Chapter 9

ExCel: Super-Resolution Imaging of *C. elegans* with Expansion Microscopy

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Abstract

Studies of *C. elegans* will benefit from a powerful method for super-resolution imaging of proteins and mRNAs at any 3-D locations throughout the entire animal. Conventional methods of super-resolution imaging in *C. elegans*, such as STORM, PALM, SR-SIM and STED, are limited by imaging depths that are insufficient to map the entire depth of adult worms, and involve hardware that may not be accessible to all labs. We recently developed expansion of *C. elegans* (ExCel), a method for physically magnifying fixed whole animals of *C. elegans* with high isotropy, which provides effective resolutions finer than the diffraction limit, across the entire animal, on conventional confocal microscopes. In this chapter, we present a family of three detailed ExCel protocols. The standard ExCel protocol features simultaneous readout of diverse molecules (fluorescent proteins, RNA, DNA, and general anatomy), all at ~70 nm resolution (~3.5× linear expansion). The epitope-preserving ExCel protocol enables imaging of endogenous proteins with off-the-shelf antibodies, at a ~ 100 nm resolution (~2.8× linear expansion). The iterative ExCel protocol allows readout of fluorescent proteins at ~25 nm resolution (~20× linear expansion). The protocols described here comprise a versatile toolbox for super-resolution imaging of *C. elegans*.

Key words Super-resolution microscopy, Expansion microscopy, *C. elegans*, Immunohistochemistry, In situ hybridization, RNA expression analysis, Molecular mapping

1 Introduction

Super-resolution microscopy enables observation of biological structures that are smaller than the diffraction limit of light (~250 nm [1]). Many super-resolution imaging methods have been adapted to *C. elegans*, including STORM [2, 3], PALM [3, 4], SR-SIM [5], STED [6], and expansion of *C. elegans* (ExCel) [7]. Among these methods, ExCel offers multiple unique advantages. Notably, ExCel enables imaging depths that are sufficient to map the entire depth of worms at any developmental stage. While worm diameter ranges from ~15 to ~60 μm, from L1 to

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adulthood, STORM, PALM, SR-SIM, and STED have imaging depths restricted to 1–20 μm; the ExCel imaging depth is >100 μm and ExCel has no requirement for any specialized imaging hardware other than a standard confocal microscope. ExCel also provides demonstrated capability to perform simultaneous imaging of multiple molecular types (protein, RNA, and DNA) in the context of anatomical structures marked by an amine-reactive stain, including multiple endogenous proteins detected with high sensitivity. Finally, ExCel enables all of the above in the context of intact animals.

ExCel is based on previously established molecular strategies of expansion microscopy (ExM) [8–17], a family of protocols for physically enlarging biological specimens with high isotropy and nanoscale precision. To perform standard ExCel, intact, cuticle-enclosed animals are first fixed and treated with small molecular weight (<1 kDa), cuticle-permeable reagents that add a hydrogel-anchorable moiety to general proteins [12] and nucleic acids [9]. Animals are then embedded into an expandable hydrogel, a process during which anchor-equipped proteins and nucleic acids are covalently linked to the hydrogel network, thus preserving their relative positions throughout the downstream expansion procedures. Next, a protease treatment, with Proteinase K, is applied to the gelled samples, which both mechanically softens the tissue for uniform expansion, and permeabilizes the cuticle for antibody access. Antibodies and in situ hybridization probes are applied at this cuticle-permeabilized state, to label fluorescent proteins (which survive the protease treatment) and nucleic acids, respectively. The stained sample is then expanded to a linear expansion factor of ~3.3–3.8×, and imaged on a standard confocal microscope at a sub-diffraction-limit effective spatial resolution of ~65–75 nm. With the standard ExCel protocol, fluorescent proteins, mRNA molecules, DNA, and general anatomical structures can be simultaneously visualized, all at sub-diffraction-limit resolutions, at any location throughout the entire anatomy of the worm.

A major limitation with the standard ExCel protocol is that fluorescent proteins are the only ones that can be detected, because their structure allows them to withstand the protease digestion, which in turn enables them to be detected by immunostaining. Under this limitation, studies of endogenous protein targets require the users to transgenically fuse the protein of interest to a fluorescent reporter. This limitation is resolved by an alternative version of ExCel, epitope-preserving ExCel, which replaces the epitope-disrupting Proteinase K treatment with an epitope-friendly combination treatment based on cuticle-disrupting collagenase and heat-mediated protein denaturation. After this combination of treatments, the majority (~70% [7]) of endogenous proteins assessed could be labeled with off-the-shelf antibodies. This protocol has slightly worse expansion isotropy (8–25% length

measurement error vs. 1–6% error from the standard ExCel protocol), a level that is still sufficient for many biological analyses that do not require precise length measurements, such as examining co-localization of two biomolecules, or the relative organization of biomolecules. Because this combination of treatments incompletely homogenizes worm tissue, a second round of hydrogel expansion is applied to the stained samples to compensate for the reduced expansion factor. Epitope-preserving ExCel achieves a final expansion factor of ~2.8×, allowing multiplexed imaging of endogenous protein targets at spatial resolutions of ~70–100 nm on a conventional confocal microscope.

A third protocol, iterative ExCel (iExCel), was developed for applications that would benefit from further sample expansion and finer spatial resolution (~25 nm). In iExCel, samples are first processed as in the standard ExCel protocol, including the strong, non-specific Proteinase K digestion, until immunostaining. An oligo-nucleotide-conjugated antibody is applied to stain for a target fluorescent protein. Then, a total of two rounds of tissue expansion are performed, where the oligonucleotide serves to both transfer stained signals across different hydrogels and also to amplify signal. Although iExCel enables finer spatial resolution, the protocol only supports the readout of fluorescent proteins in its current state. In addition, iExCel is more technically demanding, requiring longer processing time, and yielding fluorescent signals (over the same voxel size) weaker than standard ExCel. We therefore recommend that all users first confirm that the fluorescent protein of interest, expressed within the strain of interest, can be brightly observed under the standard ExCel protocol prior to applying iExCel.

2 Materials

The default storage condition, for all reagents listed in this section unless specified otherwise, is at room temperature (RT) for up to 6 months. Any storage condition that differs from the default is noted after the reagent recipe. Refer to expansionmicroscopy.org for a list of vendors and product numbers that the authors have used and had successful results with. Pay extra attention to the quality of sodium acrylate (a major component of all monomer solutions used in this protocol), whose stock quality can vary significantly across batches and between vendors (*see* **Note 1**).

2.1 Common for all ExCel Protocols

2.1.1 Fixation

1. PFA Fixative: 1 mL 10× PBS (final 1×), 2.5 mL 16% paraformaldehyde (final 4%), 6.5 mL ddH_2O (*see* **Note 2**).
2. 40× Borate Buffer Stock: 3.1 g boric acid (final 1 M), 1.0 g NaOH (final 0.5 M), add 45 mL of ddH_2O, and vortex to fully

dissolve the chemicals. Then, add ddH_2O until the volume reaches 50 mL.

3. BT: 1 mL 40× Borate Buffer Stock (final 1×), 1 mL 20% (v/v) Triton X-100 (final 0.5%), 38 mL ddH_2O.
4. BTB: 1 mL 40× Borate Buffer Stock (final 1×), 1 mL 20% (v/v) Triton X-100 (final 0.5%), 0.8 mL 2-mercaptoethanol (final 2%), 37.2 mL ddH_2O (*see* **Note 3**).
5. PBST-0.5%: 1× PBS, 0.5% (v/v) Triton X-100.

2.1.2 RNA and Protein Anchoring

1. AcX Stock: 10 mg/mL AcX (Acryloyl-X, SE; re-suspended in anhydrous DMSO) (*see* **Note 4**).
2. LabelX Stock: 100 μL of 1 mg/mL Label-IT Amine Modifying Reagent (re-suspended in the vendor-provided Reconstitution Solution), 10 μL of AcX Stock; incubate overnight at RT for the chemical reaction to complete, then store at −20 °C until needed (*see* **Note 5**).
3. RNA Anchoring Buffer: 20 mM MOPS pH 7.7, 0.1% (v/v) Triton X-100.
4. Protein Anchoring Buffer: 100 mM MES pH 6.0, 150 mM NaCl, 0.1% (v/v) Triton X-100.
5. MOPST-0.1%: 100 mM MOPS pH 7.0, 150 mM NaCl, 0.1% (v/v) Triton X-100.

2.1.3 Gelation and Expansion

1. Monomer Solution Stock: 11.36 mL 33% (w/w) sodium acrylate, 2.5 mL 50% (w/w) acrylamide, 5 mL 5% (w/w) *N*,*N*′-diallyl-tartardiamide (DATD crosslinker), 20 mL 5 M NaCl, 2.5 mL 1 M MOPS pH 7.0, 5.64 mL ddH_2O (*see* **Note 6**).
2. Non-activated Monomer Solution: 925 μL Monomer Solution Stock, 5 μL 20% (v/v) Triton X-100, 70 μL ddH_2O (*see* **Note 7**).
3. Activated Monomer Solution: 925 μL Monomer Solution Stock, 5 μL 20% (v/v) Triton X-100, 30 μL 0.5% (w/w) 4-Hydroxy-TEMPO (4-HT), 20 μL 10% (w/w) *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), 20 μL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see* **Note 8**).
4. Non-expanding Digestion Buffer: 50 mM Tris pH 8.0, 0.5 M NaCl, 40 mM $CaCl_2$, 0.1% (v/v) Triton X-100 (*see* **Note 9**).
5. TNT Buffer: 50 mM Tris pH 8.0, 1 M NaCl, 0.1% (v/v) Triton X-100.

2.1.4 General Purpose Buffer

1. 20× SSC: 3.0 M NaCl, 0.3 M sodium citrate, pH to 7.0.
2. 5× SSCT: 5× SSC, 0.1% (v/v) Tween 20.
3. PBST-0.1%: 1× PBS, 0.1% (v/v) Triton X-100.

2.1.5 Antibody Staining

1. Antibodies against the target fluorescent protein (for standard ExCel and iterative ExCel protocols) or other antigens (for the epitope-preserving ExCel protocol) (*see* **Notes 10–12**).

2.2 Standard Expansion of C. elegans

2.2.1 ExFISH-HCR (for RNA Readout)

1. Re-embedding Monomer Solution: 1 mL 40% Acrylamide/Bis-acrylamide 19:1 40% (w/v) solution (commercially available), 50 µL 1 M Tris base, 8.85 mL ddH_2O, 50 µL 10% (w/w) *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), 50 µL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see* **Note 13**).
2. Probe Set Stock Solution (*see* **Note 14**).
3. HCR Amplifier Stock Solution (*see* **Note 15**).
4. Probe Hybridization Buffer (*see* **Note 16**).
5. Probe Wash Buffer (*see* **Note 17**).
6. Probe Amplification Buffer (*see* **Note 18**).

