Examining active cells after incubation with a labeled substrate (eg Carbon-13, Nitrogen-15, Hydrogen-2 or Sulfur-33/34)

Elizabeth Trembath-Reichert, Yuki Morono, Fumio Inagaki, Victoria Orphan

Abstract

Protocol for cell separation, enumeration, and sorting of low biomass samples for SIP-NanoSIMS preparations.

Citation: Elizabeth Trembath-Reichert, Yuki Morono, Fumio Inagaki, Victoria Orphan Examining active cells after incubation with a labeled substrate (eg Carbon-13, Nitrogen-15, Hydrogen-2 or Sulfur-33/34). **protocols.io**
dx.doi.org/10.17504/protocols.io.gwubxew
Published: 24 Jan 2018

Before start

1. Clean 12 spoons
    1. soaked in bleach ~15min, then rinse with 0.2um MQ, then Sonicate 15 min, then rinse, then bring water cup with spoons into clean bench and lay out on rnase away clean foil to dry. Small end of spoon is used.
2. 12 4% PFA/PBS 50 ml tubes with labels (8 ml added)
3. 5 ml pipet and tips
4. 1 ml pipet and tips
5. 12 1.5 ml eppitubes with labels for IC
6. 50 ml tube of glycerol solution
7. 50 tube holders
8. screw driver to open stuck caps
9. Turn on static remover
10. Wash hands in front of static remover
11. Clean sample bottles with rnaseaway and place on clean foil

Protocol

Sampling

Step 1.

1. Remove cap and place top down on lower part of foil.
2. Remove 1ml to eppitube.
3. Remove 8 ml (2x4ml) to PFA tube, 2% PFA final.
4. Remove half of solids to PFA tube with small end of spoon (after use dip spoon in H2O and then place back in bleach container).

5. Add 8 ml of glycerol solution and reclose bottle.

Storage

Step 2.

1. CAS freeze glycerol bottles.
2. Store PFA tubes at 4 °C o/n.
3. Eppitubes at -20 °C.

Sample washing

Step 3.

1. Spin 50 ml PFA tubes 3500 x g for 15 min
2. Prepare 3XPBS filter sterile
3. Remove supernatant by pipet or pour off.
4. Add up to 20ml mark of PBS (25 ml for 8L4 in order to cover all rock chips)
5. Resuspend and return to fridge for 8-16 hrs (overnight)
6. Centrifuge again by repeating steps, but only need to let sit for 2 hrs.
7. Spin, decant.
8. Add 50/50 sterile EtOH/3XPBS (PBS filtered 2X, EtOH 1X) to a total volume of 5X (added 8 ml to 15R3 and MCS, added 12 ml to 32R4, and 20 ml to 8L4). Add PBS first, resuspend, then add EtOH.
9. Store 4 °C.

Cell Separation

Step 4.

1. Pre-weigh fixed sample tubes.
2. Add 8ml 2.5% NaCl (all references to NaCl = 2.5%) to 15ml tube for each sample plus two more for negative controls.
3. Add 1ml of fixed sample slurry and scrape in a few rock pieces that will fit (1g) with a sterile colony picking loop.
4. Spin 4000xg for 15min at 25degC.
5. Weigh fixed sample tubes again and return to fridge.
6. Remove supernatant by 5ml pipet.
    - *This step is to remove any remaining ethanol*
7. Add 2.2ml NaCl, 400ul detergent mix, and 400ul methanol then resuspend.
    - *Detergent mix = 100mM EDTA, 100mM sodium phosphate, 1% (v/v) tween-80*
8. Shake for 59min at 500rpm in shakemaster.
    - *Should be setting 10*
9. Sonicate for 20 cycles 200W (low setting) of 30 sec on/off (20min total)
    - *Put ice in sonicator to prevent samples from heating up.*
10. Add 500ul of 1% HF, vortex, incubate for 20 min.
    - *Make sure static remover is in front of HF hood*
11. Add 500ul 1.5M Tris-Base, vortex.
12. Load sample suspension onto density gradient using 5ml pipet.
    - *The larger rock pieces will be left in this tube. Keep the tube for second density gradient. Pipet slowly down sides of tube to reduce disturbing the density gradient as much as possible.*
13. Spin 10,000xg for 60 min, at 25degC.

