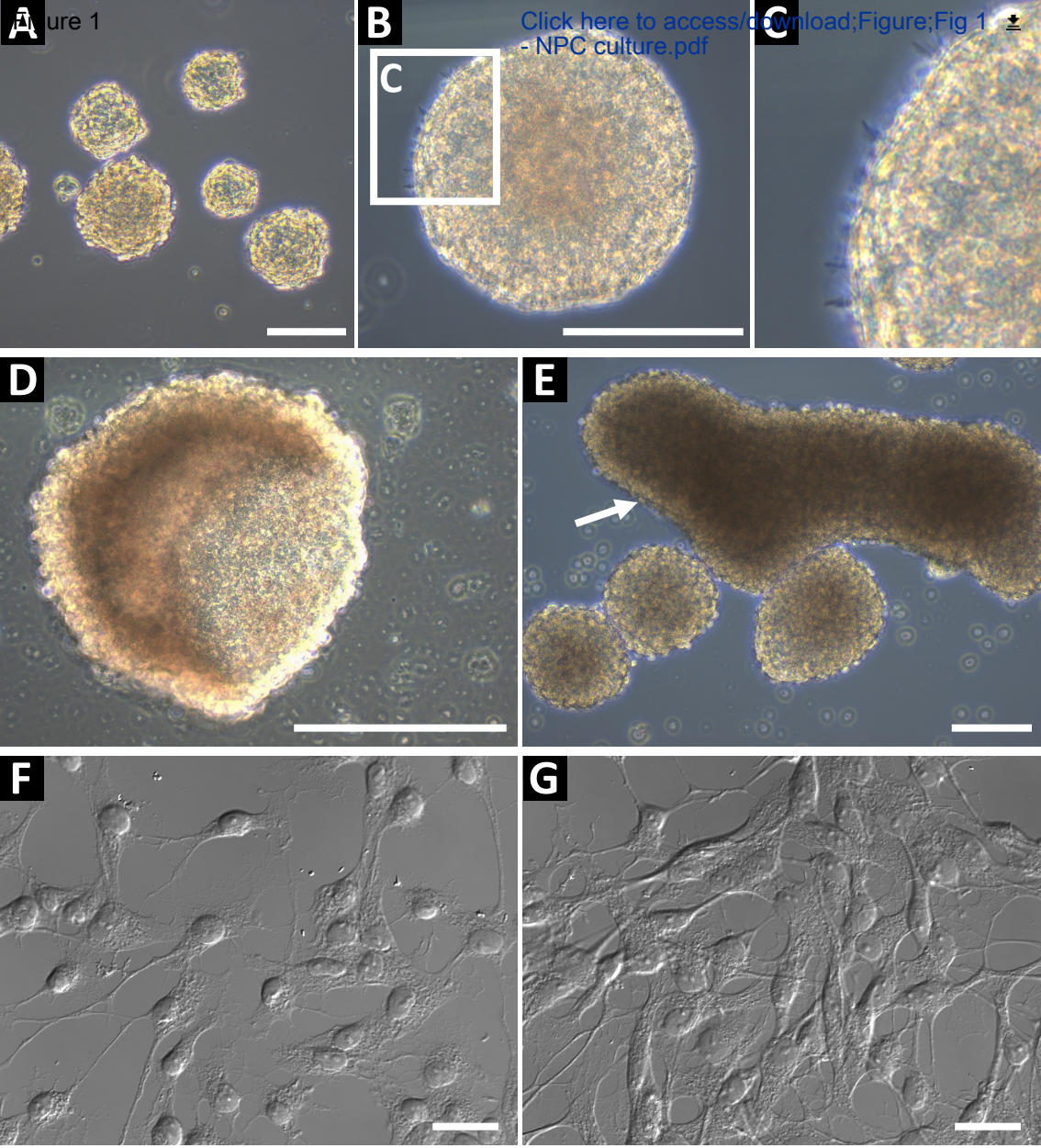
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