2.3 Epitope-Preserving Expansion of C. elegans

1. Collagenase VII Stock Buffer: 2.5 mL 1 M Tris pH 8.0, 1.5 mL 5 M NaCl, 50 µL 1 M $CaCl_2$, 45.95 mL ddH_2O.
2. Collagenase VII Dilution Buffer: 7.5 mL 1 M Tris pH 8.0, 8.5 mL 5 M NaCl, 4 mL 1 M $CaCl_2$, 30 mL ddH_2O.
3. Collagenase VII Stock Solution: Add Collagenase VII Stock Buffer to Collagenase VII powder, to a final concentration of 1 kU/mL (*see* **Note 19**).
4. TNC-40020 Buffer: 2.5 mL 1 M Tris pH 8.0, 4 mL 5 M NaCl, 1 mL 1 M $CaCl_2$, 42.5 mL ddH_2O.
5. Protein Denaturation Buffer: Added in the following order, 25 mL ddH_2O, 2.5 mL 1 M Tris base, 4 mL 5 M NaCl, 1 mL 1 M $CaCl_2$, adjust pH to 9.0 with 5 M HCl, add ddH_2O until the volume reaches 35.7 mL, vortex the solution to mix before SDS addition, 14.3 mL 20% (w/w) sodium dodecyl sulfate (SDS), vortex to mix, and store solution at 37 °C. Prolonged storage (>1 h) at RT causes SDS to precipitate, which can be reversed by heating the solution to 70–95 °C (*see* **Note 20**).
6. Epitope-preserving ExCel G2 Monomer Solution Stock: 11.36 mL 33% (w/w) sodium acrylate, 2.5 mL 50% (w/w) acrylamide, 3.75 mL 2% (w/w) *N*,*N*′-Methylenebisacrylamide (Bis crosslinker), 2.5 mL 1 M MOPS pH 7.0, 1.5 mL 5 M NaCl, 2 mL 1 M $CaCl_2$, 23.39 mL ddH_2O (*see* **Note 21**).
7. Non-activated G2 Monomer Solution: 930 µL Epitope-preserving ExCel G2 Monomer Solution Stock, 70 µL ddH_2O (*see* **Note 22**).
8. Activated G2 Monomer Solution: 930 µL Epitope-preserving ExCel G2 Monomer Solution Stock, 30 µL 0.5% (w/w)

4-Hydroxy-TEMPO (4-HT), 20 μL 10% (w/w) *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), 20 μL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see* **Note 23**).

9. DATD-cleaving solution: 3.8 mL ddH_2O, 200 μL of 500 mM sodium meta-periodate, 500 μL of 10× PBS, Adjust pH to 5.5 with 5 M HCl, add ddH_2O until the volume reaches 5.0 mL (*see* **Note 24**).

2.4 Iterative Expansion of C. elegans (iExCel)

1. DNA-conjugated Antibody Staining Buffer: 4 g of 10% (w/v) Dextran sulfate, 2 mL of 20× SSC, 1 mL of 20 mg/mL baker's yeast tRNA, 1 mL of 100% normal donkey serum, 11.9 mL ddH_2O, 100 μL of 20% Triton X-100 (*see* **Note 25**).
2. iExCel G2 Monomer Solution: 2 mL 50% (w/w) acrylamide, 1 mL 5% (w/w) *N*,*N*′-diallyl-tartardiamide (DATD crosslinker), 6.9 mL of ddH_2O, 50 μL 10% (w/w) *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), 50 μL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see* **Note 26**).
3. iExCel hybridization buffer: 4× SSC, 20% (v/v) formamide.
4. iExCel G3 Monomer Solution Stock: 11.36 mL 33% (w/w) sodium acrylate, 2.5 mL 50% (w/w) acrylamide, 3.75 mL 2% (w/w) *N*,*N*′-methylenebisacrylamide (Bis crosslinker), 20 mL 5 M NaCl, 5 mL 10× PBS, 4.39 mL ddH_2O (*see* **Note 27**).
5. Activated iExCel G3 Monomer Solution: 930 μL Bis-crosslinked Expanding G3 Monomer Solution, 30 μL 0.5% (w/w) 4-Hydroxy-TEMPO (4-HT), 20 μL 10% (w/w) *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), 20 μL 10% (w/w) Ammonium persulfate (APS; activator; add last) (*see* **Note 28**).
6. DATD-cleaving solution: Exactly the same recipe as the "DATD-cleaving solution" in Subheading 2.3.
7. Buffer A: 150 mM NaCl, 100 mM Na_2HPO_4, pH to 7.4.
8. Buffer C: 150 mM NaCl, 100 mM Na_2HPO_4, pH to 6.0.
9. Unconjugated whole IgG secondary antibody, which matches the host species of the primary antibody against the fluorescent protein (*see* **Note 29**).
10. DNA oligo-nucleotides to be conjugated with secondary antibodies. *See* Table 4 for sequences. For readout of a single fluorescent protein target, use only the B1–B2 system. For readout of two targets, additionally use the A2–A1 system.
11. LNA oligo-nucleotides. *See* Table 4 for sequences. Order at a synthesis scale of at least 250 nmol.

3 Methods

All three ExCel protocols involve at least 10 days of sample processing (from the beginning of fixation to final imaging), with daily tasks that takes ~3–6 h total, within which ~50% or more are incubation time (during which the user does not need to perform physical tasks). To facilitate planning of experiments, we have added the marker "**" at the end of certain steps throughout these protocols, to symbolize steps that we typically end a day with (as many of these steps correspond to overnight incubations). We have also added sample schedules for each of the three protocols (*see* Tables 1, 2, 3).

3.1 Standard Expansion of C. elegans (ExCel)

3.1.1 Overview

1. 3.3–3.8× linear expansion factor.
2. Supports readout of fluorescent proteins, RNAs, DNA, anatomical structures.
3. High isotropy (1–6% error over length scales between 0 and 100 μm).
4. Protocol duration, from fixation to imaging:
   (a) For readout of fluorescent proteins only: 10 days.
   (b) For readout of fluorescent proteins and RNAs: 14 days.
   (c) To add the readout of anatomical structures via an amine-reactive dye: add 1 day to the estimates above.

See Fig. 1 for the workflow of the standard ExCel protocol, and Table 1 for a sample schedule. For all experiments involving RNA readout, all reagents and samples must be kept constantly RNAse-free, as RNAse contamination can lead to degradation of RNA molecules, and can result in both reduced signal intensity and count of the fluorescent puncta during the ExFISH-HCR readout (*see* **Note 30**).

3.1.2 Fixation and Cuticle Reduction

The default centrifugation setting is 1000 × *g* for 2 min for live worms, and 400 × *g* for 2 min for worms after fixation, unless stated otherwise. Washes are performed at room temperature (RT), unless stated otherwise.

1. Prepare PFA Fixative no more than 30 min before use, and chill to 4 °C.
2. Wash worms from plates into a 15-mL conical tube by pipetting 1–3 mL M9 buffer onto the plate and gently swirling to remove worms from the worm media surface and bacterial lawn. Pipette the loosened worms into the conical tube. Repeat this process until as many worms as possible have been transferred from the plate to the tube. Fill the tube with M9 buffer to 15 mL.

Table 1
Sample schedule for the standard ExCel protocol

Day	Approx. total time	Steps
1	*5 h, 45 m*	3.1.2 Fixation and Cuticle Reduction, **steps 1–9**
2	*30 m*	3.1.2 Fixation and Cuticle Reduction, **steps 10–12**
3	*1.25–2 h*	3.1.3 Sample Allocation, **steps 1–4** 3.1.4 RNA Anchoring, **steps 1** and **2**
4	*3.5 h*	3.1.4 RNA Anchoring, **step 3** 3.1.5 Protein Anchoring, **steps 1** and **2**
5	*2 h*	3.1.5 Protein Anchoring, **step 3** 3.1.6 Selecting a method to track hydrogel orientation 3.1.7 Gelation, **step 1**
6	*5–6 h*	3.1.7 Gelation, **steps 2–14** 3.1.8 Digestion, **steps 1–3**
7	*30 m*	3.1.8 Digestion, **step 4**
8	*3 h*	3.1.8 Digestion, **steps 5–9**
9	*5 h, 30 m*	3.1.9 ExFISH-HCR: Re-embedding, **steps 1–9**
10	*1 h, 30 m*	3.1.10 ExFISH-HCR: Probe hybridization and HCR amplification, **steps 1–4**
11	*5 h*	3.1.10 ExFISH-HCR: Probe hybridization and HCR amplification, **steps 5–10**
12	*3 h*	3.1.10 ExFISH-HCR: Probe hybridization and HCR amplification, **step 11** 3.1.11 Antibody Staining, **steps 1** and **2**
13	*3 h, 30 m*	3.1.11 Antibody Staining, **steps 3** and **4**
14	*3 h, 30 m*	3.1.11 Antibody Staining, **step 5** 3.1.12 NHS-ester Staining, **step 1**
15	*4 h, 30 m (not including imaging)*	3.1.12 NHS-ester Staining, **step 2** 3.1.13 DAPI Staining **steps 1** and **2** 3.1.14 Preliminary Imaging at a Partially Expanded State, **steps 1–4** 3.1.15 Final Imaging at Fully Expanded State, **steps 1–7**

To speed up the protocol, days 2 and 3, 8 and 9, and/or 9 and 10 may be combined for a total minimum experiment length of 12 days (if fluorescent protein, RNA, and anatomical readout are all done). If anatomical readout is not performed, days 14 and 15 may also be combined for a total experiment length of 11 days

3. Spin down (at 1000 × *g* for 2 min) and carefully replace the supernatant by pipetting 15 mL of fresh M9 buffer. Repeat the M9 wash until bacteria is gone and solution is clear. (It is typically sufficient to perform two washes total.)
4. Remove the clear M9 supernatant with a pipette. Re-suspend the worm pellet in 1–2 mL of M9 buffer and transfer worms to 1.5 mL tubes. For fixing large amounts of worms, split worms

Table 2
Sample schedule for the epitope-preserving ExCel protocol

Day	Approx. total time	Steps
1	*5 h, 45 m*	3.1.2 Fixation and Cuticle Reduction, **steps 1–9**
2	*30 m*	3.1.2 Fixation and Cuticle Reduction, **steps 10–12**
3	*1–1.5 h*	3.1.3 Sample Allocation, **steps 1–4** 3.1.5 Protein Anchoring, **steps 1–2**
4	*2 h*	3.1.5 Protein Anchoring, **step 3** 3.1.6 Selecting a Method to Track Hydrogel Orientation 3.2.2 Gelation, **step 1**
5	*5–6 h*	3.2.2 Gelation, **steps 2–14** 3.2.3 Collagenase VII-mediated Cuticle Digestion, **steps 1–3**
6	*3.5 h*	3.2.3 Collagenase VII-mediated Cuticle Digestion, **steps 4** 3.2.4 Protein Denaturation, **steps 1** and **2**
7	*10 min*	3.2.4 Protein Denaturation, **step 3**
8	*6 h*	3.2.4 Protein Denaturation, **steps 4–7**
9	*2 h*	3.2.4 Protein Denaturation, **steps 8** and **9**
10	*2–3 h*	3.2.4 Protein Denaturation, **steps 10–12** 3.2.5 Antibody Staining, **step 1**
11	*3.5–4 h*	3.2.5 Antibody Staining, **steps 2** and **3**
12	*6–7 h*	3.2.5 Antibody Staining, **step 4** 3.2.6 Preliminary Imaging to Check Antibody Staining, **steps 1–5**
13	*3 h, 30 m*	3.2.7 Antibody Anchoring, **step 1**
14	*3.5 h*	3.2.7 Antibody Anchoring, **step 2** 3.2.8 Re-Embedding into an Expandable Second Gel, **steps 1** and **2**
15	*5–6 h*	3.2.8 Re-Embedding into an Expandable Second Gel, **steps 3–14** 3.2.9 Proteinase K Digestion, **steps 1–3**
16	*6 h*	3.2.9 Proteinase K Digestion, **steps 4–8**
17	*4 h*	3.2.10 Cleave DATD-crosslinked Gels #1, **steps 1–5**
18	*30 min*	3.2.11 NHS-ester Staining, **step 1**
19	*3.5–4 h (not including imaging)*	3.2.11 NHS-ester Staining, **step 2** 3.2.12 Expansion and Imaging, **steps 1–7**