- *Can use the smaller centrifuge if other one is busy, but have to spin 2hrs20min at max speed for equal force. Set temperature to 18C.*

14. Remove all supernatant above visible cloudy layer with 20G needle and 2.5ml syringe into 50ml tube.
    - *Okay to take some larger, visible particles if they are above the cloudy layer because they will be filtered out before FCM.*
    - *Technique here is to keep needle bevel facing the side of the tube and pull directly at the surface of the liquid, slowly sliding needle down as you go to keep it at this level the entire time you are pulling out liquid.*
15. See how much gradient solution remains, and add that volume of NaCl to the rock pieces you kept from step 11 and to the density gradient remnants.
16. Resuspend anything you can from the rock pieces, and then transfer that liquid to the density gradient remnant tube.
    - *So far this has been adding about 4ml of NaCl to each tube, 8ml total.*
17. Spin density gradient remnant tube for 25min at 6000xg, 25degC.
    - *This step is to try to drop all the remaining particles to the pellet and allow you to pull out any cells that may have been in that layer.*
18. Remove supernatant with 5ml pipet to same 50ml tubes as step 13.
19. Add 2.2ml NaCl, 400ul detergent mix, and 400ul methanol, then resuspend.
20. Shake for 10min at 500rpm in shakemaster.
    - Should be setting 4.
21. Sonicate for 20 cycles 200W (low setting) of 30 sec on/off (20min total)
22. Load sample suspension on top of new gradient tube as in step 11.
23. Spin 10,000xg for 60 min, at 25degC.
24. Remove all supernatant above visible cloudy layer with 20G needle and 2.5ml syringe into 50ml tube.
25. To leave overnight, add 100X dilution of 50mM EDTA (0.5mM final) and store at 4degC. Otherwise proceed to FCM preparation.
    - *Usually this is about 200ul.*

FCM Preparation

Step 5.

1. For each real sample (do not do for negative controls), build filter tower with 0.45um cellulose acetate membrane and anodisc membrane (0.02um).
2. Check filter tower seal with a couple mls TE.
3. Load sample onto tower.

- *Should take less than 30 minutes to filter the entire sample. If it takes more time than this, then should remove remaining sample and change out filters.*

4. Remove filter tower after all sample has been filtered.
5. Turn off vacuum and close port to filtration stop.
6. Add 200ul TE to wash membrane.
7. Add 200ul of 1/40 SYBR/TE and stain for 10min.
8. Turn on filtration and open port. Add 200ul TE to wash.
9. Remove anodisc membrane to 50ml tube with 4ml TE.
10. Sonicate with probe to detach cells from anodisc.
    1. Hold probe right next to filter and scan over entire membrane. Should see membrane go white and liquid turn orange. Should take about 10 sec or so sonication.

11. Transfer supernatant to 5ml tube. Add 1ml TE wash to 50ml tube and then transfer that to the same 5ml tube, for 5ml total.
12. Prepare another set of 5ml tubes and filter baskets. Remove 4.5ml of cell suspension and filter through basket into second set of 5ml tubes for FCM. CAS freeze these samples.
13. Save remaining 0.5ml of cell suspension for membrane preparation

Membrane Preparation

Step 6.

1. Build filter tower with 0.45um cellulose acetate membrane and polycarbonate membrane.
2. Test seal with TE, then add 0.5 ml sample if it is good.
    1. *If seal is bad try twisting tower.*
3. Turn off vacuum.
4. Remove tower once all liquid is gone.
5. Add 200ul 1/40 SYBR/TE and incubate 10min.
6. Turn on vacuum and wash with 200ul TE.
7. Mount entire filter with 2:1 Vecta:TE (20ul for entire filter).
8. Wash towers by sonication in 1M HCL for 15min, then rinse in 0.1M HCL and 0.2um filtered MQ. Place directly into clean bench while wet, and wrap in RNAseaway foil if not planning to use immediately.

FCM

Step 7.