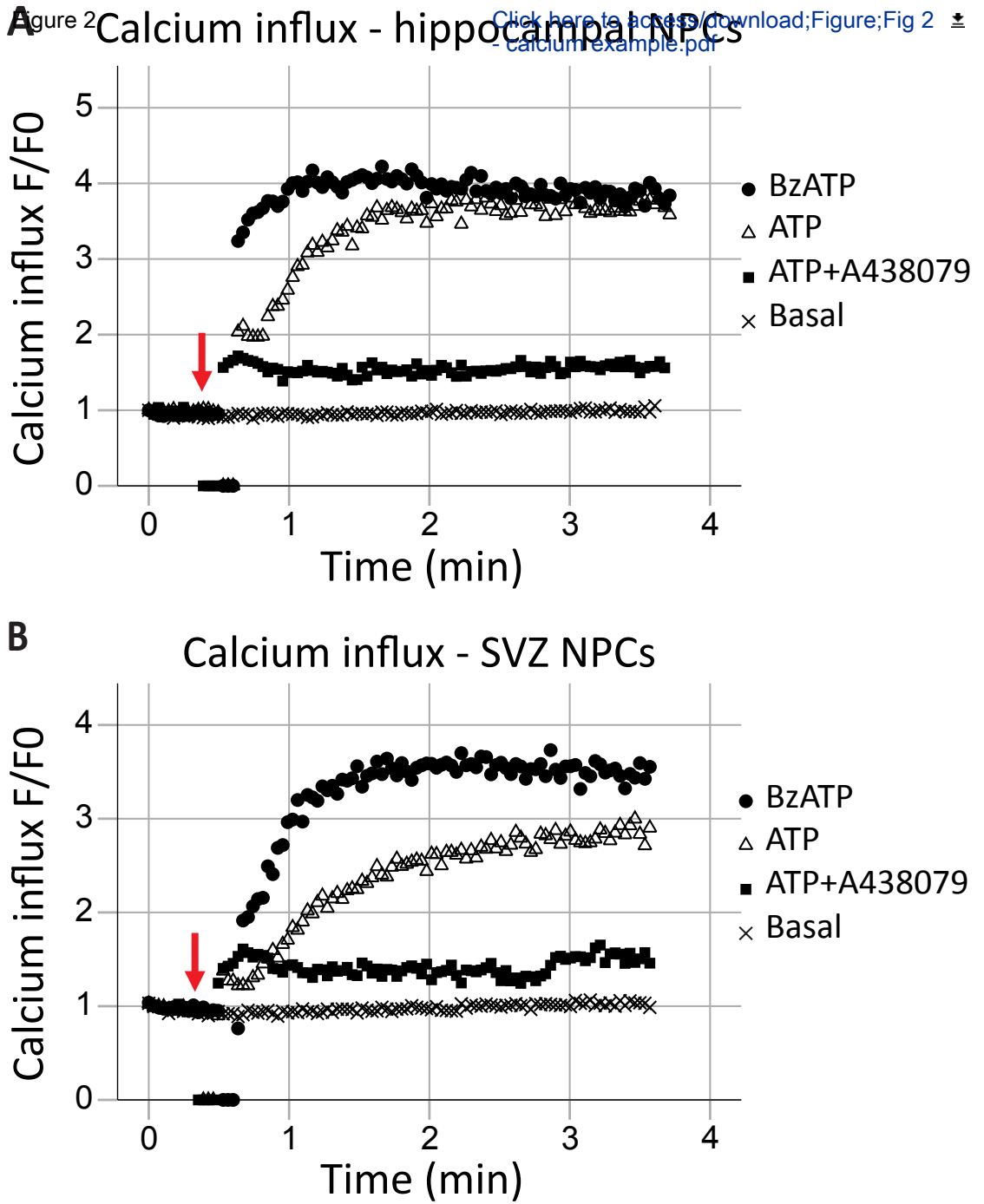

