



Version 1 ▼

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# SHInE - Simultaneous HCR, Immunohistochemistry, Nuclear staining and EdU V.1

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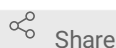
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Alexander Stockinger

## DISCLAIMER

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The authors would like to thank the members of the Tessmar- and Raible labs for fruitful discussions and support in developing and testing this protocol, our marine animal facility, and the Max Perutz Labs BioOptics facility. We would also like to acknowledge the Özpolat lab at WUSTL, particularly Duygu Özpolat and Ryan Null, for sharing protocols, experience and their probe maker tool with us.

## ABSTRACT

This protocol allows the multiplexed use of four different molecular labelling techniques in whole-mount *Platynereis* tissues. In short, gene expression (via in situ HCR 3.0), cell proliferation (via EdU labelling), proteins (via Immunohistochemistry) and nuclei (DAPI/Hoechst staining) can be assessed in whole-mount tissue samples, allowing the co-visualisation of various types of molecules in the same specimen.

This protocol combines knowledge from multiple sources (see below), and is being submitted in parallel with a manuscript detailing the applications of the method. (We will add the reference as soon as it's available.)

We successfully applied this protocol to heads and posterior regenerates of the bristleworm *Platynereis dumerilii*, at various developmental stages of the animal. Given the general nature of the detected molecules, and the wide-spread use of the individual detection techniques, we anticipate that this protocol will be well applicable to a wider range of model systems.

#### References:

- 1) Choi HMT, Calvert CR, Husain N, Huss D, Barsi JC, Deverman BE, Hunter RC, Kato M, Lee SM, Abelin ACT, Rosenthal AZ, Akbari OS, Li Y, Hay BA, Sternberg PW, Patterson PH, Davidson EH, Mazmanian SK, Prober DA, Rijn M van de, Leadbetter JR, Newman DK, Readhead C, Bronner ME, Wold B, Lansford R, Sauka-Spengler T, Fraser SE, Pierce NA. 2016. Mapping a multiplexed zoo of mRNA expression. *Development* 143:3632–3637. doi:10.1242/dev.140137
- 2) Choi HMT, Schwarzkopf M, Fornace ME, Acharya A, Artavanis G, Stegmaier J, Cunha A, Pierce NA. 2018. Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* 145:dev165753. doi:10.1242/dev.165753
- 3) Kuehn E, Clausen DS, Null RW, Metzger BM, Willis AD, Özpolat BD. 2021. Segment number threshold determines juvenile onset of germline cluster proliferation in *Platynereis dumerilii*. *Biorxiv* 2021.04.22.439825. doi:10.1101/2021.04.22.439825
- 4) Pende M, Vadiwala K, Schmidbaur H, Stockinger AW, Murawala P, Saghafi S, Dekens MPS, Becker K, Revilla-i-Domingo R, Papadopoulos S-C, Zurl M, Pasierbek P, Simakov O, Tanaka EM, Raible F, Dodt H-U. 2020. A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity. *Sci Adv* 6:eaba0365. doi:10.1126/sciadv.aba0365
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- 6) Tessmar-Raible K, Steinmetz PRH, Snyman H, Hassel M, Arendt D. 2005. Fluorescent two-color whole mount in situ hybridization in *Platynereis dumerilii* (Polychaeta, Annelida), an emerging marine molecular model for evolution and development. *Biotechniques* 39:460–464. doi:10.2144/000112023

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In submission

#### KEYWORDS

HCR, immunohistochemistry, in situ hybridisation, FISH, whole-mount, multiplexed, EdU, proliferation, Platynereis, Annelid

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#### GUIDELINES

- Please use aliquots of common stocks, including all buffers, to avoid repeated freeze-thaw cycles.
- The protocol is based on the detection of RNA, so minimize RNA degradation. It does not seem to be as critical as with sequencing experiments, but keep samples on ice whenever indicated, and work quickly in a clean setting.
- Protect samples from light once fluorescent dyes have been added to them!
- HCR signal seems to be best in the first 5 days after preparation of the samples, so ideally, image your samples right after preparing them.
- Many of the steps in this protocol can be optimized for individual samples or experimental conditions. We tried to indicate these steps. If you do come up with improved conditions for certain samples, we want to encourage you to upload a modified version of this protocol!

