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.

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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

Before start

Fixing adherent cells:

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

- 5mL 10x PBS
- 5mL 37% formaldehyde (100% formalin)
- 40mL NF H_2O

70% EtOH (lasts forever):

- 35mL 95% EtOH
- 15mL Nuclease-free (NF) H_2O

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

ClampFISH:

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

- 10% Dextran sulfate
- 20% Formamide
- 2X SSC
- NF H_2O

Wash buffer (Store at RT):

- 10% Formamide
- 2X SSC
- NF H_2O

Click components:

- BTTAA (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-cli ck-reagents/clk-067-bttaa (Store 20mM aliquot at -20°C)
- $CuSO_4$ (Store 5mM aliquot at -20°C)
- Sodium Ascorbate (store at RT)
- 2X SSC/0.25% Triton (store at RT)

Materials

T7 DNA Ligase - 750,000 units M0318L by New England Biolabs

Chamber slides, LabTek (for 2 well or 12 565 470 for 8 well) 12 565 471 by LabTek

10xPBS AM9624 by Ambion

100% formalin F1635-500ML by Sigma

95% EtOH by Contributed by users

Nuclease-free (NF) H2O by Contributed by users

T7 DNA ligase reaction buffer by Contributed by users

5'hexynyl labeled probes with adapter region (left arm)- by Contributed by users

3'NHS-azide probes with adapter region (right arm) by Contributed by users

Left and right adapters for each probe by Contributed by users

Backbone sequence by Contributed by users

Monarch PCR and DNA cleanup Kit T1030S by New England Biolabs

10% Dextran sulfate by Contributed by users

20% Formamide by Contributed by users

2X SSC by Contributed by users

Please see additional Materials for Glucose Oxidase Prep (Step 90). by Contributed by users

Protocol

Fixing adherent cells

Step 1.

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

NOTES

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Want a bit of space between cells for clean segmentation.

Fixing adherent cells

Step 2.

Aspirate media and rinse cells with 1X PBS.

Fixing adherent cells

Step 3.

Aspirate PBS and fix with fixation solution for 10 minutes.

Fixing adherent cells

Step 4.

Aspirate fixation solution and rinse cells in 1X PBS. (1/3)

Fixing adherent cells

Step 5.

Rinse cells in 1X PBS. (2/3)

Fixing adherent cells

Step 6.

Rinse cells in 1X PBS. (3/3)

Fixing adherent cells

Step 7.

Aspirate 1X PBS and apply 70% EtOH to each well.

NOTES

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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

Step 8.

Request licensing permission from: https://flintbox.com/public/project/50547/

NOTES

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Raj lab probe design server--Matlab required

Designing the probes

Step 9.

Create a Bitbucket account/username: https://bitbucket.org/account/signup/

Designing the probes

Step 10.

When licensing permission is granted, email arjunrajlab@gmail.com your Bitbucket username and request access to the repository.

Designing the probes

Step 11.

https://bitbucket.org/account/signin/?next=/arjunrajlaboratory/probedesign

Designing the probes

Step 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:

cmd COMMAND

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

Designing the probes

Step 13.

Add the source code to your MATLAB path.

Designing the probes

Step 14.

Add the **rajlab** directory and subdirectories, and be sure to hit **Save**.

Designing the probes

Step 15.

Now you have the probe design software!

Designing the probes

Step 16.

If you have a smFISH probe set for your target of interest, make a snapgene file and add features for each probe.

Designing the probes

Step 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

Designing the probes

Step 18.

Select the species and search for the gene you want.

NOTES

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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

Designing the probes

Step 19.

Click on the RefSeq version of the gene. There may be many versions of the gene corresponding to different splice variants (isoforms).

Designing the probes

Step 20.

This will bring up a bunch of tracks. Here, you can visually examine the various isoforms.

Designing the probes

Step 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

Designing the probes

Step 22.

Click on the track of the isoform (under the heading "*RefSeq Genes*").

Designing the probes

Step 23.

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

For mRNA, start by searching in the CDS (If you are unsuccessful at finding enough probes int he CDS,

you can add the UTR in your search)

DO NOT mask the repeats.

Click Submit.

Designing the probes

Step 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.

Designing the probes

Step 25.

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

Designing the probes

Step 26.