To speed up the protocol, days 2 and 3, and/or 12 and 13 may be combined for a total experiment length of 17 days (if anatomical readout by NHS-ester staining is performed). If anatomical readout is not performed, days 18 and 19 may also be combined for a total minimum experiment length of 16 days

into multiple tubes such that the pellet size in each tube is no more than ~50 μL per 1.5 mL tube. Spin down (at 1000 × *g* for 2 min) and remove as much supernatant as possible with a pipette without disturbing the worm pellet.

Table 3
Sample schedule for the iterative ExCel protocol

Day	Approx. total time	Steps
1	*5 h, 45 m*	3.1.2 Fixation and Cuticle Reduction, **steps 1–9**
2	*30 m*	3.1.2 Fixation and Cuticle Reduction, **steps 10–12**
3	*1–1.5 h*	3.1.3 Sample Allocation, **steps 1–4** 3.1.5 Protein Anchoring, **steps 1–2**
4	*2 h*	3.1.5 Protein Anchoring, **step 3** 3.1.6 Selecting a method to track hydrogel orientation 3.1.7 Gelation, **step 1**
5	*5–6 h*	3.1.7 Gelation, **steps 2–14** 3.1.8 Digestion, **steps 1–3**
6	*30 m*	3.1.8 Digestion, **step 4**
7	*4 h*	3.1.8 Digestion, **steps 5–9** 3.3.4 Antibody Staining, **steps 1** and **2**
8	*3.5–4 h*	3.3.4 Antibody Staining, **steps 3** and **4**
9	*3.5–4 h*	3.3.4 Antibody Staining, **steps 5**
10	*5 h, 30 m*	3.3.5 Expansion and Re-embedding into Non-Expanding Gel #2
11	*1 h*	3.3.6 Linker Hybridization, **steps 1–3**
12	*3.5 h*	3.3.6 Linker Hybridization, **steps 4**
13	*3.5 h*	3.3.6 Linker Hybridization, **step 5**
14	*4–4.5 h*	3.3.7 Re-embedding into Expanding Gel #3, **steps 1–7**
15	*3.5–4 h*	3.3.8 Cleave DATD-crosslinked Gels #1 and #2, **steps 1–4**
16	*1 h*	3.3.9 LNA Hybridization, **steps 1** and **2**
17	*3.5 h*	3.3.9 LNA Hybridization steps 3
18	*3.5 h*	3.3.9 LNA Hybridization, **steps 4**
19	*3.5–4 h (not including imaging)*	3.3.10 Expansion and Imaging **steps 1–4**

To speed up the protocol, days 2 and 3, 10 and 11, and/or 13 and 14 may be combined for a total minimum experiment length of 16 days

5. Place worm pellets and PFA Fixative on ice for 5 min.
6. Add 1 mL of PFA Fixative to the worm pellet. Place sample on a tube rotator to mix vigorously at RT for 30 min.
7. Incubate sample at 4 °C for 4 h without mixing.
8. Wash sample (spin down, remove supernatant, add buffer and mix thoroughly by inverting tubes) with 1 mL of BTB three times with no extended incubation time. Use the centrifugation setting of 400 × *g* over 2 min, for all centrifugation steps from this point on.

9. After the 3 quick washes with BTB, spin down, remove supernatant, and incubate sample in 1 mL of fresh BTB at 4 °C overnight. **.
10. Wash with 1 mL of BT two times with no extended incubation time.
11. Wash with 1 mL of PBST-0.5% two times with no extended incubation time.
12. Wash with 1 mL of PBS. The fixed sample can be stored in this PBS at 4 °C for up to 2 weeks. **.

3.1.3 Sample Allocation

Typically, a lot more worms are fixed compared to the quantity needed to be expanded for a given experiment. Processing too many more worms than needed can both waste reagents, and increase the labor and difficulty required to get all samples through the expansion pipeline. Before proceeding to the expansion protocol, perform the following steps to allocate the quantity of fixed worms that will be processed in the current experiment.

It is important to realize that the end product of this section is *not* individual tubes carrying an amount of worms that correspond to a single hydrogel. That splitting procedure will be performed in Subheading 3.1.7 (Gelation), but before then, it saves a lot of time and reagent to process all the to-be-gelled worms in larger batches (e.g. within a single or a few tubes). Thus, for example, if a user would like to make 20 hydrogel samples, the end product of this section will not be 20 individual tubes, but less than 5 tubes.

1. Determine the number of worm-embedded gels needed at the end, in order to achieve the experimental goal. If you follow through the next steps specified in this protocol, each gel will contain ~30–50 worms, and thus each gel may contain a sufficient number of biological replicates for statistical purposes (depending on your scientific question). However, each gel can only go through a single set of staining solutions, so animals in the same gel cannot be stained differently. Therefore, associate experimental conditions (i.e. type of antibody to apply; type of hybridization probes to apply; which spectral channels will be used for which signals) to individual gels, ideally in an organized spreadsheet. If you are trying this protocol for the first time, or are starting a new type of experiment that differs from what you have done in the past, it is recommended that you add at least 1–2 gels to the list, that correspond to the positive and/or negative control conditions (i.e. you can apply the same experimental condition as what has been previously done, which would give known outcomes, e.g. the antibodies or hybridization probes that have been demonstrated to work under ExCel [7]). Finally, for new

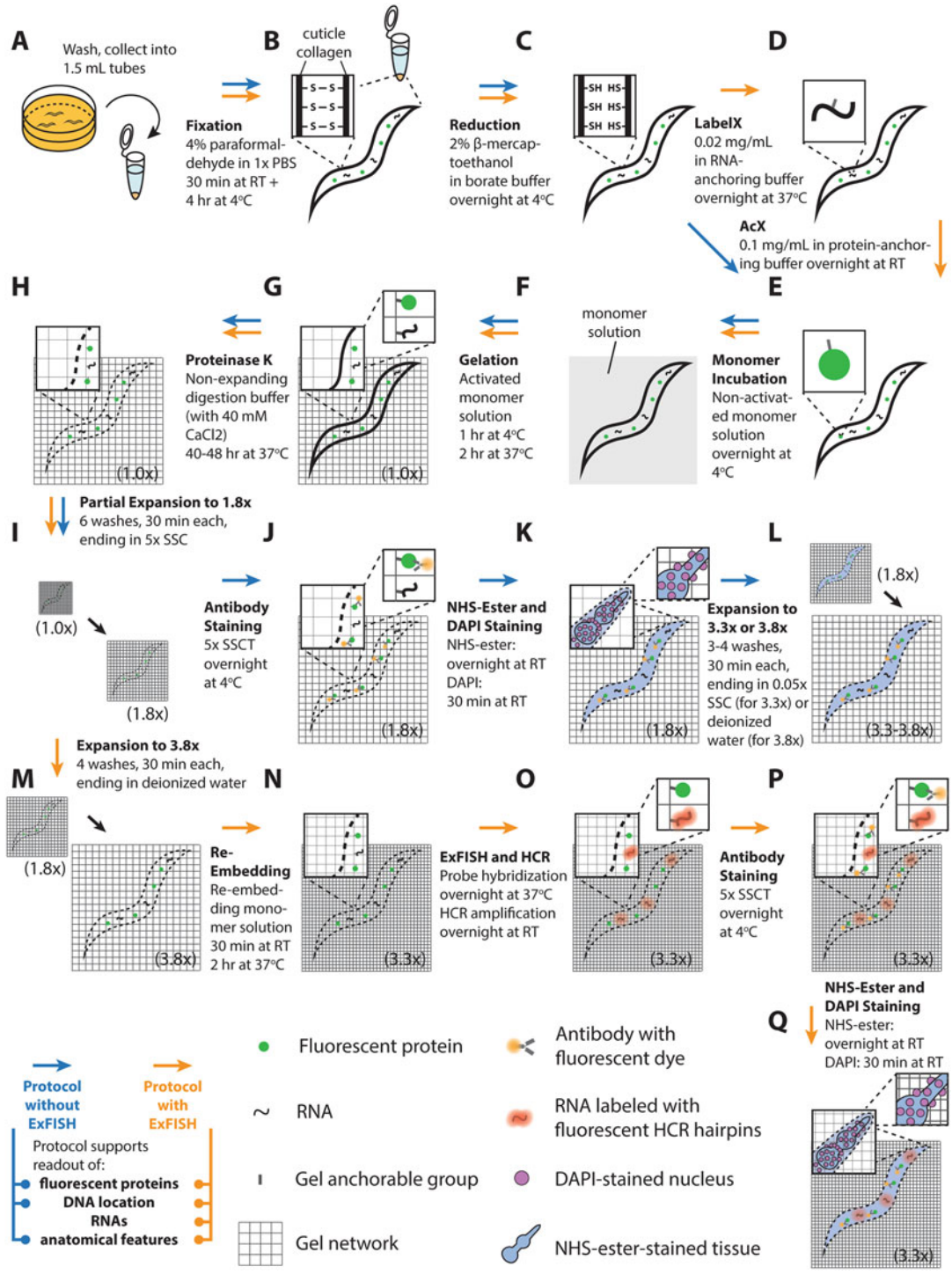

Fig. 1 Workflow for the standard ExCel protocol. A method for expanding cuticle-enclosed intact *C. elegans*. Depending on whether the user intends to visualize RNAs or not, the protocol branches into two forms. The protocol without ExFISH, which supports the readout of fluorescent proteins, DNA location (in the form of DAPI staining), and anatomical features, is indicated with blue arrows, ending in Panel **I**. The protocol with ExFISH,