For multi-color fluorescence labeling, check whether the microscope you intend to use has lasers and filters available. HCR signal in general seems to only be visible on the confocal microscope. You can also check for bleed through risk and plan accordingly, for example using this tool:

<https://www.thermofisher.com/order/fluorescence-spectraviewer>

#### Probe maker:

Probes can be ordered from Molecular Instruments, alongside the amplifier hairpins that contain the fluorophore.

To design HCR probes yourself, you can use a Python based tool developed by Ryan Null in the Özpolat lab at WUSTL. It can be found here:

[https://github.com/rwnull/insitu\\_probe\\_generator](https://github.com/rwnull/insitu_probe_generator)

and was first referenced in

Kuehn E, Clausen DS, Null RW, Metzger BM, Willis AD, Özpolat BD. 2022. Segment number threshold determines juvenile onset of germline cluster expansion in *Platynereis dumerilii*. *J Exp Zoology Part B Mol Dev Evo* 338:225–240. doi:10.1002/jez.b.23100

In short, use a target sequence and the probe generator will generate sets of probes that contain the initiator sequence of your choice.

\* Probes can either be acquired through <https://www.molecularinstruments.com>, or self-designed (we use this python tool

[https://github.com/rwnull/insitu\\_probe\\_generator](https://github.com/rwnull/insitu_probe_generator)) and then ordered as oligos, for example through <https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos/custom-dna-oligos/opools-oligo-pools>

For more detailed instructions on EdU pulse-and-chase experiments in *Platynereis dumerilii*, please refer to

Zattara, E. E. & Özpolat, B. D. Developmental Biology of the Sea Urchin and Other Marine Invertebrates. *Methods Mol Biology* 2219, 163–180 (2020).

## MATERIALS TEXT

### Buffer recipes

#### Hybridization buffer (toxic, store at -20° C):

1x concentration	For 40ml
30% formamide	12 mL formamide
5x sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
0.1% Tween 20	400 µL of 10% Tween 20
50 µg/mL heparin	200 µL of 10 mg/mL heparin
1x Denhardt's solution	800 µL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill to 40 ml with ultrapure H <sub>2</sub> O

#### Wash buffer (toxic, store at -20° C):

1x concentration	For 40ml
30% formamide	12 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
0.1% Tween 20	400 µL of 10% Tween 20
50 µg/mL heparin	200 µL of 10 mg/mL heparin
	Fill up to 40 ml with ultrapure H <sub>2</sub> O

#### Amplification buffer (store at 4° C):

1x concentration	For 40ml
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 µL of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 ml with ultrapure H <sub>2</sub> O

#### 5x SSCT (store at 4° C):

1x concentration	For 40ml
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0,1% Tween 20	400µl of 10% Tween 20
	Fill up to 40µl with ultrapure H <sub>2</sub> O

#### 1X PTW:

1X PBS with 0.1% Tween-20

#### Tissue Clearing:

1. Pende, M. *et al.* A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity. *Sci Adv* **6**, eaba0365 (2020).

## Reagents

A	B	C
Reagent	Manufacturer	Product Number
Citric acid monohydrate	Sigma-Aldrich	C1909
Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye	Invitrogen™	C10337
Denhardt's solution 50x	Invitrogen™	750018
Dextran Sulfate 50% solution	Merck Millipore	S4030
Formamide	Sigma-Aldrich	47671
Glycine	Roth	3908.3
Heparin	Sigma-Aldrich	H3393
Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water	Invitrogen™	H3570
Magnesiumchlorid hexahydrate (MgCl <sub>2</sub> )	VWR	25.108.295
Methanol	VWR	20847
Methanol, suitable for HPLC	Sigma-Aldrich	34860
Paraformaldehyde (PFA)	Sigma-Aldrich	441244
Phosphate buffered saline (PBS)	Sigma-Aldrich	P4417
Proteinase K	Sigma-Aldrich	1.245.680.100
Saline-Sodium Citrate buffer 20× Concentrate (SSC 20X)	Sigma-Aldrich	S6639
SlowFade™ Diamond Antifade Mountant	Molecular Probes™	S36972
Thymidine	Sigma-Aldrich	T9250
Tween 20	Sigma-Aldrich	P1379

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#### Optional: EdU incubation

1  

Incubate worms with **10 micromolar ( $\mu\text{M}$ )** EdU in artificial sea water (ASW) (EdU aliquots in both DMSO and H<sub>2</sub>O work fine) for your desired pulse length.

