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ClampFISH

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ABSTRACT

Non-enzymatic, high-gain signal amplification methods with single-cell, single-molecule resolution are in great need. We present click-amplifying FISH (clampFISH) for the fluorescent detection of RNA that combines the specificity of oligonucleotides with bioorthogonal click chemistry in order to achieve high specificity and extremely high-gain (>400x) signal amplification. We show that clampFISH signal enables detection with low magnification microscopy and separation of cells by RNA levels via flow cytometry. Additionally, we show that the modular design of clampFISH probes enables multiplexing, that the locking mechanism prevents probe detachment in expansion microscopy, and that clampFISH works in tissue samples.

ATTACHMENTS

[ClampFISH](#)
[protocol_20180522.pdf](#)

GUIDELINES

The protocol workflow is as follows:

1. Fixing adherent cells (Steps 1-7)
2. Designing the probes (Raj lab probe design server-- Matlab required) (Steps 8-35)
3. Making the probes (Steps 36-48)
4. ClampFISH (Steps 49-90)

For additional information and supplementary material, please see full manuscript:
<https://www.biorxiv.org/content/early/2017/11/21/222794>

MATERIALS

MATERIALS

- ☒ T7 DNA Ligase - 750,000 units **New England Biolabs Catalog #M0318L**
- ☒ Chamber slides, LabTek (for 2 well or 12 565 470 for 8 well) **LabTek Catalog #12 565 471**
- ☒ 10xPBS **Ambion Catalog #AM9624**
- ☒ 100% formalin **Sigma Catalog #F1635-500ML**
- ☒ 95% EtOH **Contributed by users**
- ☒ Nuclease-free (NF) H₂O **Contributed by users**
- ☒ T7 DNA ligase reaction buffer **Contributed by users**
- ☒ 5'hexynyl labeled probes with adapter region (left arm)- **Contributed by users**
- ☒ 3'NHS-azide probes with adapter region (right arm) **Contributed by users**
- ☒ Left and right adapters for each probe **Contributed by users**
- ☒ Backbone sequence **Contributed by users**
- ☒ Monarch PCR and DNA Cleanup Kit - 50 preps **New England Biolabs Catalog #T1030S**
- ☒ 10% Dextran sulfate **Contributed by users**
- ☒ 20% Formamide **Contributed by users**
- ☒ 2X SSC **Contributed by users**
- ☒ Please see additional Materials for Glucose Oxidase Prep (Step 90). **Contributed by users**

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID:
12472

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SAFETY WARNINGS

! For safety warnings and hazard information, please refer to the SDS (Safety Data Sheet).

BEFORE START INSTRUCTIONS

Fixing adherent cells:

Fixation solution (can leave in hood at RT for months):

- 5mL 10x PBS
- 5mL 37% formaldehyde (100% formalin)
- 40mL NF H₂O

70% EtOH (lasts forever):

- 35mL 95% EtOH
- 15mL Nuclease-free (NF) H₂O

Making the probes:

Materials:

1. 5'hexynyl labeled probes with adapter region (left arm)--resuspend in NF H₂O 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 H₂O to 400 µM
3. Left and right adapters for each probe--resuspend in NF H₂O to 400 µM
4. Backbone sequence--resuspend in NF H₂O to 400 µM

ClampFISH:

ClampFISH hybridization buffer (Store at -20°C):

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

Wash buffer (Store at RT):

- 10% Formamide
- 2X SSC
- NF H₂O

Click components:

- BTAA (Order through iLab at AECOM Chem bio facility): <http://www.einstein.yu.edu/research/shared-facilities/chemical-biology/Ligands-for-CuAAC/> Or from Jena bioscience: <https://www.jenabioscience.com/click-chemistry/click-reagents-by-chemistry/auxiliary-cu-i-click-reagents/clk-067-bttaa> (Store 20mM aliquot at -20°C)
- CuSO₄ (Store 5mM aliquot at -20°C)
- Sodium Ascorbate (store at RT)
- 2X SSC/0.25% Triton (store at RT)

Fixing adherent cells

- 1 Grow cells to 50-70% confluence in multi-well chamber slides.

Note

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.
00:10:00 Fixation
- 4 Aspirate fixation solution and rinse cells in 1X PBS. (1/3)
- 5 Rinse cells in 1X PBS. (2/3)
- 6 Rinse cells in 1X PBS. (3/3)
- 7 Aspirate 1X PBS and apply 70% EtOH to each well.

Note

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

- 8 Request licensing permission from: <https://flintbox.com/public/project/50547/>

Note

Raj lab probe design server--Matlab required
- 9 Create a Bitbucket account/username: <https://bitbucket.org/account/signup/>
- 10 When licensing permission is granted, email arjunrajlab@gmail.com your Bitbucket username and request access to the repository.
- 11 <https://bitbucket.org/account/signin/?next=/arjunrajlaboratory/probedesign>
- 12 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:

Command

```
hg clone https://srouhanifard@bitbucket.org/arjunrajlaboratory/probedesign
```

- 13 Add the source code to your MATLAB path.
- 14 Add the **rajlab** directory and subdirectories, and be sure to hit **Save**.
- 15 Now you have the probe design software!
- 16 If you have a smFISH probe set for your target of interest, make a snappgene file and add features for each probe.
- 17 To make a **targetname**.txt file with the target RNA sequence (Steps 17-29), first, go to UCSC genome browser: <http://genome.ucsc.edu/cgi-bin/hgGateway>
- 18 Select the species and search for the gene you want.

