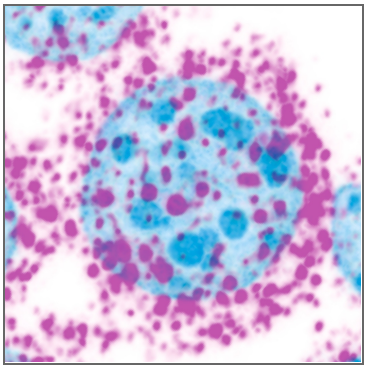

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origamiFISH (Cell Culture)

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ABSTRACT

This is the origamiFISH protocol for cell culture work, as outlined in the associated paper "Universal, label-free, single-molecule visualization of DNA origami nanodevices across biological samples using origamiFISH" (PMID: 37500778; Nat. Nanotechnol.; 2023). This protocol is meant to complement the methods section of the original paper, in providing a more detailed workflow for origamiFISH. We hope this will allow easier implementation of origamiFISH by other laboratories.

MATERIALS

1. Poly-D-lysine (PDL): Sigma #P0899 or ThermoFisher #A3890401

2. Coverslips: can be purchased from any supplier. A few options are: EMS #72230-01, NeuVitro #GG-12-15H

3. PFA: EMS #15710

4. Coverslip rack: EMS #70366-16

5. SSC: ThermoFisher #15557044

6. Forceps: any #3 or #5 forcep appropriate for coverslip handling. A few suggestions: FST #11231-30, FST #11252-30

7. Hybridization buffer: can be ordered directly from Molecular Instruments website for the desired volume. Make sure to select the appropriate hybridization buffer for cell culture work.

8. Wash buffer: can be ordered directly from Molecular Instruments website for the desired volume. Make sure to select the appropriate hybridization buffer for cell culture work.

9. Amplification buffer; can be ordered directly from Molecular Instruments website

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for the desired volume.

10. origamiFISH probes: sequences can be found in Supplementary Table 1 of the origamiFISH paper. The oligos can be ordered directly from IDT as 25nmol or 100nmol format STD - we typically order as an oligo plate. We typically make 100μM stock solutions of each oligo for long-term storage, and then aliquots of probe pool working stocks (i.e. containing all probes) at 1μM. This makes it such that you can add 1μL of working stock probe pool per 100μL hybridization buffer for the probe solution.

11. HCR hairpins: can be ordered directly from Molecular Instruments website for the desired volume and color combinations. The origamiFISH probe sequences provided above correspond to the B2 and B3 HCR initiators. Therefore, if you would like to perform origamFISH in Alexa 647, purchase the B2-647 or B3-647 hairpins and use with the corresponding probes. Likewise if you would like to perform origamiFISH in Alexa 488, purchase the B2-488 or B3-488 hairpins. If you want to perform dual origamiFISH and staple-initiated origamiFISH, simply use B2 for one, and B3 for the other.

12. Fluoromount-G: Southern Biotech #0100-01

DNA origami (DN) Uptake

1 NOTE: DN uptake can occur in any vessel of choice to fit experimental needs. We typically perform DN uptake in a 24-well plate containing 12mm PDL-treated #1.5 glass coverslips. PDL treatment ensures cell adherence, and #1.5 glass coverslips ensure compatibility with downstream confocal microscopy. This is the most economical option for sample processing, but does require that the user be familiar with coverslip handling, as movement of coverslips is necessary throughout the protocol. Coverslips can be treated in house (see "PDL Treatment" step below), or purchased commercially. If an easier alternative is preferred, we recommend performing DN uptake and origamiFISH in 24-, 48-, or 96-well #1.5 glass coverslip bottom plates from Mattek. This may additionally be a good option for high-throughput sample processing and imaging.

2 On the day before DN uptake, plate cells such that they will be ~70-80% confluent by the following day.

3 At time of uptake, remove cell media and replace with the desired concentration of DNs in

complete media (or media of choice). The volume of media can be modified by the user. We find that 200 - 300µL is appropriate for 24-well plates. If a 200µL volume is used, rock the plate back and forth a few times to ensure even coverage.

4 Incubate in a TC incubator for the desired timeframe. We typically perform uptake for anywhere between 1 minute to 4 hours.

5 Following uptake, remove DN-containing media and fix immediately in 4% PFA for 10 minutes at room temperature (RT). Although any brand of 4% PFA can be used, we recommend EM grade PFA for optimal sample quality. We typically store unused 16% PFA (but not 4% PFA) at -20˚C and reuse up to three freeze-thaw cycles.

6 Following fixation, rinse the sample once with 1x PBS, followed by 3x washes for 10-15 minutes each. Although it is recommended to wait the full 10 minutes for washes as is field standard in protocols, we often perform 5x quick rinses instead with no difference in results. Likewise, longer washes are also fine. The point is to remove the PFA. However that is done will be ok.

PDL treatment for glass coverslips

7 NOTE: Glass coverslips should be treated with poly-D-lysine prior to cell uptake. Similar procedures can be followed for uncoated glass bottom plates.

8 Clean coverslips by immersing in 1N HCl overnight. We typically do this in a coverslip rack placed in a beaker, and process up to 50 coverslips at a time as excess coverslips can be stored at 4˚C for 1-2 months.

9 After HCl treatment, wash coverslips extensively with water. We typically perform this step by completely submerging the coverslip racks with water at least 5 times.

10 After the last wash, place the coverslips in 70% ethanol and bring into the tissue-culture BSC to perform the remaining steps. Note that cleaned coverslips can be stored in ethanol or dry if a stopping point is desired.