2 

1h

Optional: Chase out EdU with **3 millimolar (mM)** thymidine for **01:00:00** ; then replace thymidine solution with fresh ASW and incubate for desired chase length

#### Day 1: Fixation, Dehydration

3 **Dissect tissue of interest**

Anesthetize worms in a 50% mix of 7.5% (w/v) MgCl<sub>2</sub> and ASW

Dissect the tissue of interest; transfer it to a 1,5ml microcentrifuge tube pre-filled with 1ml ASW **On ice**

*Note: the protocol is also compatible with whole animals, but will require larger volumes and more reagent accordingly.*

4 

1h

#### Fix the samples

Replace the ASW in the tube with 1ml 4%PFA/1XPTW (carefully pipet away the ASW; samples should sink to the tube bottom). Fix samples for **01:00:00** at **Room temperature** shaking gently on a rocking platform

*Make sure the samples are swimming back and forth freely in the tube; dislodge stuck samples by gently flicking the tube.*

5 

11m

## Dehydrate and store samples

Dehydrate the samples through a series of increasing MeOH concentrations diluted in 1X PTW.

Perform these steps on ice, and wait until the samples have settled down at the bottom of the well after each increase in MeOH concentration (takes around 1-3 minutes)

- 25% MeOH in PTW ⌚ 00:03:00
- 50% MeOH in PTW ⌚ 00:03:00
- 75% MeOH in PTW ⌚ 00:03:00
- Wash in 100% MeOH ⌚ 00:01:00
- Wash in 100% MeOH ⌚ 00:01:00

6 

Store samples at -20° C for at least ⌚ **Overnight** . Longer storage is possible (several months in our hands, but even longer storage might be possible).

Day 2: Rehydration, digestion, probe hybridization

1h 45m

12m

7 

**Re-hydrate samples** through a series of decreasing MeOH concentrations in 1X PTW on ice.

- 75% MeOH in PTW ⌚ 00:03:00
- 50% MeOH in PTW ⌚ 00:03:00
- 25% MeOH in PTW ⌚ 00:03:00
- Wash 2x in 1X PTW ⌚ 00:03:00

8 

**Perform proteinase K treatment** with 1ml of Proteinase K in PTW according to table below, at ⌚ **Room temperature** .



8.1 




This is a **critical, time-sensitive step** and all reagents needed for washes after digestion should be prepared in advance. Concentrations and digestion



times **should be adjusted to individual tissues** and possibly differences in proteinase K batches.

Tissue	PK concentration	time
Heads, Blastemas, adult tissues	100µg/mL	5'
Larvae, <1dpf	100µg/mL	30 seconds
Larvae, 3dpf	100µg/mL	2'




- 8.2 Briefly rinse 2x with 1 ml of glycine wash buffer to stop digest. Work  **On ice** <sup>5m</sup> from here on.  
Wash once for  **00:05:00** with 1x PTW.

- 8.3  <sup>20m</sup>  
Post-fix in 1mL 4%PFA diluted in 1xPTW for  **00:20:00** shaking while still  **On ice** .

- 8.4 Wash samples 2x  **00:05:00** in 1ml PTW. <sup>5m</sup>

## 9 <sup>20m</sup>

### Optional: tissue clearing

If clearing with DEEP-CLEAR is wanted, perform it at this step. Worm tissue can be cleared at  **37 °C** for  **00:15:00** at 300 rpm on a heat block. Clearing time should be optimised for different tissues. Wash the samples in PTW thoroughly after clearing (3x  **00:05:00** ).

*Note: in Platynereis tissues, the effects of clearing depend on the tissue sampled. Opaque tissues such as eyes benefit from clearing, while naturally transparent tissues like blastemas don't.*

*For details on tissue clearing, please refer to*

Pende, M.*et al.* A versatile depigmentation, clearing, and labeling method for exploring

## 10 Probe hybridization with HCR probe(s) of choice.

*Note: a weak probe signal might be improved by increasing the probe concentration; several labs have reported successfully reusing the probe solution (simply freeze the probe mix after use)*

10.1



1h 5m

Incubate in 1ml 50% hybridization buffer in PTW for 00:05:00 at

Room temperature

Incubate in 300µl 100% hybridization buffer for 01:00:00 at 37 °C on a heat block .

