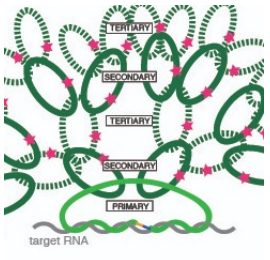
Jun 01, 2018

ClampFISH

DOI
https://dx.doi.org/10.17504/protocols.io.qeydtfw



Sara H Rouhanifard[1], Ian A Mellis[1], Margaret Dunagin[1], Sareh Bayatpour[1], Orsolya Symmons[1], Allison Cote[1], Arjun Raj[1]

[1]University of Pennsylvania

Human Cell Atlas Metho...

Sara Rouhanifard

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.qeydtfw

External link: https://www.biorxiv.org/content/early/2018/05/07/222794

Protocol Citation: Sara H Rouhanifard, Ian A Mellis, Margaret Dunagin, Sareh Bayatpour, Orsolya Symmons, Allison Cote, Arjun Raj 2018. ClampFISH . **protocols.io** https://dx.doi.org/10.17504/protocols.io.qeydtfw

Manuscript citation:

Exponential fluorescent amplification of individual RNAs using clampFISH probesSara H Rouhanifard, Ian A Mellis, Margaret Dunagin, Sareh Bayatpour, Orsolya Symmons, Allison Cote, Arjun Raj

doi: https://doi.org/10.1101/222794

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

Created: May 25, 2018

Last Modified: June 01, 2018

Protocol Integer ID: 12472

Keywords: clampFISH, fluorescent detection of rna, clampfish probe, clampfish signal, modular design of clampfish probe, oligonucleotide, rna levels via flow cytometry, specificity of oligonucleotide, probe detachment in expansion microscopy, clampfish, detection with low magnification microscopy, rna, low magnification microscopy, amplifying fish, expansion microscopy, rna level, fluorescent detection, microscopy, molecule resolution, flow cytometry, works in tissue sample

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

PDF

ClampFISH protocol_2...

220KB

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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F1635-500ML
- 95% EtOH
- Nuclease-free (NF) H2O
- T7 DNA ligase reaction buffer
- 5'hexynyl labeled probes with adapter region (left arm)-
- 3'NHS-azide probes with adapter region (right arm)
- Left and right adapters for each probe
- Backbone sequence
- Monarch PCR and DNA Cleanup Kit - 50 preps **New England Biolabs Catalog #**T1030S
- 10% Dextran sulfate
- 20% Formamide
- 2X SSC
- Please see additional Materials for Glucose Oxidase Prep (Step 90).

Safety warnings

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

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-click-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)

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 snapgene 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 int he 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

```
tgttgatgttgtggcactttggtggctctg Podxl_exon_30mer_target_1
```

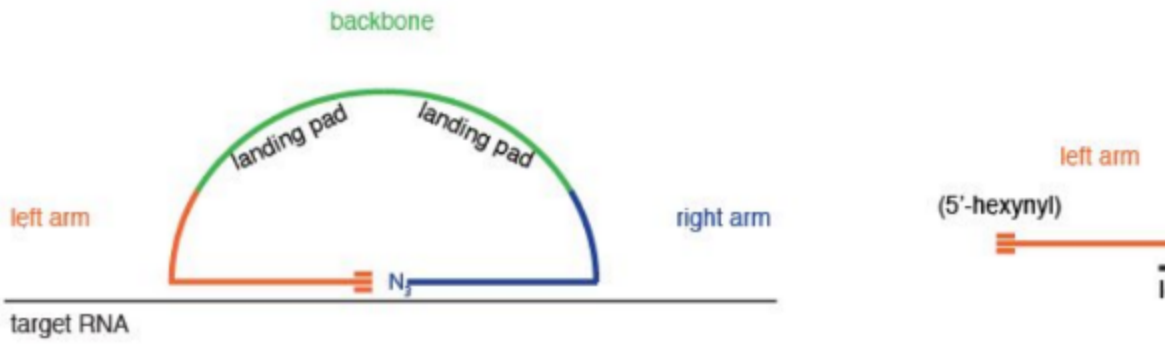
29 If you split this sequence in half, it will give you the sequence for the region of the probe that binds to the target:

	right arm: **tgttgatgttgtggc**	left arm: **actttggtggctctg**

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:



Command

```
1. PODXl_P1_left: /5Hexynyl/actttggtggctctgACATCATAGT
2. PODXl_P1_right: /5Phos/aagtgactgttgttgatgttgtggc/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 µL 400 µM left arm
- 1.5 µL 400 µM left adapter
- 1.5 µL 400 µM right arm
- 1.5 µL 400 µM right adapter

1 µL 400 µM backbone

10 µL 2X reaction buffer

1 µL NF H2O

Note

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.

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 H_2O).

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 $CuSO_4$ 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.

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 Another round of hybridization

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 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.