Fixing adherent cells:

Materials

1. Chamber slides, LabTek (12 565 471 for 2 well or 12 565 470 for 8 well)
2. 10xPBS, Ambion AM9624
3. Fixation solution: 5mL 10x PBS, 5mL 37% formaldehyde (100% formalin: Sigma, F1635-500ML), 40mL NF H_2O (can leave in hood at RT for months).
4. 70% EtOH: 35mL 95% EtOH, 15mL Nuclease-free (NF) H_2O (lasts forever).

Method

1. Grow cells to 50-70% confluence in multi-well chamber slides. Want a bit of space between cells for clean segmentation.
2. Aspirate media and rinse cells with 1X PBS.
3. Aspirate PBS and fix with fixation solution for 10 minutes.
4. Aspirate fixation solution and rinse cells 3X in 1X PBS.
5. Aspirate 1X PBS and apply 70% EtOH to each well. Samples can be stored in EtOH in 4°C for long periods of time, but we should ensure that the 70% EtOH is topped up on a regular basis.

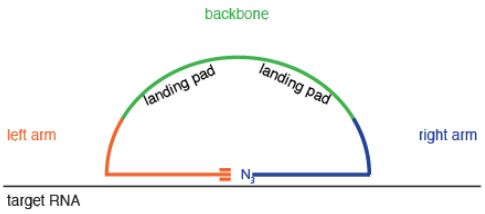
Designing the probes (Raj lab probe design server--Matlab required)

1. Request licensing permission from: https://flintbox.com/public/project/50547/
2. Create a Bitbucket account/username: https://bitbucket.org/account/signup/
3. After the licensing permission is granted, email arjunrajlab@gmail.com your Bitbucket username and request access to the repository.
4. https://bitbucket.org/account/signin/?next=/arjunrajlaboratory/probedesign
5. Get the source code:
    a. At the top right, click “clone” then copy the text command that pops up.
    b. Open up Terminal (Mac) and navigate to the desired directory, then type the command, for example:
    ```
    hg clone
    https://srouhanifard@bitbucket.org/arjunrajlaboratory/probedesign
    ```
6. Then add the source code to your MATLAB path.
7. After adding the **rajlab** directory and subdirectories, be sure to hit Save.
8. Now you have the probe design software!
9. If we have a smFISH probe set for your target of interest, make a snapgene file and add features for each probe.
10. Make a targetname.txt file with the target RNA sequence
    a. Go to UCSC genome browser: http://genome.ucsc.edu/cgi-bin/hgGateway
    b. Select the species and search for the gene you want.
        i. take note the genome build (e.g. hg19, mm10, etc) you are using. It might be important to use the latest or a previous version of a genome, depending on your project/application
    c. Click on the RefSeq version of the gene. There may be many versions of the gene corresponding to different splice variants (isoforms).

d. This will bring up a bunch of tracks. Here, you can visually examine the various isoforms.
e. If you don't have a preference or knowledge of which isoform is the one you should use, then pick an isoform that is the greatest common factor in the sense that it has the most sequence shared between all isoforms.
f. Click on the track of the isoform (under the heading "RefSeq Genes").
g. click on "Genomic Sequence..."
h. For mRNA start by searching in the CDS.
i. If you are unsuccessful at finding enough probes in the CDS, you can add the UTR in your search.
j. For intron FISH, you obviously want the introns.
k. DO NOT Mask repeats.
l. Click submit
m. This brings up a big sequence with multiple FASTA entries. Copy this into a text file (for example "myseq.txt"). On the Mac, do this by first hitting command-A to select everything, then command-C to copy, then open TextEdit, then paste, and then hit command-shift-T to convert to a plain text file.

11. Open the folder that contains your file in a matlab window
12. Run this script in Matlab:
    findprobesHD('targetname.txt', 20, 'outfilename', …
    'targetname_30mer_target', 'species', 'mouse', 'oligolength', 30, …
    'allowableGibbsFE', [-50, -30], 'targetGibbsFE', -40)
13. From the output file, map the new probe sequences on your snapgene file. Try to choose probes that are non-overlapping with the smFISH probes, but this is not critical.
14. The 30mer sequence output will look like this:
    `tgttgatgttgtggcactttggtggctctg  Podxl_exon_30mer_target_1`
15. If you split this sequence in half, it will give you the sequence for the region of the probe that binds to the target:
    `right arm:  left arm:`
    `tgttgatgttgtggc actttggtggctctg`
16. Next, add the **adapter sequences** and the modifications:
    1. `PODXl_P1_left: /5Hexynyl/actttggtggctctg`**`ACATCATAGT`**
    2. `PODXl_P1_right: /5Phos/`**`aagtgactgt`**`tgttgatgttgtggc/3AzideN/`

    Here's a diagram of how each probe will bind to the target and how the full probe will be built:



Notes about ordering probes: When ordering click probes, choose “100 nmole DNA Oligo” for both left and right arms and note that the right arm (3’NHS-azide probes with adapter region) require HPLC purification. For the 5’hexynyl modification, standard desalting is sufficient. The probes used in this paper were ordered from IDT; however, Gene Link can provide discounted pricing for the same products.