Fig. 1 (continued) which additionally supports readout of RNAs, is indicated with orange arrows, ending in panel **q**. For all steps after hydrogel formation (Panels **g**–**q**), the linear expansion factor of the hydrogel-specimen composite is shown in parentheses. (**a**–**q**) Steps of the protocol, with the bold text indicating the title of the step. (**a**) Live animals are collected into 1.5-mL Eppendorf tubes. (**b**) Animals are fixed in 4% paraformaldehyde in 1× PBS, for 30 min at room temperature, and then 4 h at 4 °C. (**c**) Fixed specimens are incubated with BTB overnight at 4 °C. This step chemically reduces the disulfide bonds between collagen fibers in the cuticle, which could enhance diffusion of chemical reagents. After this step, specimens proceed to either a sequential treatment of LabelX and AcX (Panel **d** and **e**) for the protocol with ExFISH (orange arrows), or solely with a treatment of AcX (Panel **e**) for the protocol without ExFISH (blue arrow). (**d**) Specimens are incubated with LabelX overnight at 37 °C. This step equips nucleic acids with a polymer-anchorable moiety. (**e**) Specimens are incubated with AcX at a concentration of 0.1 mg/mL in protein anchoring buffer overnight at RT. This step equips proteins with a polymer-anchorable moiety. (**f**) Specimens are incubated in non-activated monomer solution overnight at 4 °C. (**g**) Hydrogel polymerization by incubating the specimens in activated monomer solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, AcX-modified proteins and LabelX-modified nucleic acids are covalently anchored to the hydrogel network. (**h**) Specimens are treated with Proteinase K at 8 U/mL, in non-expanding digestion buffer for 2 days (40–48 h) at 37 °C. The $CaCl_2$ in the buffer keeps the hydrogel at the unexpanded state, to avoid partial expansion of the internal tissue before the cuticle is thoroughly digested. (**i**) Specimens are partially expanded from a linear expansion factor of 1.0× to 1.8×, with 6 serial washes lasting 30 min each, incrementally reducing salt concentrations each time (i.e. lowering Tris, NaCl, and $CaCl_2$ concentrations bit by bit throughout each of the six washes, ending with 5× SSC). We use this serial washing procedure to lower the speeds of expansion, which we reasoned could potentially result in more isotropic expansion after Proteinase K digestion, by permitting more time for the gel network to uniformly stretch out the embedded tissue. After this step, specimens proceed to either further expansion (Panel **m**) for the protocol with ExFISH (orange arrow) or antibody staining (Panel **j**) for the protocol without ExFISH (blue arrow). (**j**) To visualize fluorescent proteins, specimens are stained with fluorescent antibodies against fluorescent proteins in 5× SSCT overnight at 4 °C, to amplify their fluorescent signal. (**k**) To visualize anatomical features and DNA, specimens are stained with an NHS-conjugated dye and DAPI, respectively. NHS staining is performed at 2 μM in NHS staining buffer overnight at RT. DAPI staining is performed at 5 μg/mL in 5x SSCT for 30 min at RT. (**l**) Specimens are expanded from a linear expansion factor of 1.8× to 3.3×or 3.8×, with 3–4 serial washes lasting 30 min each, reducing the amount of SSC each time, until the final specimen is immersed in 0.05× SSC (for 3.3×) or deionized water (for 3.8×). As characterized previously [7], 3.8× expansion provides better effective resolution (~65 nm), but has faster decay of immunostained signal (dropped by ~35% over 3 h); 3.3× expansion provides ~75 nm effective resolution, with a signal decay of <10% over 3 h. After expansion, specimens were ready for imaging. (**m**) Specimens are expanded from a linear expansion factor of 1.8× to 3.8×, with 4 serial washes lasting 30 min each, reducing the amount of SSC each time, until the final specimen is immersed in deionized water. (**n**) Specimens ae re-embedded into another non-expandable hydrogel to lock up its size at the expanded state. The re-embedding monomer solution contains charged molecules. Therefore, the linear expansion factor slightly drops from 3.8× to 3.3× during this step. After re-embedding, the linear expansion factor remains at ~3.3×, regardless of the immersing environment. (**o**) To visualize RNA molecules, we first perform in situ hybridization (ExFISH) of RNA-detection probes to the target RNA, overnight at 37 °C, and then perform hybridization-chain-reaction (HCR) to amplify the hybridized signal, overnight at RT. (**p**) To visualize fluorescent proteins, specimens are stained with fluorescent antibodies against fluorescent proteins in 5× SSCT overnight at 4 °C, to amplify their fluorescent signal. (**q**) To visualize anatomical features and DNA, specimens are stained with an NHS-conjugated dye and DAPI, respectively (conditions described in step **k** above). After staining, specimens were ready for imaging. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

users who are not fully familiar with handling hydrogel samples, and who thus have a greater chance to lose samples due to unexpected failures (e.g. breaking gels, failing to embed worms or gels due to pre-mature gelation, etc.), it is recommended to add another ~2 gels to the list, as backup samples.

2. Once you determine the number of gels needed (from **step 1**), multiply that number by ~30–50 worms/gel (new users can aim for ~50–70 worms/gel, as there could be some loss during the gelation step, which is reduced as technique improves), to get the total number of worms that need to be processed in the current experiment.
3. Transfer the total number of worms, determined in **step 2**, from the stock tube of fixed worms, into a single new 1.5 mL tube. If you need to transfer hundreds of worms, it would be difficult to count accurately with a single pipetting attempt, so use the following approach instead. First, mix the contents in the stock tube of fixed worms well, with a pipettor (such that worms are evenly distributed throughout the solution). Then, pipette out 100 μL of the well-mixed content into a separate, new 1.5 mL tube. Estimate how many worms were transferred, by holding the new tube against a lamp or light source to facilitate visualization of worms via naked eye. Then, back-calculate the volume of well-mixed content from the stock tube, that is needed to achieve the total number of worms calculated in **step 2**. Transfer additional volume of well-mixed content from the stock tube into the new tube, until the volume in the new tube reaches the calculated volume, i.e. until the total number of worms in the new tube reaches the desired number.
4. Store the stock tube back into 4 °C. Spin down the tube of allocated worms. Estimate the size of the worm pellet. If the worm pellet appears clearly larger than ~30 μL, re-suspend the pellet back into the supernatant with a pipettor, and then split the evenly mixed content into multiple tubes, with the goal of having each tube to contain $\leq$30 μL of worm pellet after spin down. Repeat this distribution step until the goal specified in the previous sentence is achieved. This distribution step ensures that the reagents applied in the next several steps are always at molar excess compared to their biochemical substrates (i.e. total biomolecules in the total mass of worm) within each tube, and make final results more consistent.

Reaction volumes in the following sections are specified for a single 1.5 mL tube of worms (whose pellet size has been distributed to be $<$30 μL/tube). If you end up with multiple tubes after the worm pellet distribution, you can process all of them in parallel, but be sure that each tube receives the full volume specified in the next

sections (e.g. each tube gets a full 1 mL of Protein Anchoring Buffer for **step 1** in the "Protein Anchoring" section, and so forth).

3.1.4 RNA Anchoring (Optional, for RNA Readout)

1. Spin down and remove PBS supernatant. Pre-incubate worms with 1 mL of RNA Anchoring Buffer at RT on a tube rotator for 1 h.
2. Spin down and remove buffer, then incubate worms with LabelX at 0.01 mg/mL (1:100 dilution of LabelX stock) in 1 mL of RNA Anchoring Buffer, overnight at RT on a tube rotator. **.
3. Wash worms with 1 mL of PBST-0.1% three times, 30 min each at RT on a tube rotator.

3.1.5 Protein Anchoring (for Fluorescent Protein Readout)

1. Spin down and remove PBS supernatant. Pre-incubate worms with 1 mL of Protein Anchoring Buffer at RT on a tube rotator for 1 h.
2. Spin down and remove buffer, then incubate worms with AcX at 0.1 mg/mL (1:100 dilution of AcX stock) in 1 mL of Protein Anchoring Buffer, overnight at RT on a tube rotator. **.
3. Wash worms with 1 mL of MOPST-0.1% three times, 30 min each at RT on a tube rotator.

3.1.6 Selecting a Method to Track Hydrogel Orientation

During the initial gelation step of any ExCel protocol (to be described in Subheading 3.1.7), the worms, which have diameters in the range of ~10–60 μm, settle to the bottom surface of the casted gel, which has a thickness of ~180 μm (set by the #1.5 cover glass, which serves as the spacer of the gelling chamber). Then, during the repeated washes throughout the ExCel protocols, the hydrogel sample could get flipped, which results in worms located on the top surface of the hydrogel. If the hydrogel is in such an upside-down orientation during a re-embedding process, or during the final imaging step, a short-working-distance objective (<1–1.5 mm; most high NA and magnification objectives are in this category) might not be able to reach parts or all of the expanded animals. Thus, it is necessary to ensure that the hydrogel sample is in the correct orientation (i.e. worms are on the bottom surface), at the following time points: (a) Prior to any expanding step; (b) Prior to any re-embedding step; (c) Prior to the final imaging step.

If the re-embedding or the final imaging step is preceded by hydrogel expansion, an orientation check needs to be performed prior to the expansion process (we will remind the users again at these later time points in the protocol), because if the gel is on the wrong side, expanded gels are typically too fragile to get flipped. If you have found that the gel is on the wrong side after expansion,

perform the expansion wash steps in reverse to shrink before flipping the gel. Do not try to flip an expanded gel, as it is likely to rip apart.

To track the orientation of the hydrogel (i.e. the surface of the gel the animals are located on), use either of the following two procedures after gelation, and before the gel is lifted off from the chamber. (a) Take a low-magnification image of the gelled sample **before** opening the chamber, to record the orientation of worms in the gel (to be described in **step 12** in Subheading 3.1.7). The image can be based on transillumination or fluorescence (if the fluorescent protein(s) are expressed in a way that allows delineation of the border of the worm). Later, to determine whether the gelled sample has been flipped or not, take another image of the gel, and compare this image to the initial image. (b) During gel trimming (to be described in **step 14** in Subheading 3.1.7), trim the gel into a shape that makes it feasible to identify the side of the gel. For example, a clearly-trimmed 4-edge shape (with inner angles 90°, 90°, 135°, 45°, consecutively) allows one to distinguish whether the gel has been flipped or not, because if flipped, the gel can never resume its original shape by rotation.

Both methods work well when executed properly (and are also mutually compatible; i.e. it is possible to do both on the same sample, if desired). The first method requires microscopy access whenever gel orientation needs to be checked, and might also require more experience and practice with microscopy (when checking the gel orientation, one has to be able to find expanded worms by transillumination and/or dim residual fluorescence). Select a method (or both methods) before proceeding to the next section.

3.1.7 Gelation

To ensure that the final state expanded worm could be captured within the working distance of a high-NA lens, perform either or both of the two methods for tracking hydrogel orientation (review Subheading 3.1.6). This is critical when performing epitope-preserving ExCel and Iterative ExCel (where failure to track the hydrogel orientation at various stages might result in no image at all at the end, because the worms could end up at a center region [on the *z*-axis] within the final hydrogel sample, instead of regions closest to the sample surface. In those cases, the user will be unable to optically access the worms via a short-working-distance lens.), and also recommended for standard ExCel, for optimal chance of successful imaging.