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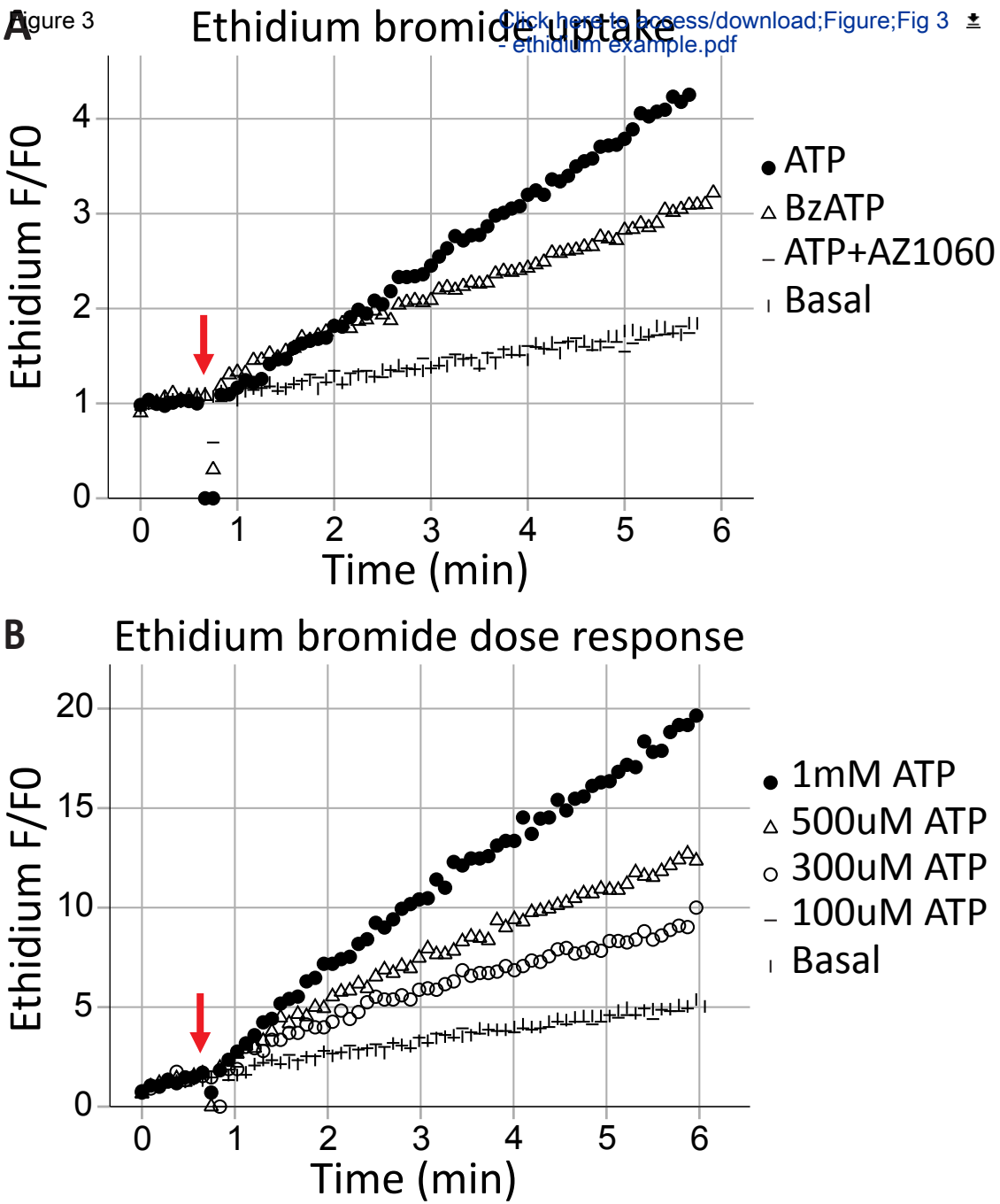
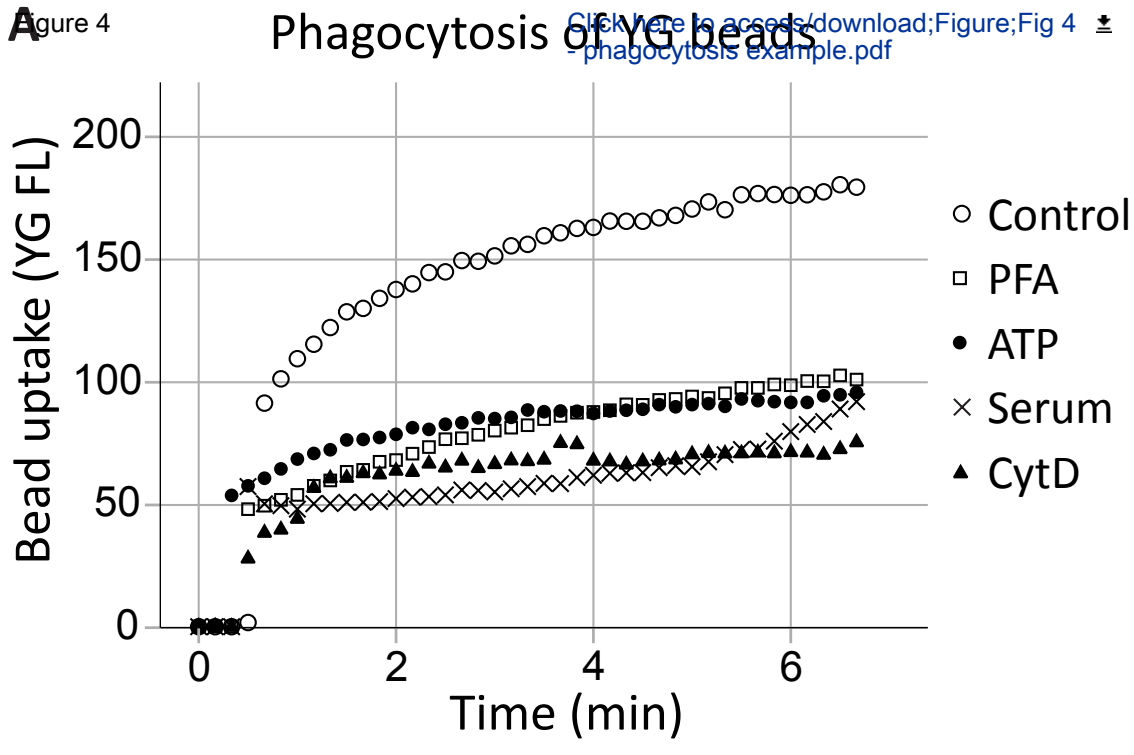