Run this script in Matlab:

cmd COMMAND

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

Designing the probes

Step 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.

Designing the probes

Step 28.

The 30mer sequence output will look like this:

cmd COMMAND

```
tgttgatgttgtggcactttggtggctctg Podxl_exon_30mer_target_1
```

Designing the probes

Step 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:	left arm:
tgttgatgttgtggc	**actttggtggctctg**

right arm: left arm:

tgttgatgttgtggc actttggtggctctg

Designing the probes

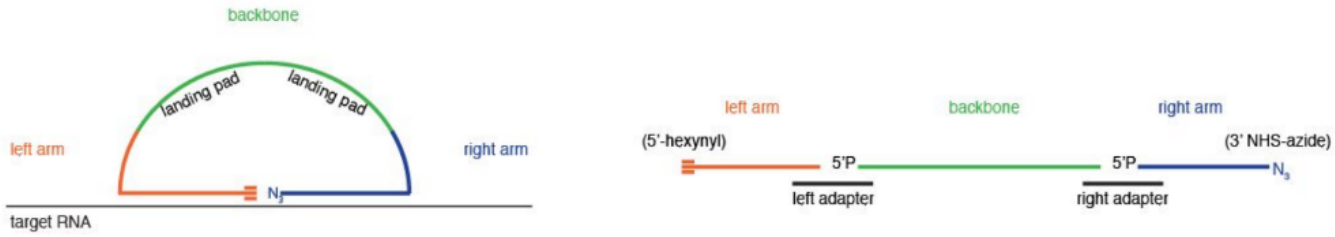
Step 30.

Now, 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:



cmd COMMAND

```
1. PODXl_P1_left: /5Hexynyl/actttggtggctctgACATCATAGT
2. PODXl_P1_right: /5Phos/aagtgactgt□tgttgatgttgtggc/3AzideN/
```

NOTES

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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

Step 31.

Combine reaction components without enzyme.

AMOUNT

1.5 µl Additional info: 400 µM left arm

AMOUNT

1.5 µl Additional info: 400 µM left adapter
AMOUNT
1.5 µl Additional info: 400 µM right arm
AMOUNT
1.5 µl Additional info: 400 µM right adapter
AMOUNT
1 µl Additional info: 400 µM backbone
AMOUNT
10 µl Additional info: 2X reaction buffer
AMOUNT
1 µl Additional info: NF H2O

NOTES
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Notes on making the probes:

**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 H_2O

Heat to 37°C for 5.5 hours, heat inactivate at 65°C 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 µl of 400 µM "Right arm" (with 3'OH), 5 µl of 4 mM NT analog (N6-(6-Azido)hexyl-3'-dATP from Jena Biosciences), 4 µl of TdT enzyme, 10 µl of Transferase buffer, 26 µl NF H_2O

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 µl to make ~400 µM stock oligo.

Making the probes

Step 32.

Heat reaction components to 70°C for 3 min.

TEMPERATURE
70 °C Additional info: Heating reaction components

Making the probes

Step 33.

Leave reaction components at room temperature for 5 min.

Making the probes
Step 34.

Add 2 µl of T7 DNA ligase (diluted 1:10 in NF H_2O).

AMOUNT
2 µl Additional info: T7 DNA ligase

Making the probes
Step 35.

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

Making the probes
Step 36.

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

NOTES
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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.

Making the probes
Step 37.

Add 80 µl of TE to the samples.

AMOUNT
80 µl Additional info: TE

Making the probes
Step 38.

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

AMOUNT
700 µl Additional info: Binding buffer

Making the probes
Step 39.

Spin down for 1 min.

Making the probes
Step 40.

Discard liquid.

Making the probes

Step 41.

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

AMOUNT
200 µl Additional info: Wash buffer

Making the probes

Step 42.

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

AMOUNT
200 µl Additional info: Wash buffer

Making the probes

Step 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.

AMOUNT
60 µl Additional info: Elution buffer

ClampFISH - Primary probe hybridization

Step 44.

Thaw clampFISH hybridization buffer.

ClampFISH - Primary probe hybridization

Step 45.

Label your samples - you can label the top part of the chamber, this will not be imaged.

ClampFISH - Primary probe hybridization

Step 46.

Make clampFISH hybridization mix: Use 50µl/well clampFISH hybridization buffer + 0.5 µl of each clampFISH primary probe.