Note

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
- 19 Click on the RefSeq version of the gene. There may be many versions of the gene corresponding to different splice variants (isoforms).
- 20 This will bring up a bunch of tracks. Here, you can visually examine the various isoforms.
- 21 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
- 22 Click on the track of the isoform (under the heading "*RefSeq Genes*").

23 Click on '*Genomic Sequence...*'.

For mRNA, start by searching in the CDS (If you are unsuccessful at finding enough probes in the CDS, you can add the UTR in your search)

DO NOT mask the repeats.

Click Submit.

24 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.

25 Open the folder that contains your file in a matlab window.

26 Run this script in Matlab:

Command

```
findprobesHD('targetname.txt', 20, 'outfilename', ...  
'targetname_30mer_target', 'species', 'mouse', 'oligolength', 30, ...  
'allowableGibbsFE', [-50, -30], 'targetGibbsFE', -40)
```

27 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.

28 The 30mer sequence output will look like this:

Command

```
tgttgatgttggtgcactttggtggctctg Podxl_exon_30mer_target_1
```

29 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: tgttgatgttggtgc	left arm: actttggtggctctg
--------------------------------------	-------------------------------------

right arm:	left arm:
<u>tgttgatgttggtgc</u>	<u>actttggtggctctg</u>

30 Now, add the adapter sequences and the modifications:
1. PODXL_P1_left: /5Hexynyl/**actttggtggctctg**ACATCATAGT
2. PODXL_P1_right: /5Phos/aagtgact**tgttgatgttggtgc**/3AzideN/

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

Command

1. PODXI_P1_left: /5Hexynyl/actttggtggctctgACATCATAGT
2. PODXI_P1_right: /5Phos/aagtgactgttgatgttggtgc/3AzideN/

Note

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.

Making the probes

- 31 Combine reaction components without enzyme.

- 1.5 μ L 400 μ M left arm
- 1.5 μ L 400 μ M left adapter
- 1.5 μ L 400 μ M right arm
- 1.5 μ L 400 μ M right adapter
- 1 μ L 400 μ M backbone
- 10 μ L 2X reaction buffer
- 1 μ L NF H₂O

Note

Notes on making the probes:

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

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

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

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

5 μ L of 400 μ M "Right arm" (with 3'OH), 5 μ L of 4 mM NT analog (N6-(6-Azido)hexyl-3'-dATP from Jena Biosciences), 4 μ L of TdT enzyme, 10 μ L of Transferase buffer, 26 μ L NF H₂O

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

- 32 Heat reaction components to 70°C for 3 min.

70 °C Heating reaction components

00:03:00 Heating reaction components

- 33 Leave reaction components at room temperature for 5 min.

00:05:00 Reaction components at room temperature

- 34 Add 2 µl of T7 DNA ligase (diluted 1:10 in NF H₂O).

2 µL T7 DNA ligase

- 35 Incubate at room temp in the dark for a minimum of 1 hr. (overnight is fine)

01:00:00 Incubation

- 36 Column purify using Monarch PCR and DNA cleanup Kit according to manufacturer's instructions (Steps 42-48).

Note

****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.****

- 37 Add 80 µl of TE to the samples.

80 µL TE

- 38 Add 700 µl of binding buffer to sample and apply to column.

700 µL Binding buffer

- 39 Spin down for 1 min.

00:01:00 Spinning down

- 40 Discard liquid.

- 41 Wash with 200 µl of Wash buffer and discard liquid. (1/2)

200 µL Wash buffer

- 42 Wash with 200 µl of Wash buffer and discard liquid. (2/2)

200 µL Wash buffer

- 43 Remove column and add to a new microfuge tube. Apply 60 µl of elution buffer to the center of the column. Wait 1 min, then spin down.

60 µL Elution buffer

ClampFISH - Primary probe hybridization

- 44 Thaw clampFISH hybridization buffer.
- 45 Label your samples - you can label the top part of the chamber, this will not be imaged.
- 46 Make clampFISH hybridization mix: Use 50µl/well clampFISH hybridization buffer + 0.5 µl of each clampFISH primary probe.
50 µL clampFISH hybridization buffer
0.5 µL of each clampFISH primary probe
- 47 Aspirate 70% ethanol from chamber slides.
- 48 Add Wash buffer to each well (500 µl 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.
- 49 Aspirate off wash buffer.
- 50 Pipet 50 µl of clampFISH hybridization mix (prepared in step 51) into the middle of the well.
50 µL clampFISH hybridization mix
- 51 Place cover glass on top, use forceps to pat it down and remove bubbles.
- 52 Humidify the hybridization chamber (kimwipe with wash buffer inside chamber is good enough).
- 53 Use parafilm to seal the plate.
- 54 Incubate at 37°C for a minimum of 4 hours, or overnight.
37 °C Incubation
04:00:00 Incubation

ClampFISH - Secondary probe hybridization (also use this for tertiary probe hybridization)

55 Take samples from incubator, remove parafilm.

56 Add wash buffer to each well.

Note

The buffer will seep underneath the cover glass and lift it, making it easier to remove. Use tweezers to fully remove cover-slip.