Due to variability in sodium acrylate qualities, it is recommended that all users perform a pilot gelation procedure (which takes <1 h) whenever a new batch of Monomer Solution Stock is made from a previously un-validated bottle of sodium acrylate powder (*see* **Note 1**). This procedure allows the user to determine

whether the 4 °C incubation time (for **step 6** in this section) in this gelation step needs to be reduced, to accommodate for a faster gelation timing for a specific sodium acrylate stock. Without doing this, the user might face the undesirable situation where pre-mature gelation occurs during the 4 °C incubation, which can trap the worms in hydrogel pre-maturely, in a tube rather than on a slide, and prevent them from being processed further with the ExCel protocols.

For users who are less experienced with this protocol, it is important to note that this section involves a time-sensitive procedure that, if not performed quickly and correctly, will destroy the sample (again, due to the hydrogel solidifying before the user has time to load them into chambers). The chance of such failure is much reduced if the user only make a few hydrogel (e.g. up to ~5) at a time, and is further reduced if the user pre-splits the entire batch of to-be-fixed worms, into individual tubes, each of which carries the amount of worms that correspond to a single hydrogel. This pre-splitting step is specified as the optional **step 2** in the following protocol.

1. Spin down and remove MOPST-0.1%, then incubate worms in 1 mL of Non-activated Monomer Solution overnight at 4 °C. **.
2. (Optional; this step is a pre-splitting step that takes a longer time to perform, but reduces the chance of experimental failure during the time-sensitive gelation procedure later). If worms were split into several tubes in Subheading 3.1.3, **step 4**, regroup them into a single container (e.g. a 15 mL tube). Then, mix the content well with a pipette, and evenly distribute the content into *N* individual 1.5-mL tubes, where *N* is the number of hydrogel samples determined in Subheading 3.1.3, **step 1**. The number of worms inside each tube should be 30–50 worms (or 50–70 worms, whichever value used in Subheading 3.1.3, **step 2**). All following steps apply to each individual tube.
3. Prepare 1 mL of Activated Monomer Solution with all reagents except for APS (Monomer Solution Stock + Triton X-100 + 4-HT + TEMED; do not add the activator APS yet), and thoroughly mix the solution by a vortex. Chill the solution to 4 °C on a cold block. It is important to ensure that the solution is fully chilled to 4 °C before proceeding, because starting with a warmer solution will lead to faster gelation, which might cause sample loss (*see* **step 8**).
4. Spin down worms. Remove supernatant.
5. Activate the Monomer Solution from **step 3** by adding 20 μL of 10% APS, and thoroughly mix the solution by a vortex. Start

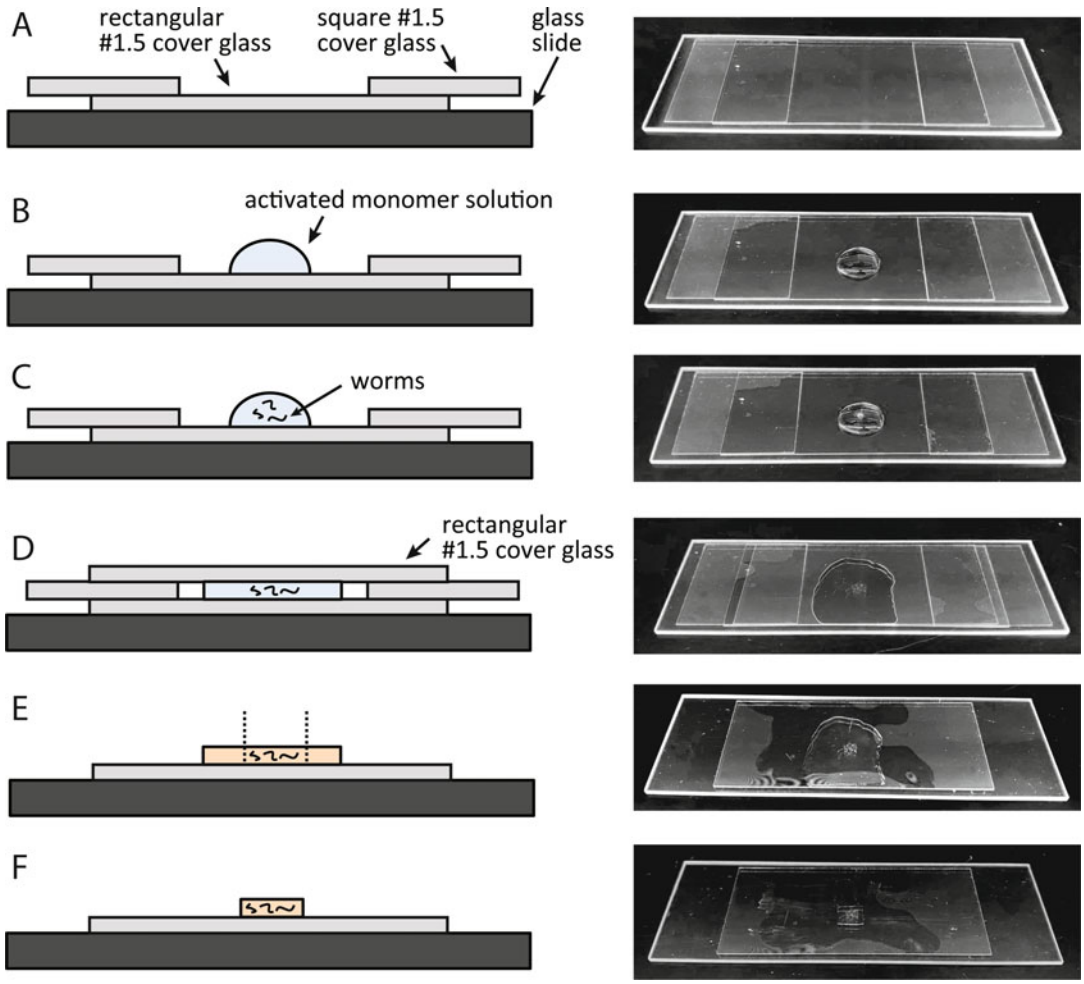

Fig. 2 Gelation procedure for ExCel protocols. (**a**) To construct a gelling chamber, a #1.5 cover glass is adhered to a glass slide, with a drop of water pipetted onto the glass slide as adhesive. Then, two #1.5 cover glasses are adhered to opposite ends of a glass slide to serve as spacers. (**b**) Activated monomer solution is added to the center of the chamber. (**c**) Worms are loaded to the center of the droplet. (**d**) A rectangular cover glass is placed on top of the spacers. (**e**) The gelling chamber is incubated at 37 °C for 2 h to accelerate the formation of the hydrogel polymer. Afterwards, the top and side cover glasses are removed to facilitate trimming of the gelled sample. (**f**) Trimmed samples proceed to downstream digestion, staining, and expansion procedures. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

a timer right after the APS is added. Perform these steps immediately before adding solution to worms.

6. Add 1 mL of Activated Monomer Solution to worms. Incubate sample at 4 °C for 30 min, on a pre-chilled cold block.
7. Near the end of incubation, construct gelling chambers (Fig. 2a). Adhere a rectangular cover glass (e.g. 22 × 50 mm) to a glass slide with a drop of water. Then adhere two pieces of

#1.5 square cover glasses (e.g. 22 × 22 mm) at separate ends of the rectangular cover glass, again using drops of water as adhesive. These square cover glasses function as spacers of the gelling chamber. If gelling chambers are constructed more than 5 min before the incubation will be finished, cover them to prevent dust accumulation.

8. Immediately after the 30-min incubation, spin down worms. Without disturbing the pellet, transfer ~30 μL of the supernatant as a droplet on the rectangular cover glass, between the spacers (Fig. 2b). Remove the rest of the supernatant and leave only ~30 μL along with the worm pellet. Note that the monomer solution should be completely liquid at this stage. If not, pre-mature gelation has occurred, and we do not recommend proceeding with the samples anymore. If pre-mature gelation occurs constantly, reducing the incubation time in **step 6** to 20 min should resolve the problem (*see* **Note 1**).
9. Carefully re-suspend the pellet in the leftover volume with a pipette, without creating bubbles. Load all the volume of the tube into a pipette. Push out any air in the tip of the pipette. Insert the pipette tip to the center of the droplet on the rectangular cover glass. Slowly push out the content so that the worms are highly concentrated at the center of the droplet (Fig. 2c). We find that the ideal number of worms in a gel is ~30–50. If more worms are in the droplet, they may be added to another gel on another gelling chamber.
10. Allow 30–60 s for worms to settle near the bottom of the chamber. If splitting a large pellet into multiple samples at this step, the time it takes to distribute worms across ~5 chambers should be enough to allow worms to settle. Close the chamber by carefully and slowly placing a rectangular cover glass (e.g. 22 × 50 mm) on top of the spacers, minimizing bubble formation (Fig. 2d). One way to achieve this is to use a tweezer to clip on one end of a rectangular cover glass, hold the cover glass above the chamber by ~5 mm (in parallel to the glass slide; no contact yet), press down the un-clipped end of the rectangular cover glass until it contacts the spacer, release the clipping force so that the cover glass just rests on the lower branch of the tweezer, and then slowly lower the tweezer until the cover glass contacts the other spacer. It is desirable to avoid worms from overlapping with one another (which can increase the difficulty with imaging), so if worms appear too concentrated, it can be useful to gently spread out the worms by repeatedly lifting and lowering the cover glass slowly, BEFORE closing it completely. If you have already closed the chamber and the worms appear concentrated, we recommend continuing without attempting to open and redistribute worms, as this is likely to introduce bubbles.

11. Incubate the gelling chamber at 37 °C for 2 h. Maintain high humidity with one of the two following methods: (1) in the incubator, keep a small open beaker of warm water near your gelling chambers; (2) place the gelling chambers in a humidified chamber (e.g. a 0.75-quart storage container, such as Amazon.com #B01M2BTKYB), with the gelling chamber sitting on top of a stack of two lids of 6-, 24-, or 96- well plates, with a small amount (10–20 mL) of deionized water at the bottom of the sealed container.
12. At the end of the 2-h incubation at 37 °C, remove samples from 37 °C, and perform a round of pre-expansion imaging. There are two goals of this imaging session: (A) to record the initial orientation of the hydrogel, which enables tracking of the hydrogel orientation at later stages; the importance of this procedure is described in Subheading 3.1.6; (B) to take pre-expansion images of a few (e.g. ~3) animals, which can later become useful for validation purposes, or in case troubleshooting becomes necessary at a later stage.

    To perform (A), take large (i.e. across multiple fields of view as a grid, and ideally automatically stitched by the imaging software) images (bright field or fluorescence; the latter of which is typically more useful [if the strain contains reasonably bright and/or widespread expression of fluorescent protein(s)], because the fluorescent signals persist to much later stages in the protocol than brightfield outlines [with immunostaining-based signal amplification], whose scattering becomes increasingly dim after sample expansion, but still can be useful if the strain does not contain strong native fluorescence) of your gels at low magnification (e.g. via a 4× objective). Record whether the sample is imaged through the glass slide (i.e. right-side-up) or through the coverslip, which is necessary for correctly getting the gels to be flipped into the same orientation, during later stages.