1. Turn on system START button
2. Turn on powerstrips on table legs
3. Turn on blue and red switches (sheath fluid controls?) and watch to see pressure increase on input and output tanks.
4. Turn on blue laser box switch (shelf above floor).
5. Slap tank on table leg around for a few minutes, purging (opening and closing black valves at the top and bottom of the canister) every 30 seconds or so. This is to dislodge any air bubbles from the lines.
6. Open chamber door and place a catch tray in the sample area.
7. Very gently remove nozzle cap solution.
8. Touch the start drop button to begin water flow.
9. Turn on the two light buttons.
10. Prepare cleaning solutions of 50% bleach, MQ, and 70% ethanol (rough is fine). Filter sterilize.
11. Go to menu.
12. Pour bleach into cap of 50ml tube, and hold under nozzle. Push plunger button and hold for 20-30 seconds. Push again to turn off.
13. Pour out cap contents into blue bucket and rinse with MQ water then place back on 50ml bleach tube.

14. Repeat with MQ water to rinse.
15. Finish off with Ethanol.
16. Return to normal flow and close sample chamber door.
17. Turn on the de-blubbler for 5 minutes.
    - while waiting, turn on laser key. Turn on laser on monitor as well by laser control panel (both on button and open shutter)
    - Keep laser power around 34%
    - Turn on Summit program on computer desktop
    - Create files for today by opening bead program and most recent program run from my fold and saving these with today's date.
18. May need to adjust pinhole here, but we didn't need to do it.
19. Prepare fluorescent beads (in fridge). Give a little shake to resuspend and add about 10 drops to cell sorting tube from supply shelf.
20. Place into sample holder and then into machine.
21. Go to second panel (cell blobs). Make sure axes are set to FL2 x-axis, H, 1 and FSC y-axis, H, 7
22. Turn on acquisition mode and cycle mode.
23. Push start on monitor to begin flow of beads into sorter and turn on the vibration mode.
24. Monitor pressure difference between sheath and sample, to remain between 0.1 and 0.5.
25. Press boost button until you see the first beads.
26. Turn knob until you get about 100-200 events per second (EPS).
27. Tune laser parameters such that the intensity is as high as possible on monitor.
28. Now look at computer screen and try to get the peak shape to be as sharp as possible and having the CV below 2 or 3 for each panel.
29. Once satisfied, save.
30. Stop machine. Open sample holder (up arrow) Remove sample and do a backflush to remove excess beads (swirly button). Put MQ 50ml tube in sample holder and into machine. Run MQ for a few minutes.
31. Move to third panel (dropplets).
32. Mark end of first separated drop.
33. Run debubbler for 1.5 minutes and then check to see if red line is in EXACT same place. If yes, proceed, if no repeat.
34. Go to 4th panel (spreading laser lines).
35. Place charging plates in sample holder on either side of laser without getting them wet. Turn charge on.
36. Change voltage to around 3500 V

37. Turn on each stream individually, then turn on all at once.
38. Play with phase to find the worst condition, then go 180 degrees from there and that should be the best condition. Turn off charge and wipe down plates if they got wet in this process.
39. Run defanning to find sharpest center stream.
40. Go back to 3rd panel and run intelisort (head picture)
41. Go back to 4th panel and turn on all streams and make sure still looks good.
42. Go back to 3rd panel and press up/down arrows with drops in the middle button to put into cruise control. Can be messed up by weather change!
43. To start a sample, brief sonication in waterbath (hold in for 30 seconds).
44. Set streams to center and right 2. Put right 2 at 42%.
45. Start sample run on computer FIRST, then on machine. Set vibrate mode.
46. Boost until see first detections.
47. Record a few seconds to make sure regions are drawn correctly on computer program. Stop sample.
48. Set up filtration tower with cellulose acetate membrane, and ½ ITO coated polycarbonate membrane ontop of a water bubble. Place ID mark with tweasers to help find sample.
49. Align filter setup with pink tape.
50. Close chamber door and double click stream button to put one drop on membrane and test position.
51. Turn sample back on and set EPS to 15-20,000.
52. Clear soft logic in sorting menu on computer and make sure R24 and R19 are selected.
53. Differential pressure and EPS may need to decrease in order to account for dilute samples. Tracking the success rate will let you know if you need to tune these parameters.
54. Remove sample and filter.
55. Find sorted cells on LDM and mark their location using the tweezer mark as a guide for where to look.
56. Store filter dry until NanoSIMS analysis.