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
A438079	Tocris	2972/10	
ATP	Sigma-Aldrich	A2383	
AZ10606120	Tocris	3323/10	
bzATP	Sigma-Aldrich	B6396	
cytochalasin D	Sigma-Aldrich	C8273	
FACSCalibur	Becton Dickinson		
Fluo-8AM	AAT-Bioquest	21080	Fluo-4AM and Fura-Red AM have also been used successfully
Fluoresbrite YG Microspheres	Polysciences Inc	17154-10	1.00 µm, yellow-green
Glutamine	ThermoFisher Scientific	25030081	200 mM
HBSS	ThermoFisher Scientific	14170112	
heparin	Sigma-Aldrich	H3149	
NeuroCult Basal Medium	Stemcell Technologies	5700	Mouse and rat
NeuroCult Proliferation Supplement	Stemcell Technologies	5701	Mouse and rat
oxidized ATP	Sigma-Aldrich	A6779	
Pluronic F-127	Sigma-Aldrich	P2443	pluronic acid
Recombinant Murine EGF	Peprotech	315-09	

Recombinant Murine FGF-basic	Peprotech	450-33	
tetramethylammonium hydroxide	Sigma-Aldrich	T7505	
Time Zero Module	Cytek Biosciences		
Tissue culture flasks	BD Falcon (Corning)	353108 (T25), 353136 (T75)	Blue vented screw cap
TrypLE Express	Gibco	12604013	
Trypsin-EDTA (0.25%)	ThermoFisher Scientific	25200056	with phenol red
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Notes have been revised and either moved to discussion or included as their own method step.

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Steps revised significantly

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12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Steps for video have been highlighted.

15. Figures 2-4: Please indicate the time unit for the x-axis.

This has been corrected

16. Discussion: Please move lines 357-369 to the Discussion section. Please also discuss critical steps within the protocol.

Done. Some crucial steps have been added – though these were mainly the Notes that have been moved from the method to the discussion.

17. References: Please do not abbreviate journal titles.

This has been corrected

18. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors present a video script demonstrating time-resolved FACS measurements of P2X7 receptor-dependent changes of the intracellular Ca2+ concentration ([Ca2+]i), ethidium fluorescence and latex bead bead phagocytosis. It is potentially an interesting project but several questions remain.

Major Concerns:
I am not aware if there are already videos published showing time-resolved FACS measurements. In life science it is a rather standard method, especially the measurement of [Ca2+]i and the uptake of fluorescent dyes.

To the best of our knowledge, no video has been published showing time-resolved FACS measurements. Our manuscript with detailed protocol and video would contribute to this gap in the literature. In addition, there are very few reports detailing the use of adult neural progenitor cells for functional P2X7 analysis. This manuscript details their culture and use for P2X7 research, and hopefully will lead to further research being conducted in this specific field.