    To perform (B), take additional images at a higher magnification (e.g. via a 10×–40× objective), over areas of interest (e.g. fluorescent-protein-expressing regions) on individual animals. The working distances of some these high-magnification lenses might be too small to go through the glass slide, which requires you to flip the sample upside-down, and image through the cover slip side. If possible, record where the imaged animals are located, on the low-magnification map that you have acquired in task (A). Typically, acquiring images for ~three animals are sufficient for general purposes of validation and troubleshooting (e.g. to confirm whether the anti-fluorescent protein staining, which is applied at a later stage, works as expected).

13. After pre-expansion imaging, prepare the digestion solution (*see* **step 1** of the next section). The digestion solution should be applied to the samples immediately after **step 14** (gel trimming) to avoid gel drying out.
14. With a tweezer or a razor blade, remove the top cover glass and spacers and leave the gel on the bottom cover glass (Fig. 2e). The gel can occasionally come off with the top cover glass. In that case, leave the gel on the cover glass and dispose the rest of the chamber. In the case that one part of the gel comes off with the top glass while another part of the gel remains on the bottom cover glass, place the top cover glass back, and try opening from the other side of the chamber (i.e. if you approached from the right spacer, now approach from the left spacer). Carefully try to remove one side of the chamber without ripping or stretching the gel. If necessary, a paint brush may be used to gently coax the gel off of the surface with less area stuck to it.

    With a razor blade, trim away excessive gel (Fig. 2f). The size of the trimmed gel should be such that, when fully expanded, the gel can fit flatly into the imaging container (a good size is ~7 mm × 7 mm, which can fit into a 6-well plate when expanded). If the worms were successfully kept concentrated at the center of the gel, it should be relatively easy to retain most worms in the trimmed gel. If the worms were more spread out, you may want to trim into two gels near to, or smaller than, the suggested 7 × 7 mm size. To enable tracking of the hydrogel orientation, the user can trim the gel into a 4-edge parallelogram shape (with inner angles 90°, 90°, 135°, 45°, consecutively), which allows the user to later distinguish whether the gel has been flipped or not (review Subheading 3.1.6).

3.1.8 Digestion

Based on our experience, the extent of digestion seems to be quite sensitive to the quality of Proteinase K. Use Proteinase K ordered within 2 months. Reaction volumes in the following sections are specified for a single hydrogel. Scale up if you are processing multiple gels.

1. After the 2-h gelation incubation and before opening the gelling chambers, prepare 1 mL of digestion solution per gel by adding Proteinase K to Non-expanding Digestion Buffer to a final concentration of 8 U/mL (1:100 dilution).
2. Add ~50 μL of digestion solution to the trimmed gel immediately after trimming (the trimmed gel should still be on the coverslip). Let the gel sit immersed in the digestion solution for 5 min at RT.

3. Add ~950 μL of digestion solution to a well in a 24-well plate for each gel. Prepare a flat-tip paint brush (if performing RNA readout with ExFISH-HCR, decontaminate the paintbrush from RNAse by rinsing with RNAse Zap and deionized water; *see* **Note 30**), then slowly insert the brush under the gel until most of the bristles are covered by the gel, lift the gel with the brush, keeping the gel horizontal and level with the slide, and transfer the gel into the well by lowering it into the Digestion Buffer. Allow the gel to float off of the brush, and gently wave it off in the liquid only if necessary. Try to avoid flipping the gel over, as you will want to keep track of which side the worms are closer to, for ease of imaging after expansion. Incubate sample overnight at 37 °C. ******.
4. Replace solution with 1 mL of **freshly prepared** digestion solution. Incubate sample overnight at 37 °C. ******.
5. Now that the worms have been embedded, we use "wash" to mean simply exchange the immersing solution in the 24-well plate, without the centrifuging; the solution exchange is typically performed by a P1000 pipettor; whenever solution is being removed from the well that contains the gelled sample, always confirm the exact location of the hydrogel inside the well first, by identifying the slightly scattering boundary or body of the trimmed gel, before inserting the pipette into the well and start retrieving solution; without this careful procedure, the gel can get sucked into the pipettor, which creates a typically very obvious obstruction to the fluid flow into the pipette tip, and which can damage the gelled sample. We recommend tilting the plate under bright light to identify the edge of the gel, placing the pipette tip in a corner of the well where the gel is not present, then slowly removing solution so as not to suck up the gel. If the gel is sucked up, it can be pushed back out and you may continue with the protocol, but your sample may be damaged (worms may break or come out of their gelled places). Maintain this careful process throughout the rest of the protocol. Wash gels once with 1 mL of TNT-20 Buffer (TNT Buffer + 20 mM $CaCl_2$) for 30 min at RT.
6. Wash once with 1 mL of TNT-10 Buffer (TNT Buffer + 10 mM $CaCl_2$) for 30 min at RT.
7. Wash twice with 1 mL of TNT Buffer for 30 min each at RT.
8. Wash once with 1 mL of 5× SSC-NaCl Buffer (5× SSC + 500 mM NaCl) for 30 min at RT.
9. Wash once with 1 mL of 5× SSC for 30 min at RT. ******.

3.1.9 ExFISH-HCR (for RNA Readout): Re-Embedding

ExFISH-HCR requires hybridization between DNA probes and RNA targets, which have reduced affinity in low ionic strength environment (e.g. deionized water, which is necessary to fully

expand the gelled sample). This trade-off can be resolved by first casting the expanded sample into a non-expanding acrylamide gel, which locks up the expansion factor of the first gel. The re-embedded gel can then be processed and imaged in higher-salt environments that favor hybridization and maximize HCR amplicon stability.

1. Place a rectangular glass slide into a 4-well rectangular dish. Transfer each sample onto the glass slide with a paint brush, the same way you transferred it from the gelling chamber to the 24-well plate. Briefly image each gel to be sure it matches earlier images taken immediately after gelation (as instructed in Subheading 3.1.6, and **step 12** in Subheading 3.1.7). This will ensure that worms, which were at the bottom of the gel during gelation, remain at the bottom of the gel (i.e. the gel is in its original orientation, instead of being up-side-down) for easier imaging of the entire volume once it is expanded.

   If gels are flipped, flip back over gently with an RNAse-decontaminated paintbrush (washed with RNAse Zap and deionized water; *see* **Note 30**). It is essential to perform this step now, as gels become more delicate as they are expanded.

2. Expand sample by washing serially in 2.5× SSC, 0.5× SSC, 0.05× SSC and ddH_2O using the same careful pipetting as in the 24-well plate, for 30 min each at RT. Use 3 mL for each washing step. Note that as the gel expands it becomes more delicate and easier to accidentally pipette up when removing washing buffers. Because of this we recommend you continue to use a P1000 pipettor, instead of a Pipet-Aid-type pipettor, to remove liquids despite the higher wash volume. Also take care to keep the expanded gel on top of the glass slide when removing liquids, and avoid letting it hang off of an edge of the slide between washes. This is made easier by following our recommended gel size of 7 × 7 mm during the trimming step after initial gelation.

3. Incubate the sample in 3 mL of re-embedding monomer solution for 30 min at RT with gentle circular shaking. The amount of re-embedding monomer solution should be just sufficient to barely reach the top surface of the gel (80–100% of gel height; even if the solution does not cover the gel at a still state, as long as the solution washes over the sample during the circular shaking, there will be sufficient diffusion of the monomers into the sample), but not much more than that. Oxygen in the ambient environment slows down the gelation reaction to allow complete diffusion of the gel monomers over the 30 min incubation; too much monomer solution can cause pre-mature gelation at the bottom of the well, due to reduced access to ambient oxygen. This is the reason we recommend that shaking

is gentle and circular and does not result in the gel being exposed to air, or the solution to become pooled at one end of the well (if it is tilted, for example).

4. During the incubation, prepare two spacers per gel of three times the height of initial gel spacers. For example, if #1.5 cover glass was used during the initial gelation step, use a stack of three #1.5 cover glasses adhered to each other with a small drop of ultrapure water between the coverslips. It is advisable to prepare these in advance to minimize oxygen exposure of your gels between pre-incubation and re-embedding.
5. After 30 min of incubation, transfer all monomer solution from the well to a separate 50-mL tube and set aside at RT for later use (in the next step). During the solution transfer, position the expanded gel on the center of the glass slide, by tilting the 4-well plate and/or gently moving the gel with a flow of monomer solution (using a pipette). The edges of the rectangular gel should be parallel to the edges of the slide and not be close to, or hanging off from, the edge of the glass slide. Use a blade or forceps to carefully transfer the glass slide from the 4-well plate onto a sheet of Kimwipe, to remove droplets on the bottom of the glass slide. Trim away excessive gel if it is large enough that it is at risk of hanging off the slide.
6. Place spacers prepared in **step 4** on both ends of the glass slide, with the gel in the center. Add a small amount (~50 μL) of the saved monomer solution onto the gel, and slowly place a rectangular cover slip on the gel, to enclose the gelling chamber in the same style as performed in the initial gelation. Avoid bubbles. Use a pipette aimed in the space created by your spacers to completely fill the chamber with extra monomer solution, such that the gel is fully enclosed by monomer solution, with minimal contact to ambient oxygen.
7. Incubate the gelling chamber at 37 °C for 2 h, in a humidified chamber (e.g. a 0.75-quart storage container, such as Amazon.com #B01M2BTKYB, with the gelling chamber sitting on top of a stack of two lids of 6-, 24-, or 96- well plates, and with a small amount (10–20 mL) of deionized water at the bottom of the sealed container).
8. Remove top cover glass, following the same guidelines as in the original gelation step for removing the cover glass, and trim away excessive gel. You should be able to see the border of the initial expanded gel to use as a guide. We recommend splitting large gels into smaller ones with no more than 1.5 cm per dimension, which simplifies downstream processing.
9. Transfer trimmed gels into individual wells in a 24-well plate containing 1 mL of 5× SSC using a paintbrush as described in previous steps of this protocol. Wash three times with 5× SSC, 10 min each. **.

3.1.10 ExFISH-HCR (for RNA Readout): Probe Hybridization and HCR Amplification

ExFISH-HCR readout is performed with HCR v3.0 kits from Molecular Instruments (https://www.molecularinstruments.com/). It requires five items: (1) Probe Set Stock Solution (a.k.a. "HCR Probe Set" on the website) against the target mRNA; (2) HCR Amplifier Stock Solution (a.k.a. "HCR amplifier" on the website); (3) Probe Hybridization Buffer; (4) Probe Wash Buffer; (5) Probe Amplification Buffer. Thaw HCR Probe Set Stock and HCR Amplifier Stock Solutions at RT before adding to buffers and samples. For steps conducted at RT and 37 °C, bring the kit buffers to the working temperature before adding to your samples. Refer to Subheading 2.2.1 for instructions and notes of ordering and storing these reagents.