11 Remove the ethanol and allow coverslips to dry COMPLETELY.

12 Place dried coverslips into 24-well plates using forceps, 1 coverslip per well.

13 Add ~300-500µL of 0.1 mg/mL PDL solution to each well. Sometimes the coverslips float after PDL solution is added. If this happens, push it down using forceps...

14 Incubate in PDL solution overnight at RT.

15 Remove the PDL solution - if desired this solution can be stored and reused up to three times. Wash the coverslips with water extensively. We typically wash 5 times.

16 Treated coverslips can either be used directly, or stored at 4˚C for 1-2 months dry or in water.

Permeabilization

17 Following fixation, samples should be permeabilized prior to performing origamiFISH. Although, in cases where dual IHC is desired and where ethanol will interfere with antibody stainings, we have found that origamiFISH can also be performed without ethanol permeabilization with slight decreases in signal.

18 Fixed samples can be permeabilized in 70% ethanol for either 1 hour at RT, or overnight at -20˚C. If origamiFISH cannot be performed right away, the sample can also be left at -20˚C in ethanol for an extended amount of time (make sure to seal the plate with parafilm or a plate sealing film to prevent evaporation).

However, if absolute consistency in results is desired (i.e. for quantifications between conditions), we recommend processing samples directly after overnight -20˚C permeabilization. If storage is needed prior to this step, the samples can also be placed in 1x PBS at 4˚C, until overnight permeabilization can be performed.

Hybridization

19 NOTE: If using 12mm glass coverslips, we recommend practicing coverslip handling prior to proceeding with the hybridization step. In short, you should be able to pick up the coverslip using forceps and handle with ease (i.e. transferring coverslips between wells, flipping the coverslip etc).

20 Remove the sample from the freezer shortly before hybridization (you can use a kimwipe to wipe the outside and bottom of the plate if there is frost/condensation, in order to see the samples better). Remove the 70% ethanol and allow the coverslip to dry completely (i.e until the 'shine' on the coverslip is gone and it becomes visibly more matt) - this is important as ethanol will react with the hybridization buffer. Alternatively, the sample can also be rinsed with PBS a few times instead of waiting for it to dry.

21 To perform denaturation, a few options are possible. The point here is to place the sample in hybridization buffer (Molecular Instruments) for heat denaturation. This can either be done by adding ~200 - 300µL of hybridization buffer to the sample directly, or by adding 100µL of hybridization buffer to an empty well first, and placing the coverslip on top, cell side down. The only difference is the cost of the experiment.

22 After introducing hybridization buffer to the sample, place the plate on a 50˚C hot plate or in a 50˚C incubator for 10-15 minutes.

23 Remove the hybridization buffer and replace with probe solution containing 1-10nM origamiFISH probes in hybridization buffer. We did not find that origamiFISH probe concentration made a differentiable difference to signal, as expected from RNA smFISH literature. Therefore, we typically used 10nM (per probe concentration, not total concentration) to avoid pipetting less than 1µL solutions. Similar to the denaturation step, this can either be done in 300µL of hyb buffer placed directly into the well, or by placing a 100µL drop of hyb buffer on top of which the coverlip is placed, cell-side down.

24 Probe hybridization is performed overnight at 35˚C in an incubation chamber. We typically fill the empty wells with water to create a humidified chamber and to prevent buffer evaporation.

25 The following day, wash the samples 3x in wash buffer (Molecular Instruments) at 37˚C, for 15 minutes each. We recommend performing a quick rinse with wash buffer prior to starting the washes to remove as much of the probes as possible. If the coverslip was hybridized cell side down, it should be flipped before starting the washes.

26 Rinse the sample in 2x SSCT (0.1% Triton-X in 2x SSC buffer).

HCR signal amplification

27 As the samples are being washed, prepare the HCR hairpins for amplification.

28 Prepare metastable hairpins following manufacturer's instructions (Molecular Instruments), by heating the respective hairpins (in separate tubes to prevent premature amplification) to 95˚C and cooling slowly to RT over 30 minutes. We typically perform the heating step in a PCR machine.

29 We typically use 2μL of each HCR hairpin (i.e. 2μL of H1 and 2μL of H2) per sample, dissolved in 100μL of amplification buffer. Due to the small volume, this is the one step where we would strongly recommend placing the coverslip onto the drop of amplification buffer, cell side down. Unless cost is not a consideration, in which case the volume of hairpin and amplification buffer can be increased for easier sample handling.

30 Amplification is performed in the dark for 3 hours at RT.

NOTE: Make sure the samples are wrapped in aluminum foil or protected from light for all of the remaining steps.

31 Remove the amplification buffer and wash the samples in 2x SSCT for 3x 15 minutes. If the samples were amplified cell-side down, it should be flipped before starting the washes.

Sample mounting

32 DAPI staining can be performed according to manufacturer's instructions if desired. We typically do this for 5-10 minutes at RT at 1:3000 concentration in 2x SSCT.

33 The coverslip can then be mounted onto a slide for imaging. We typically do this in Fluoromount-G mounting media although it can certainly be substituted with other mounting media of choice. We typically place the coverslips onto a kimwipe (cell-side up) until it is dried - the edges of the coverslip can be dabbed onto the kimwipe to speed this process up.

We then place a small drop of mounting media a slide, and place the coverslip, cell-side down, onto the mounting media until it spreads out evenly. The coverslip can be pushed down gently to ensure even spread. We do not typically use an exact volume of mounting media and so have not provided here. This is because trying to pipette an exact volume of viscous mounting media typically introduces bubbles. As a result, we typically pipette in excess (~70µL) and introduce a small drop onto the slide, enough to cover the coverslip without pooling in excess.