The kinetics of [Ca2+]i does not reflect the kinetics of receptor activation. The latter is much faster (within about 10 ms). [Ca2+]i change rather reflects the integral of the Ca2+ influx and is additionally dependent on membrane potential, buffering and other Ca2+ transport processes. A [Ca2+]i increase can even be absent although P2X7 receptors have been activated (see Loehn et al Cell calcium 29, Pippel et al. Cell Calcium 57).

One paragraph has been added in the Discussion to address this concern.

Furthermore, in several cells (as mentioned by the authors) P2Y receptors which release Ca from intracellular stores may become activated by ATP too. It should be mentioned how P2X7- and P2Y-dependent Ca2+ signals can be differentiated.

One paragraph has been added in the Discussion.

Is it verified that the introduced phagocytosis assay really reflects phagocytosis and not binding of the beads to the cells? The very fast increase of bead fluorescence under control conditions after addition of the beads is suspicious.

The nature of this real-time bead uptake method as a measurement of phagocytosis has been extensively studied. We found that bead uptake was completely abolished at low temperature (4^{o}C), at which phagocytosis is completely blocked. This indicates there is little adhesion of beads to cells in such constantly stirred condition. We have also found that 5-10% serum is able to completely block bead uptake at 37^{o}C in both a stirred condition and a static condition under microscopy (Gu et al, Blood, 2010; J Biol Chem, 2012). Thus, it is unlikely the rapidly increased cellular fluorescence intensity is due to

nonspecific binding of beads to the cell. In addition, inhibitors for phagocytosis, e.g. cytochalasin D and Latrunculin A, are used routinely to assess nonspecific binding. This is the method we have been applied for many years and we are very happy to share our knowledge with other researchers.

Minor Concerns:
What is the idea behind the complicated preparation of the P2X7 expressing neural progenitor cells? Why haven't macrophages been used? They are much easier to be prepared.

This manuscript aims to provide researchers the means to continue P2X7 research in adult neural progenitor cells, which have very different applications and clinical implications to macrophages. Macrophages may be substituted if the sole purpose of the research is to examine P2X7 receptor activity, though in macrophages this has already been established. The field is shifting to explore the function of these receptors in other cell types, and subsequently their potential as a therapeutic target.

In Figures 2 to 4, the time unit is not shown. Which extracellular solutions were used?

Unit has been added. The extracellular solutions are detailed in the method. Na^{2+} medium is used for the phagocytosis assays, while K^{+} medium is used for calcium and pore formation assays.

How is the application of definite concentrations of agonists or antagonists managed? The substances always become diluted by the amount of water in the cell suspension to which they are added.

The volume in the FACS tube is known, and the exact volume of a stock to add to a known volume to end up with the desired final concentration was calculated (C_1V_1 formula).

All abbreviations must be explained.

Addressed

Figure 3: Ethidium uptake takes place at 300 µM ATP, and not only at 500 and 1000 µM. 3B demonstrates concentration dependence and not does dependence.

The ethidium uptake occurring at 300 µM is not statistically significant. As JoVE required that representative results only are shown, repeats and data analysis calculations have not been included.

Fig. 4: Why oxATP was used as blocker (which may be harmful to cells) and not the more "modern" blockers like AZ10606120?

OxATP is the only inhibitor of P2X7 receptor which not only irreversibly blocks P2X7 channel/pore function but also inhibits its phagocytic function.

P6 L290: To determine significance between treatments, take an average of the fluorescence intensities over the final 10-20 seconds". This is an unclear statement.

Statement has been reworded

P1 L87: "Plate reader methods, which are rapid and allow for up-scale, do not allow for the observation of kinetics." Why not?

Most of plate readers do not have the adapter for agonist injection, constantly stirring and temperature control which are essential for real-time measurement. Additionally, the high background of each well can make it difficult to measure small changes.

Reviewer #2:

Manuscript Summary:

The technique proposed by the authors is quite simple, with an excellent cost benefit, although other works have even used flow cytometry to show the functionality of P2X7 receptor, especially in ethidium bromide incorporation or calcium signaling assays. However, none used in a combined way to show the three properties of the P2X7 receptor live., becoming a good tool for the study of this receptor in different cellular models.

The methodology of both neurospheres culture and preparation for flow cytometry is well detailed, but some informations are missing as stated below.

Minor Concerns:

1) During calcium influx assay, the authors used 1 mM of ATP or 300 µM BzATP which are known to be a high concentration of agonists and can induce pore formation. How could the authors guarantee that calcium influx is being mediated by P2X7 channel opening but not by pore formation? What would happen in calcium influx if lower concentrations of ATP and/or BzATP was used?

