Refined protocol for generating STAP cells from mature somatic cells.

We have found this to be an effective protocol for generating STAP cells in our lab, regardless of the cell type being studied. The refined protocol below takes elements from the two most effective approaches described in our Jan 31, 2014 article published in Nature (Obokata et. Al., Stimulus triggered fate conversion of somatic cells into pluripotency. Nature 505. 641-647, 2014). We have found this refinement to be useful as a generic protocol to generate STAP cells from a "generic" source of cells. It is very important that each step be performed precisely as described. The protocol is extremely simple, but will vary slightly, if you are starting with tissue rather than a cell suspension. It also will vary depending upon the cell type or tissue with which you are starting. It is important to not skip any steps. It is especially important to triturate the cell suspension for a minimum of 30 minutes, until the suspension can be easily triturated up and down the reduced bore pipettes of the smallest orifices. We first describe the protocol when starting with a suspension of cells, and then describe additional steps necessary when starting with a soft tissue.

A. Generating STAP cells when starting with a suspension of mature somatic cells:

A1. Add the live somatic cells to be treated, as a cell suspension to a centrifuge tube, and then centrifuge at 1200 rpm for 5 minutes.

Note: Trypsin-EDTA, 0.05 % (Gibco: 25300-054) can be added to the tissue culture dish containing cells, for 3- 5 minutes, to release adherent cells to be added to the centrifuge tube.

A2. Aspirate the supernatant down to the cell pellet.

A3. Resuspend the resulting pellet at a concentration of 1x 10^6 cells/ml in of Hanks Balanced Saline Solution (HBSS Ca^+Mg^+ Free: Gibco 14170-112) in a 50 ml tube.

Note: We recommend working with a volume of 2-3ml of the cell suspension in a 50ml tube.

A4. Precoat a standard 9" glass pipette with media (so the cells do not stick to the pipette - we use: Fisher brand 9" Disposable Pasteur Pipettes: 13-678-20D). Triturate the cell suspension in and out of the pipette for 5 minutes to dissociate cell aggregates and any associated debris. This can be done with a fair amount of force.

A5. As a final **extremely important** step in the trituration process, make two fire polished pipettes with very small orifices as follows:

Heat the standard 9” glass pipette over a Bunsen burner and then pull and stretch the distal (melting) end of the pipette, until the lumen collapses and the tip breaks off, leaving a closed, pointed glass tip. Wait until the pipette cools, and then break off the closed distal tip until a very small lumen is now identifiable. Repeat this process with the second pipette, but break the tip off a little more proximally, creating a slightly larger distal lumen. The larger lumen should be about 100-150 microns in diameter, while the other pipette should have a smaller lumen of about 50-70 microns.

Now triturate the cell suspension through the pipette with the larger lumen for 10 minutes. Follow this with trituration through the pipette having the smaller lumen (50-70 microns) for an additional 15 minutes. Continue to triturate the suspension until it passes easily up and down the fire polished pipette of the smaller bore. **This is a very important step. Do not skip this step, or take a shortcut**. **Again, remember to precoat each pipette with media**. Also, during trituration, try to avoid aspirating air and creating bubbles or foam in the cell suspension.

A6. Add HBSS to the suspension to a total volume of 20ml, centrifuge at 1200rpm for 5 minutes and then aspirate the supernatant.

A7. Resuspend the cells in HBSS at a **pH of 5.4**, at cell concentration of 2 million cells/ml, then place in an incubator at 37ºC for 25 minutes.
Note: The pH of the HBSS will increase with the addition of the cell suspension, so we start with an HBSS solution of lower than the desired final pH of 5.6.
Also note: When making the solution acidic, mildly pipette it using a 5ml pipette for 10 seconds immediately after adding the acid to the Hanks Solution. **The resulting pH of 5.6 of cell suspension is very important in the creation of STAP cells**. HBSS has a very weak buffering capacity, so any solution transferred from the supernatant of the previous suspension will affect the pH of the HBSS drastically. The instructions below will show how to create HBSS with the optimum pH of 5.6-5.7 for STAP cell generation.

First, titrate the pH of pre-chilled HBSS (at 4ºC) with 12N HCl to a pH of 5.4. This is done by slowly adding 11.5µl of 12N HCl to 50ml of HBSS. After confirming this pH, sterilize the solution by filtering through a 40 micron syringe filter or bottle top filter of, into a new sterile container for storage. Please confirm the final pH of 5.6-5.7 through an initial test experiment with an appropriate number of cells. Because the pH of the HBSS is so important, it is highly recommended that the pH of the solution be checked, re-titrated and re-sterilized prior to each use.

A8. After 25 minutes in the acid bath, centrifuge the suspension at 1200rpm for 5 minutes.

A9. Aspirate the supernatant and resuspend the resulting pellet in 5ml of what we term “sphere media” (DMEM/F12 with 1% Antibiotic and 2 % B27 Gibco 12587-

010) and place at a concentration of 1x 10^5 – 1 x 10^6 cells/cm^2, within a non-adherent tissue culture dish in the presence of the following supplements: b-FGF (20ng/ml), EGF (20ng/ml), heparin (0.2%, Stem Cell Technologies 07980). LIF (1000U) should be added if the cells are murine). **Note**: Supplements such as bFGF, EGF and heparin may not be necessary.

IMPORTANT: After the cells are placed in tissue culture dishes, they should be gently pipetted using a 5ml pipette, twice/day for 2 minutes, for the first week, to discourage them from attaching to the bottom of the dishes. This is important to generate good sphere formation. Add sphere media containing the supplements described every other day. (Add 1ml/day of sphere media to a 60mm culture dish, or 0.5ml/day to a 35mm dish.)

B. Generating STAP cells when starting with soft tissues that contain many RBCs.

B1. Place the excised, washed sterile organ tissue into an 60mm petri dish containing 50-500µl of collagenase, depending on the size of the tissue. Add a sufficient volume of the collagenase to wet the entire tissue.

Note: Different types of collagenase or other enzymes are better for digestion of different organ tissues. (The spleen may not need to be exposed to any digestive enzymes.)

B2. Mince and scrape the tissue for 10 minutes using scalpels and scissors to increase surface area that is exposed to the collagenase, until the tissue appears to become gelatinous in consistency.

B3. Add additional collagenase to the dish to make the total volume = 0.5ml, and place in a incubator/shaker for 30 minutes at 37ºC at 90rpm.

B4. Add 1.5ml of HBSS to the dish (yielding a total volume of 2.0ml) and then aspirate the entire contents via a 5ml pipette and place into a 50ml tube.

B5. **Now proceed to triturate as previously described above (step A4-5) when starting with mature somatic cells.**

B6. After trituration is completed (through step A5 when using a culture dish of mature somatic cells), add 3ml of HBSS, yielding a volume of 5ml, to the 15ml tube and then **slowly** add 5ml of Lympholyte **to the bottom of the tube** to create a good **bilayer**.

Note: It is important to add this as described to create a bilayer and avoid mixing of the two solutions.

B7. Centrifuge this tube at 1000g for 10 min. Rotate the tube 180º and recentrifuge at 1000g for an additional 10 min. This will cause the erythrocytes to form a pellet at the bottom of the tube.

B8. Using a standard 9” glass pipette, aspirate the cell suspensions layer between HBSS and Lympholyte and place in a new 50 ml tube.

B9. Add HBSS to the suspension to a total volume of 20ml of HBSS and then thoroughly mix the suspension by pipetting via a 5ml pipette for 1 minute.

B10. Centrifuge the solution at 1,200rpm for 5 minutes and aspirate the supernatant.

B11. For the next steps see A7-9.