*Careful when replacing media to not lose any samples. The buffer is very viscous; wait until samples settle down to the bottom of the tube.*

10.2



Meanwhile prepare the probe solution:

- 1pmol of each probe mix (1µl of 1µM stock) in 250µl hybridization buffer
- heat the probe solution to 37°C (for small volumes, we use the heat block the samples are already on; for larger volumes, we use a water bath)

10.3



Replace the hybridization buffer with the prepared probe solution and incubate the samples Overnight at 37 °C .

Day 3: Washes

1h 16m 30s

11



1h 10m

**Wash out the HCR probes:**

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:05:00 with 1ml 5X SSCT at Room temperature

Wash for 🕒00:05:00 with 1ml 5X SSCT at 🌡 Room temperature

**From here on, the protocol progresses differently for each combination of methods**  
(HCR only, HCR & EdU, HCR & IHC, HCR, EdU & IHC)

**Please select the step case below accordingly:**

Step 11 includes a Step case.

**HCR only**

**HCR & EdU**

**HCR & IHC**

**HCR, EdU & IHC**

Day 3: HCR probe detection 1h 16m 30s

step case

### HCR only

This is the bare-bones protocol to just stain your samples with HCR.

## 12 HCR Amplification:

12.1 Wash with 1ml PTW for 🕒00:05:00 at 🌡 Room temperature 35m  
Equilibrate samples in 300µl amplification buffer for 🕒00:30:00 at  
🌡 Room temperature

12.2 **Meanwhile:** 31m 30s  
Prepare the HCR hairpin mix.

- Use 15 pmol per hairpin (5µl of 3µM stock) - move them to individual 1,5ml microcentrifuge tubes\*
- Heat the hairpins to 🌡 95 °C for 🕒00:01:30 by adding them to a pre-heated heatblock
- Let the hairpins cool to 🌡 Room temperature protected from light for 🕒00:30:00
- Mix the cooled hairpins with amplification buffer to a total volume of 250µl

*\* NOTE: for each amplifier, TWO different hairpins have to be used! (e.g., B1H1 and B1H2 are a pair of hairpins compatible with the B1 amplifier sequence) - heat those in separate tubes and pool only directly before adding them to the sample!*

*\* NOTE: several probe pairs can be co-detected in one sample by using different sets of amplifiers with different fluorophores.*

- 12.3 Incubate the samples in 250 µl hairpin mix 🕒 **Overnight** protected from light at 🌡 **Room temperature** .

*Note: amplification buffer is viscous; samples will eventually equilibrate and sink to the bottom of the tube, but careful pipetting is recommended to not lose any samples.*

Day 4: Amplification Termination

1h 25m

1h 40m

### 13 Terminate the amplification:

Wash in 1ml 5X SSCT for 🕒 **00:05:00** at 🌡 **Room temperature**

Wash in 1ml 5X SSCT for 🕒 **00:05:00** at 🌡 **Room temperature**

Wash in 1ml 5X SSCT for 🕒 **01:00:00** at 🌡 **Room temperature**

Wash in 1ml 5X SSCT for 🕒 **00:05:00** at 🌡 **Room temperature**

Wash in 1ml PTW for 🕒 **00:05:00** at 🌡 **Room temperature** shaking on rocking platform

Staining of nuclei (DAPI/ Hoechst) can be performed here by adding the dye directly to PTW and increasing the incubation time to 🕒 **00:15:00** .

Wash in 1ml PTW for 🕒 **00:05:00** at 🌡 **Room temperature** shaking on rocking platform

### 14 Mount the samples:

Mounting strategies may differ based on sample type/ size.

For whole mount *Platynereis* blastemas or heads, the following works well:

Equilibrate the sample in a small volume of mounting medium (in our hands, SlowFade Diamond worked best)

Put four small pieces of "pattafix" or similar poster mounting clay (Blu-tack or similar) into the corners of the "mounting area" of a microscope slide to build a stage.

Put the sample in the middle of the stage, and add a small volume (~10µl) of mounting medium on top of it.

Gently press down a cover glass, using a second microscope slide to keep it even.