1. Use a spreadsheet to organize which gel samples will be stained with which probe sets, and then with which HCR amplifiers (according to the HCR initiators that are associated with the to-be-applied probe sets; the initiator associated with the probe set is also printed on the manufacturer's packaging [marked as B1 to B5]).
2. Pre-incubate sample in 500 μL of the Probe Hybridization Buffer for 1 h at 37 °C. Note that buffers included in the HCR protocol are viscous, so take your time pipetting to ensure that you've picked up the correct volume.
3. Prepare 500 μL of probe solution by adding the HCR Probe Set Stock Solution to the Probe Hybridization Buffer for a final concentration of 4 nM. Mix the contents well with vortexing.
4. Incubate sample with 500 μL of the probe solution overnight at 37 °C. **.
5. Wash sample with 1 mL of the Probe Wash Buffer four times, 30 min each at 37 °C.
6. Wash sample with 1 mL of 5× SSCT three times, 30 min each at RT.
7. Pre-incubate sample in 500 μL of the Probe Amplification Buffer for 30 min at RT.
8. During the incubation period in **step 7**, aliquot 2.5 μL of each HCR Amplifier Stock Solution (provided at 3 μM; for each HCR initiator, there are two separate tubes of HCR Amplifier Stock Solution—H1 and H2; both solutions need to be separately aliquoted; for example, if a gel sample is stained with 3 different probe sets, whose corresponding HCR initiators are B1, B2, and B4, then a total of 6 aliquots of HCR Amplifier Stock Solution – B1H1, B1H2, B2H1, B2H2, B4H1, B4H2—should be aliquoted at 2.5 μL each) into separate PCR tubes. Incubate these aliquots at 95 °C for 90 s, on a pre-warmed PCR machine. Let these aliquots sit in a dark environment at RT for 30 min.

9. Prepare HCR amplifier solution by adding 2.5 μL of each 95 °C-treated HCR Amplifier Stock Solution to 500 μL of amplification buffer. Mix the contents well with vortexing.
10. Incubate sample in 500 μL of HCR amplifier solution in a dark environment overnight at RT. To avoid photobleaching the fluorophores on the HCR hairpin, the sample should be kept in a dark environment from this point on. This can be achieved by keeping the sample-containing plate in aluminum foil, throughout all downstream reactions and washes, and only temporarily remove the foil during sample handling (e.g. pipetting the immersion fluid in and out of the wells) or imaging. Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil. **.
11. Wash sample with 1 mL of 5× SSCT for two times, 1 h each at RT.

3.1.11 Antibody Staining (for Fluorescent Protein Readout)

1. Pre-incubate sample in 1 mL of 5× SSCT for 30 min at RT.
2. Incubate sample with primary antibody against fluorescent protein, at desired concentration (to determine this, the user can follow manufacturer's instruction, earlier literature, or use a default concentration of 10 μg/mL) in 500 μL of 5×SSCT overnight at 4 °C. Depending on the resulting level of background and true signal, adjustments to the primary and secondary antibody concentrations may be helpful for optimizing image quality, with repeated trials of this protocol. **.
3. Wash sample with 1 mL of 5× SSCT three times, 1 h each at RT.
4. Incubate sample with secondary antibody with a fluorophore, at desired concentration (a good default concentration is 10 μg/mL; this concentration can be adjusted) in 500 μL of 5× SSCT in a dark environment overnight at 4 °C. To avoid photobleaching the fluorophores on the secondary antibody, the sample should be protected from light from this point on (if not already in such state, as instructed in **step 10** of Subheading 3.1.10, i.e. the ExFISH-HCR: Probe hybridization and HCR amplification section). This can be achieved by keeping the sample-containing plate in aluminum foil, throughout all downstream reactions and washes, and only temporarily remove the foil during sample handling (e.g. pipetting the immersion fluid in and out of the wells) or imaging. Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil. **.
5. Wash sample with 1 mL of 5× SSCT three times, 1 h each at RT. **.

3.1.12 NHS-Ester Staining (Optional, for Morphology Readout)

1. Incubate sample with 2 μM of the NHS ester of a fluorescent dye (e.g. Alexa Fluor 546 NHS Ester or Alexa Fluor 647 NHS Ester) in 1 mL of NHS-ester staining buffer (5× SSCT, pH 6.0), in a dark environment overnight at RT. To avoid photobleaching the fluorophores, the sample should be protected from light from this point on (if not already in such state). Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil. **.
2. Wash sample with 1 mL of 5× SSCT three times, 30 min each at RT.

3.1.13 DAPI Staining (Optional, for DNA Readout)

1. Incubate sample with 5 μg/mL DAPI in 1 mL of 5× SSCT for 30 min in a dark environment at RT. To avoid photobleaching the fluorophores, the sample should be protected from light from this point on (if not already in such state). Unless otherwise noted, all incubations and washes in the following steps are performed in a dark environment, e.g. inside aluminum foil.
2. Wash sample with 1 mL of 5× SSCT three times, 10 min each at RT.

3.1.14 Preliminary Imaging at a Partially Expanded State (Only for Samples that Have Not Been Re-Embedded)

For samples that have not been re-embedded, they are currently at a ~2× expanded state after the immersion in 5× SSCT. Although a round of preliminary imaging at this point is not necessary for the final imaging, we highly recommend doing this, in order to (1) confirm that the staining performed in the sections above (Subheadings 3.1.9–3.1.13) works as expected (e.g. positive and/or negative control conditions produce a pattern of staining consistent to past results), (2) for experimental conditions with no past results to compare to, get a sense of the localization of the stains, because the user will later need to find these signals at an expanded state, which yields dimmer per-pixel fluorescent intensity and larger physical space to search through, and which could thus make imaging quite challenging without some prior knowledge on where the stains are located, and (3) if pre-expansion images were acquired at the post-gelation stage (in **step 12** of Subheading 3.1.7, i.e. the Gelation section), it is a good time to check whether the hydrogel is on its original orientation (i.e. with worms at the bottom surface of the hydrogel) or upside-down, by taking another low-magnification map of the hydrogel sample at this stage, and compare how the animals spatially distribute across the hydrogel sample, between the pre-expansion image and the current image (refer to Subheading 3.1.5 to review the importance of doing this).

1. Transfer sample to a container suitable for imaging, e.g. a glass-bottom 6-well plate. For samples that have not been re-embedded, expand sample by serial washes with reducing

salt concentration as follows. Note that as the gel expands it becomes more fragile, and use the methods described in previous steps to avoid sucking the gel into the pipettor when removing liquid during wash steps.

2. Remove as much liquid from the well as possible. The liquid should be removed to the extent that no more solution that can be taken up by a P1000 or a P200 pipettor, and the gel does not appear to be sliding around when the plate is slightly tilted (e.g. ~20°).
3. Perform imaging. The standard imaging workflow that we recommend is to first use a low-magnification objective (e.g. 4×) to acquire a single *z*-plane map of the gel sample (ideally by automated acquisition and stitching of multiple evenly spaced fields of view, which is available in most imaging software), which helps to identify and locate all animals that can be imaged. Next, use the low-magnification map of the gel sample to navigate (via automated stage control, or manually) to the animals and regions of interest. Then, use a high-magnification objective (e.g. 20× or 40×) to acquire z-stacks through the regions of interest.
4. After imaging, add 2 mL of 5× SSC to the well, to re-hydrate the sample. Keep sample at 4 °C until ready for final imaging. ******.

3.1.15 Final Imaging at Fully Expanded State

1. If a short working distance lens will be used, ensure that the hydrogel sample is in the same orientation as casted during the gelation step, so that worms are located at the bottom of the hydrogel (*see* Subheading 3.1.6 to review this concept). If a hydrogel sample is upside-down, use a flat-tip paintbrush to flip the hydrogel sample. This can be achieved by fully inserting the paintbrush to the bottom of the gel (which is floating in the 5× SSC in the well), horizontally lifting the paintbrush along with the gel, inverting the paintbrush, lowering the paintbrush until the gel makes contact with the buffer in the well, and finally retrieving the paintbrush without causing the gel orientation to flip back. If unsure about whether a flip was successful, re-image the sample until the sample is confirmed to be on the original orientation (i.e. worms at the bottom of the gel).

   For samples that have not been re-embedded, proceed to **step 2**. Otherwise, proceed to **step 5**.
2. Wash sample with 2 mL of 2.5× SSC for 30 min at RT.
3. Wash sample with 2 mL of 0.5× SSC for 30 min at RT.
4. Wash sample with 2 mL of 0.05× SSC for 30 min at RT.
5. Remove as much liquid around the expanded gel as possible. The liquid should be removed to the extent that no more solution that can be taken up by a P1000 or a P200 pipettor,

and the gel does not appear to be sliding around when the plate is slightly tilted (e.g. ~20°).

6. Perform imaging. The standard imaging workflow that we recommend is to first use a low-magnification objective (e.g. 4×) to acquire a single z-plane map of the gel sample (ideally by automated acquisition and stitching of multiple evenly spaced fields of view, which is available in most imaging software), which helps to identify and locate all animals that can be imaged. Next, use the low-magnification map of the gel sample to navigate (via automated stage control, or manually) to the animals and regions of interest. Then, use a high-magnification objective (e.g. 20× or 40×) to acquire z-stacks through the regions of interest.
7. To store the sample, re-immerse the sample in 2 mL of 5× SSC at 4 °C after imaging. If the sample was just expanded for imaging, shrink the sample by serial 30 min washes with increasing salt concentration (the steps you used to expand, in reverse order). If the sample was re-embedded (HCR performed for RNA readout), you can apply 5× SSC directly after imaging. HCR amplicons and IHC staining are stable for up to a week.

3.1.16 Agar Immobilization (Optional, for Vibration-Free Imaging)

If too much sample vibration or drifting is observed under a high-resolution objective (e.g. ≥40×), affecting image quality, immobilize the expanded gel with 2%(w/w) low-melt agarose solution prepared by dissolving 2 g of agarose in 98 mL of ddH_2O, heating to a near-boil in a microwave, and mixing by vortex until the agarose powder completely dissolves. Wait 5 min for the solution to cool and for the bubbles to settle to the top of the solution, then add the agarose solution (retrieved from the lower fraction of the solution, with no or little bubbles) to the edges of the expanded gel (after water is completely removed from the well, leaving only the gel at the center of the well) drop by drop (allow ~3 s in between drops), starting at opposite corners, then proceeding to opposite sides. After ~2 min, slowly add more low-melt agarose solution, again in a dropwise manner, to cover the entire gel. Wait for ~2 more minutes for the agarose to solidify, and proceed to imaging.

3.1.17 Example of Final Images

An example of confocal images acquired after the standard ExCel protocol is shown in Fig. 3. In this example, a transgenic L2 hermaphrodite animal expressing *tag-168p::GFP* is sequentially stained with ExFISH-HCR against the egfp mRNA, antibody against GFP, NHS ester of a fluorescent dye (Atto 647N NHS ester; against amines; for anatomical features) and DAPI (for DNA location). The sample is then expanded to 3.3× linear expansion factor, and imaged under confocal microscopy under a 40× objective (water immersion, with a numerical aperture of 1.15).