Making the probes:

Materials

1. 5’hexynyl labeled probes with adapter region (left arm)--resuspend in NF H_2O to 400 µM
    a. Could include internal fluor in the left arm if desired (Cy3 or Cy5 from IDT)
2. 3’NHS-azide probes with adapter region (right arm)--resuspend in NF H_2O to 400 µM
3. Left and right adapters for each probe--resuspend in NF H_2O to 400 µM
4. Backbone sequence--resuspend in NF H_2O to 400 µM
5. T7 DNA ligase (NEB, M0318L)
6. T7 DNA ligase reaction buffer
7. NF H_2O
8. Monarch PCR and DNA cleanup Kit, NEB T1030S

**For cost savings, can enzymatically add 5’phosphate rather than ordering it directly from IDT:

10 µl DNA 4 nmol, 35 µl 10x buffer, 7µl 80 mM ATP (home made, fresh), 3.5 µl t4 PNK 10U/µl, Fill up to 350 µl H2O

Heat to 37C for 5.5 hours, heat inactivate at 65C for 20 min. Ethanol precipitate and resuspend in 10 µl to make 400 µM stock.

**For cost savings, can enzymatically add 3’azide rather than ordering directly from IDT:

5 ul of 400 µM “Right arm” (with 3’OH), 5 ul of 4 mM NT analog (N6-(6-Azido)hexyl-3'-dATP from Jena Biosciences), 4 ul of TdT enzyme, 10 µl of Transferase buffer, 26 µl NF H_2O

Heat for 90 min, 37C then inactivate enzyme for 10 min, 70C. Should be near >95% conversion. Monarch column purify to remove free NT and resuspend in 5 µl to make ~400 µM stock oligo.

Method

1. Combine reaction components without enzyme
    a. .75 ul of 400 µM left arm
    b. .75 ul of 400 µM left adapter
    c. .75 ul of 400 µM right arm
    d. .75 ul of 400 µM right adapter
    e. .5 ul of 400 µM backbone
    f. 5 µl of 2X reaction buffer
    g. .5 µl NF H_2O
2. Heat reaction components to 70 degrees for 3 min, then leave at room temperature for 5 min.
3. Add 1 µl of T7 DNA ligase (pre-diluted 1:10 in NF H_2O)
4. Incubate at room temp in the dark for a minimum of 1 hr. (overnight is fine)
5. Column purify using Monarch PCR and DNA cleanup Kit according to manufacturer's instructions:
    a. Add 40 µl of TE to the samples
    b. Add 350 µl of binding buffer to sample and apply to column
    c. Spin down for 1 min and discard liquid
    d. Wash 2X with 200 µl of Wash buffer and discard liquid
    e. Apply 30 µl of elution buffer to the center of the column
    f. **Note that these quantities may overload a single column. This protocol was used for all experiments presented at the time of publication. Spreading the sample over multiple columns may increase the final yield.**

ClampFISH:

Materials

ClampFISH hybridization buffer: (Store at -20)

10% Dextran sulfate
20% Formamide
2X SSC
NF H2O

Wash buffer (Store at RT)

10% Formamide
2X SSC
NF H2O

Click components:

BTTAA (Order through iLab at AECOM Chem bio facility): http://www.einstein.yu.edu/research/shared-facilities/chemical-biology/Ligands-for-CuAAC/)
Also found that Jena bioscience is selling it now too!
https://www.jenabioscience.com/click-chemistry/click-reagents-by-chemistry/auxiliary-cu-i-click-reagents/clk-067-bttaa

(Store 20mM aliquot at -20)
$CuSO_4$ (Store 5mM aliquot at -20)
Sodium Ascorbate (store at RT)
2X SSC/0.25% Triton (store at RT)

Primary probe hybridization:

1. Thaw clampFISH hybridization buffer. Label your samples - you can label the top part of the chamber, this will not be imaged.
2. Make clampFISH hybridization mix: Use 50µl/well clampFISH hybridization buffer + 0.5 µl of each clampFISH primary probe
3. Aspirate 70% ethanol from chamber slides
4. Add Wash buffer to each well (500ul per well for 8-well chamber slides). Pipet the wash buffer against the wall of the chamber, to ensure you don't dislodge the cells from the bottom of the well.
5. Aspirate off wash buffer.
6. Pipet 50 µl of clampFISH hybridization mix (prepared in step 2) into the middle of the well. Place cover glass on top, use forceps to pat it down and remove bubbles.
7. Humidify the hybridization chamber (kimwipe with wash buffer inside chamber is good enough) Use parafilm to seal the plate.
8. Incubate at 37°C for a minimum of 4 hours, or overnight.