57 Aspirate and replace wash buffer.

58 Seal the plate with parafilm.

59 Put the plate to 37°C for 20 minutes.

37 °C Incubation

00:20:00 Incubation

60 Aspirate and replace wash buffer.

61 Seal the plate with parafilm.

62 Put the plate to 37°C for 20 minutes.

37 °C Incubation

00:20:00 Incubation

63 Make clampFISH hybridization mix: Use 50µl/well clampFISH hybridization buffer +1 µl of clampFISH secondary probe (MM2 series). Use tertiary probes when applicable.

50 µL clampFISH hybridization buffer

1 µL clampFISH secondary probe

64 Aspirate off wash buffer.

65 Pipet 50 µl of clampFISH hybridization mix (prepared in step 68) into the middle of the well.

50 µL clampFISH hybridization mix

66 Place cover glass on top, use forceps to pat it down and remove bubbles.

- 67 Humidify the hybridization chamber (kimwipe with wash buffer inside chamber is good enough).
- 68 Use parafilm to seal the plate.
- 69 Incubate at 37°C for a minimum of 2 hours
37 °C Incubation
02:00:00 Incubation
- 70 Take samples from incubator, remove parafilm.
- 71 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.
- 72 Aspirate and replace wash buffer.
- 73 Seal the plate with parafilm, put to 37°C for 20 minutes.
37 °C Incubation
00:20:00 Incubation
- 74 Aspirate and replace wash buffer.
- 75 Seal the plate with parafilm, put to 37°C for 20 minutes.
37 °C Incubation
00:20:00 Incubation
- 76 Aspirate wash buffer and replace with 2X SSC.

ClampFISH - Click reaction (done after every secondary probe hybridization)

- 77 Prepare click reaction components:

a. Mix the CuSO₄ and the BTAA first, then add 2X SSC/0.25% triton. Lastly, add 1 mL of NF H₂O 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.

	[stock]	volume added	[final]
CuSO₄	5 mM	3.75 µl	75 µM
BTAA (ligand)	20 mM	1.875 µl	150 µM
Sodium Ascorbate	100 mM	6.25 µl	2.5 µM
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.

🔥 37 °C Incubation

🕒 00:20:00 Incubation

ClampFISH - Click reaction

78 Aspirate click reaction and replace with wash buffer.

79 Prepare for another round of hybridization (same as earlier steps) and repeat until reached the desired amplification. (Tertiary is P9 series)

⇒ go to step #55 undefined

ClampFISH- smFISH on the terminating backbone

80 To perform smFISH on the terminating round of clampFISH for detection, first, prepare hybridization solution:
1 µl of 5 µM probe in 50 µl of smFISH hybridization buffer (10% Formamide/2X SSC/10% Dextran Sulfate)

🔥 1 µL 5 µM probe

🔥 50 µL smFISH hybridization buffer

Note

For FAQs about the Raj lab smFISH protocol: <https://sites.google.com/site/singlemoleculernafish/faq>

81 Now, 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.

82 Incubate at 37°C for at least 6 hours.

🔥 37 °C Incubation

🕒 06:00:00 Incubation

83 Wash with Wash buffer (10% formamide/2X SSC). (1/2)

84 Wash with Wash buffer (10% formamide/2X SSC). (2/2)

Note

For FAQs about the Raj lab smFISH protocol, see: <https://sites.google.com/site/singlemoleculernafish/faq>

- 85 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

1. 850 μ L nuclease-free (NF) H₂O
2. 40 μ L 10% glucose
3. 100 μ L 20X SSC
4. 10 μ L 1M Tris @ pH8 (this helps with oxidase proton products)
5. 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----

1. *This is required to reduce photobleaching of Cy5 by dissolved oxygen.*
2. *Glucose + Oxygen in sample $\rightarrow$ (GluOx) $\rightarrow$ less O₂ in sample.*
3. *Glucose oxidase stock enzyme is aliquoted out and frozen for storage.*
4. *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.*
5. **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 40 μ L of 10% glucose to 960 μ L antifade-premix.
 - Split anti-fade buffer into 100 & 900 μ L volumes.
1. 50 μ L is for enzyme, 900 μ L 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 μ L of oxidase enzyme and 1 μ L of catalase to the 100 μ L anti-fade buffer.
1. Add 100 μ L of glucose oxidase+catalase mix to sample.
 2. Use clean tweezers to add clean coverslip to squash & reduce O₂.