The P2X7 channel dilates in 20-30 sec in the presence of its agonist ATP or BzATP. Calcium influx can go through both the channel and pore formed by P2X7 receptors. Currently, there is no method which can separate the two processes. In practice, we only take consideration of the first 20 sec of calcium influx curve following the addition of ATP as an estimate of P2X7 channel function.

We have additionally observed calcium influx by live cell microscopy following applications of ATP as low as 0.1 µM, though as this additional method is not included here and is beyond the scope of this manuscript.

2) Please describe better YG latex beads: what is best particle size? Is there any specification for the beads? Are they diluted as manufacturer's protocol?

This information is provided in the protocol (1 µm beads are used). Optimal size of the latex bead was previously established and this reference is provided. Specifications (brand name) is provided in the table of materials.

3) I did not understand the NOTE in item 5.5: which is phagocyte:target ratio? The protocol said that was 1 x 10^6 cells/mL + 5 µL of 1 µm YG latex beads, isn't it?

This comment has been revised. For adult neural progenitor cells, 5 µL of YG beads is adequate, though this may need to be adjusted in other cell types.

4) Please, include the parameters of FACSCalibur Configuration and Settings such as filters, lasers and channels. Was done any compensation?

As JoVE is not able to publish brand names, the term 'FACS Calibur' has been removed, and discussion of the specifics of the flow machine is not applicable.

For the reviewers information, FACSCalibur comes with two lasers: 488nm blue laser and 635nm red diode laser. The voltage settings in different machine may differ. Our three methods only describe single colour usage therefore no compensation is needed. Ca^{2+} influx and YG bead uptake signals are collected in the FL1 (488) channel and ethidium uptake is collected in the FL2 channel, all in linear mode.

For Ca2+ influx: The FL3 voltage is adjusted to give a linear mean channel fluorescence intensity of ~700 for the gated population. No compensation is required between FL1 and FL3.
For ethidium uptake: The FL2 voltage is set at around 595V with a gain of 5.0, at which the linear mean channel fluorescence intensity for Quantum PE standard beads with MESF 300747 is 48±1 (256 linear scale) and the peak channel for right reference standard PE high level beads (MESF ~560,000) is 100±1 (256 linear scale). The compensation of FL1-FL2 and FL2-FL1 is 7% and 8% respectively.
For YG beads uptake: Linear MFI of YG fluorescence is collected in FL1 (voltage: 380-420, gain: 2.0).

5) Please specify more about the use of Time Zero Module (Cytek Biosciences).

As JoVE is not able to publish brand names, the term 'Time Zero Module' has been removed, and discussion of the specifics of is not applicable. A general description has been added to the introduction.

For the reviewers information:
Time Zero Module (Cytek, https://www.cytebio.com) and a circulating water bath device allows precise temperature control and stirring of cells suspension to which a stimulus is delivered within one second, allowing uninterrupted measurement of cellular response. It consists of two modules, the Time Zero module with water-jacket tube holder and the Air Supply module. An additional circulating water bath is also needed if temperature control is required. To install this device, the Air Supply module has to be connected to the air pressure system of the flow cytometer via a three-way valve, the sample nozzle has to be connected with a soft tubing and the short tubes (2.5 mL) have to be used instead of regular 5 mL FACS tubes. These changes may take 10-15 min to setup and another 10-15 min to clean up after each run. Since most flow cytometers are shared core facilities, other users may be affected by these changes. If subsecond cell response is not crucial for the study, an alternative way is to unscrew the sample platform (takes about 10 seconds) and fit the water-jacket tube holder on the sample bar of a BD flow cytometer (Fig. 1). The Air Supply module is therefore not needed, and regular 5 mL tubes can be used. However, the tube has to be physically removed and replaced after the addition of stimulus, which incurs a delay of 2-5 seconds before recording. In either case, a tiny stir bar (1x3 mm) has to be placed in the bottom of tube in order to mix cells. A major advantage of the Time Zero system is the device for magnetic stirring of the reaction cuvette, which maximizes the number of cell-cell interactions as well as rapidly

mixing agonist or probe into the suspension. It is also a good idea to leave a small amount of water inside the water-jacket tube holder to ensure good thermal conductivity to the tube.