Secondary probe hybridization (also use this for tertiary probe hybridization):

9. Take samples from incubator, remove parafilm
10. Add wash buffer to each well. The buffer will seep underneath the cover glass and lift it, making it easier to remove. Use tweezers to fully remove cover-slip.
11. Aspirate and replace wash buffer
12. Seal the plate with parafilm, put to 37°C for 20 minutes.
13. Aspirate and replace wash buffer
14. Seal the plate with parafilm, put to 37°C for 20 minutes.
15. Make clampFISH hybridization mix: Use 50µl/well clampFISH hybridization buffer+1 µl of clampFISH secondary probe (MM2 series). Use tertiary probes when applicable
16. Aspirate off wash buffer.
17. Pipet 50 µl of clampFISH hybridization mix (prepared in step 15) into the middle of the well. Place cover glass on top, use forceps to pat it down and remove bubbles.
18. Humidify the hybridization chamber (kimwipe with wash buffer inside chamber is good enough) Use parafilm to seal the plate.
19. Incubate at 37°C for a minimum of 2 hours
20. Take samples from incubator, remove parafilm
21. Add wash buffer to each well. The buffer will seep underneath the cover glass and lift it, making it easier to remove. Use tweezers to fully remove cover-slip.
22. Aspirate and replace wash buffer
23. Seal the plate with parafilm, put to 37°C for 20 minutes.
24. Aspirate and replace wash buffer

25. Seal the plate with parafilm, put to 37°C for 20 minutes.
26. Aspirate wash buffer and replace with 2X SSC

Click reaction:

27. Prepare click reaction components:
    a. Mix the $CuSO_4$ and the BTTAA first, then add 2X SSC/0.25% triton. Lastly, add 1 mL of NF H_2O to dry sodium ascorbate aliquot to bring concentration to 100 mM, then add desired amount to reaction. Make 250 µl per well, or scale accordingly.

reagent	**[stock]**	**volume added**	**[final]**
CuSO4	5 mM	3.75 µl	75 µM
BTTAA (ligand)	20 mM	1.875 µl	150 µM
Sodium Ascorbate	100 mM	6.25 µl	2.5 mM
2X SSC/0.25% triton X		238.125 µl	
final volume:		**250** µl	

    b. Aspirate 2X SSC and replace wells with click reaction mixture
    c. Incubate for 20 minutes at 37°C
28. Aspirate click reaction and replace with wash buffer
29. Prepare for another round of hybridization (same as earlier steps) and repeat until reached the desired amplification. (Tertiary is P9 series)
30. To perform smFISH on the terminating round of clampFISH for detection:
    a. Prepare hybridization solution: 1 ul of 5 µM probe in 50 µl of smFISH hybridization buffer (10% Formamide/2X SSC/10% Dextran Sulfate)
    b. Aspirate Wash buffer from samples and add hybridization solution to the center of the well and put a glass coverslip on top to make the probe evenly spread.
    c. Incubate at 37 degrees for at least 6 hours
    d. Wash 2X with Wash buffer (10% formamide/2X SSC)
    e. For FAQs about the Raj lab smFISH protocol: https://sites.google.com/site/singlemoleculernafish/faq
31. To complete the experiment, aspirate Wash buffer and replace with 2X SSC. Add DAPI to visualize nuclei on microscope. If using Cy5 for detection, mount slides with anti-fade buffer:
    a. "Anti-Fade" buffer: 1mL volume
        i. 850 µL nuclease-free (NF) H_2O
        ii. 40 µL 10% glucose
        iii. 100 µL 20X SSC
        iv. 10 µL 1M Tris @ pH8 (this helps with oxidase proton products)
        v. We typically prepare a pre-mix containing water, SSC and Tris, as well as a stock of 10% glucose. We then combine the pre-mix with the glucose just before we add it to the sample.
    b. **----Glucose Oxidase Prep----**

i. *This is required to reduce photobleaching of Cy5 by dissolved oxygen*
ii. *Glucose + Oxygen in sample --(GluOx)--> less O_2 in sample*
iii. *Glucose oxidase stock enzyme is aliquoted out and frozen for storage*
iv. *Note: Glucose oxidase tends to "go bad" with repeated freeze/thaw cycles. In order to keep it working, aliquot it after the first thaw, so you'll need to go through less than 5-10 freeze-thaws per aliquot.*
v. ***Materials needed: Glucose oxidase (Sigma, G2133-10KU), Catalase (Sigma, C3515-10MG), 2X SSC, 10% Glucose.***

c. During 2nd wash, prepare "Anti-Fade" buffer if there's a Cy5-labelled probe. If not, you can proceed and image in 2xSSC.

- add 40µl of 10% glucose to 960µl antifade-premix.
- Split anti-fade buffer into 100 & 900 µL volumes.
    1. 50 µL is for enzyme, 900 µL is for equilibrating sample.
- Vortex catalase in its amber vial from 4°C.
- Take out glucose oxidase from -20°C only when ready to add.
- Add 1 µL of oxidase enzyme and 1 µL of catalase to the 100 µL anti-fade buffer.
    1. Add 100 µL of glucose oxidase+catalase mix to sample.
    2. Use clean tweezers to add clean coverslip to squash & reduce O_2.