AMOUNT
50 µl Additional info: clampFISH hybridization buffer
AMOUNT
0.5 µl Additional info: of each clampFISH primary probe

ClampFISH - Primary probe hybridization

Step 47.

Aspirate 70% ethanol from chamber slides.

ClampFISH - Primary probe hybridization

Step 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.

ClampFISH - Primary probe hybridization

Step 49.

Aspirate off wash buffer.

ClampFISH - Primary probe hybridization

Step 50.

Pipet 50 µl of clampFISH hybridization mix (prepared in step 51) into the middle of the well.

AMOUNT

50 µl Additional info: clampFISH hybridization mix

ClampFISH - Primary probe hybridization

Step 51.

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

ClampFISH - Primary probe hybridization

Step 52.

Humidify the hybridization chamber (kimwipe with wash buffer inside chamber is good enough).

ClampFISH - Primary probe hybridization

Step 53.

Use parafilm to seal the plate.

ClampFISH - Primary probe hybridization

Step 54.

Incubate at 37°C for a minimum of 4 hours, or overnight.

TEMPERATURE

37 °C Additional info: Incubation

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

Step 55.

Take samples from incubator, remove parafilm.

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

Step 56.

Add wash buffer to each well.

NOTES

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

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

Step 57.

Aspirate and replace wash buffer.

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

Step 58.

Seal the plate with parafilm.

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

Step 59.

Put the plate to 37°C for 20 minutes.

TEMPERATURE

37 °C Additional info: Incubation

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

Step 60.

Aspirate and replace wash buffer.

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

Step 61.

Seal the plate with parafilm.

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

Step 62.

Put the plate to 37°C for 20 minutes.

TEMPERATURE

37 °C Additional info: Incubation

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

Step 63.

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

AMOUNT

50 µl Additional info: clampFISH hybridization buffer

AMOUNT

1 µl Additional info: clampFISH secondary probe

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

Step 64.

Aspirate off wash buffer.

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

Step 65.

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

AMOUNT

50 µl Additional info: clampFISH hybridization mix

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

Step 66.

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

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

Step 67.

Humidify the hybridization chamber (kimwipe with wash buffer inside chamber is good enough).

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

Step 68.

Use parafilm to seal the plate.

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

Step 69.

Incubate at 37°C for a minimum of 2 hours

TEMPERATURE

37 °C Additional info: Incubation

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

Step 70.

Take samples from incubator, remove parafilm.

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

Step 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.

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

Step 72.

Aspirate and replace wash buffer.

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

Step 73.

Seal the plate with parafilm, put to 37°C for 20 minutes.

TEMPERATURE

37 °C Additional info: Incubation

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

Step 74.

Aspirate and replace wash buffer.

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

Step 75.

Seal the plate with parafilm, put to 37°C for 20 minutes.

TEMPERATURE

37 °C Additional info: Incubation

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

Step 76.

Aspirate wash buffer and replace with 2X SSC.

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

Step 77.

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.

	[stock]	**volume added**	**[final]**
$CuSO_4$	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 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.

TEMPERATURE

37 °C Additional info: Incubation

ClampFISH - Click reaction

Step 78.

Aspirate click reaction and replace with wash buffer.

ClampFISH - Click reaction

Step 79.

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

GOTO

Another round of hybridization -> go to step #55

ClampFISH- smFISH on the terminating backbone

Step 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)

AMOUNT

1 µl Additional info: 5 µM probe

AMOUNT

50 µl Additional info: smFISH hybridization buffer

NOTES

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

ClampFISH- smFISH on the terminating backbone

Step 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.

ClampFISH- smFISH on the terminating backbone

Step 82.

Incubate at 37°C for at least 6 hours.

TEMPERATURE

37 °C Additional info: Incubation

ClampFISH- smFISH on the terminating backbone

Step 83.

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

ClampFISH- smFISH on the terminating backbone

Step 84.

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

NOTES

Lenny Teytelman 25 May 2018

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

ClampFISH- smFISH on the terminating backbone

Step 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 µL nuclease-free (NF) H_2O
2. 40 µL 10% glucose
3. 100 µL 20X SSC
4. 10 µL 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 *--(GluOx)--> less O_2 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 µl of 10% glucose to 960 µl 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.

Warnings

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