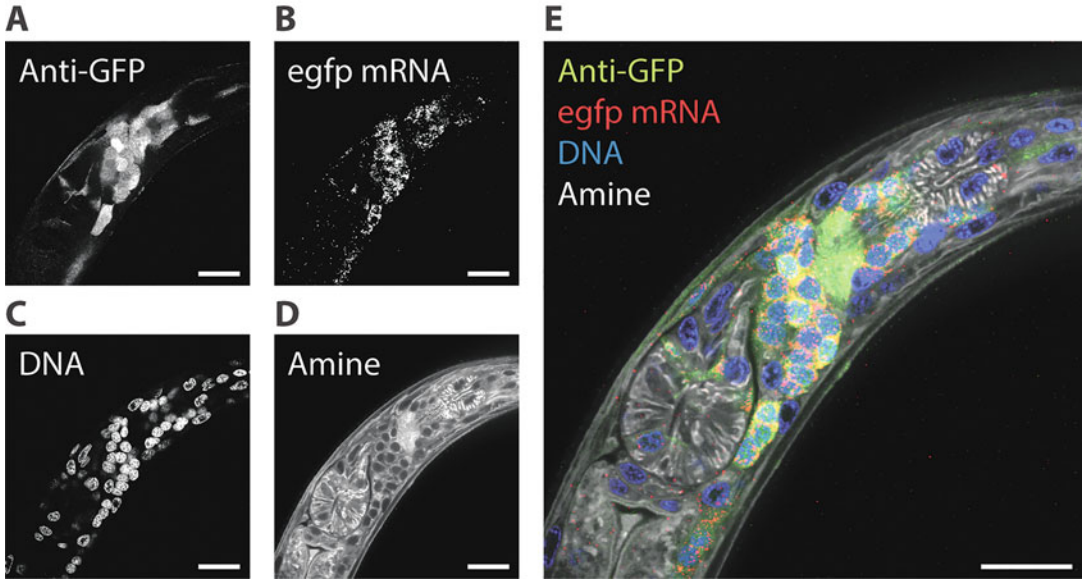

Fig. 3 Example of confocal images acquired after the standard ExCel protocol. The pharyngeal region of a representative, standard-ExCel-processed L2 hermaphrodite animal, stained sequentially with ExFISH-HCR against the egfp mRNA, antibody against GFP, NHS ester of a fluorescent dye (Atto 647N NHS ester; against amines; for anatomical features) and DAPI (for DNA location), as schematized in Fig. 1a–i, m–q. (**a**–**d**) Single-channel images of each staining modality. (**e**) Merged composite image from combining (**a**–**d**). Strain expressed tag-168p::GFP. Images are single-*z*-plane confocal micrographs acquired under a 40× objective (water immersion, with a numerical aperture of 1.15). Brightness and contrast settings: each channel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: 3.3×. Scale bars: 10 μm (in biological units, i.e. post-expansion lengths are divided by the expansion factor of the worm). (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

3.2 Epitope-Preserving Expansion of C. elegans

3.2.1 Overview

1. 2.5–3.5× linear expansion factor.
2. Supports readout of a majority of endogenous and exogenous epitopes tested (~70% of epitopes that can be detected by non-IgM-class antibodies).
3. Moderate isotropy (8–25% error over length scales between 0 and 100 μm).
4. Protocol duration, from fixation to imaging: 18 days.

See Fig. 4 for the workflow of the epitope-preserving ExCel protocol, and Table 2 for a sample schedule. To start the Epitope-Preserving ExCel protocol, first follow the standard ExCel protocol in Subheading 3.1 for Fixation and Cuticle Reduction (Subheading 3.1.2), Sample Allocation (Subheading 3.1.3) and Protein Anchoring (Subheading 3.1.5). Then, proceed to Subheading 3.2.2.

3.2.2 Gelation

Same as in gelation procedure in the standard ExCel protocol (Subheading 3.1.7), but in **step 13**, prepare Collagenase VII Solution instead of digestion solution. Also, to ensure that the final state expanded worm could be captured within the working distance of a high-NA lens, perform either or both of the two methods for tracking hydrogel orientation (*see* Subheading 3.1.6).

Reaction volumes in the following sections are specified for a single hydrogel. Scale up if you are processing multiple gels.

3.2.3 Collagenase VII-Mediated Cuticle Digestion

1. Right after the samples are transferred to 37 °C for the 2-h incubation, thaw a frozen aliquot of Collagenase VII Stock Solution (500 μL per aliquot; at 1 kU/mL) by incubating the frozen aliquot at 4 °C until the 2-h gelation incubation is complete.
2. After the 2-h gelation incubation and before opening the gelling chambers, prepare 1 mL of Collagenase VII Solution by adding 500 μL of Collagenase VII Stock Solution to 500 μL of Collagenase VII Dilution Buffer.
3. Add ~50 μL of Collagenase VII Solution to the trimmed gel immediately after trimming (the trimmed gel should still be on the coverslip). Let the gel sit immersed in the solution for 5 min at RT.
4. Add ~950 μL of Collagenase VII Solution to a 2-mL Eppendorf tube (which has a flatter bottom compared to the 1.5-mL tubes; e.g. VWR 20170-170; if not available, a 1.5-mL tube can work too). With a flat-tip paint brush, slowly insert the brush under the gel until most of the brush is inserted, vertically lift the gel with the brush, and transfer the gel into the tube. Incubate sample overnight at 37 °C. ******.
5. Wash sample (i.e. replace the incubating solution) three times with 1 mL of TNC-40020 Buffer for 1 h each at RT.

3.2.4 Protein Denaturation

In this section, the hydrogel sample will be immersed in the Protein Denaturation Buffer, which contains a near-saturating concentration of SDS. As a result, at various steps of this section (especially during the washing steps after the 95 °C incubation of **step 4**), white precipitates of SDS can form inside the hydrogel. As long as you perform the extensive washes thoroughly, as specified in the steps below, the SDS precipitates should be completely removed from the hydrogel samples at the end of this section, and have never been observed to affect the final results. (But of course, if not completely washed away by the specified procedure, SDS can in principle disrupt antibody-antigen recognition, and result in reduced fluorescent signals from the immunostaining performed in the next section).

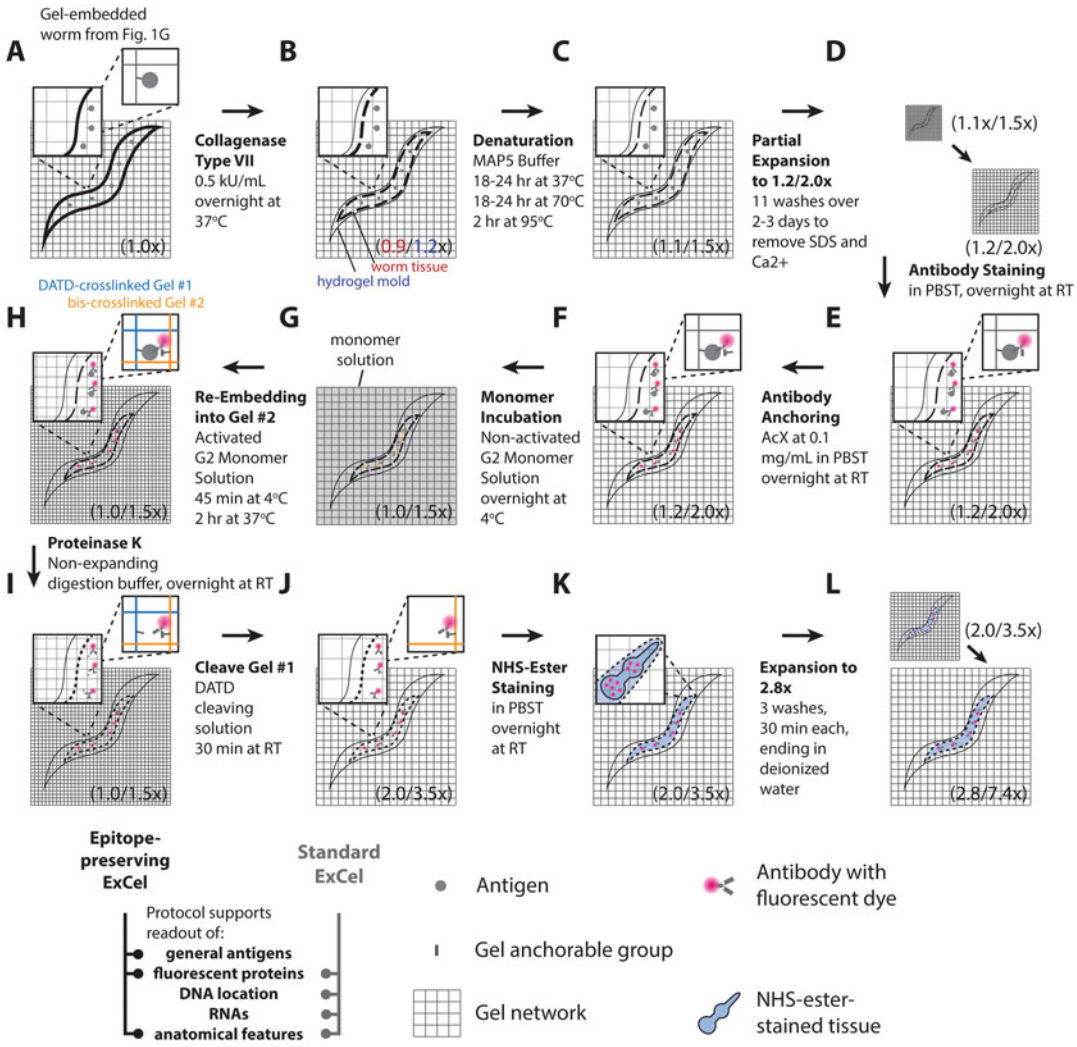

Fig. 4 Workflow for the epitope-preserving ExCel protocol. A method for expanding cuticle-enclosed intact *C. elegans*, while permitting readout of a majority of antigens that are detectable through non-IgM-class antibodies (~70%; estimated from the immunostaining results from a panel of non-IgM antibodies [7]). Sample processing prior to Panel **a** is identical to the workflow for the standard ExCel protocol without ExFISH (as shown in blue arrows in Fig. 1) until, and including, the gelation step (Fig. 1a–c, e–g). The linear expansion factor of the hydrogel-specimen composite is shown in parentheses. For stages in which the worm tissue expands to a less extent than the surrounding hydrogel, which occurs due to incomplete homogenization of mechanical strength of the fixed worm tissue, the expansion factors of the worm and of the hydrogel are shown in front of and after a slash sign, respectively. (**a**–**l**) Steps of the protocol, with the bold text indicating the title of the step. (**a**) Hydrogel polymerization is performed on the specimen, by first incubating the specimens in activated monomer solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, AcX-modified proteins are covalently anchored to the hydrogel network. (**b**) Specimens are treated with chromatography-purified collagenase type VII at 0.5 kU/mL, in a calcium-containing tris-buffered saline overnight (18–24 h) at 37 °C. During this treatment, the hydrogel expands by ~1.2× linearly, whereas the worm slightly reduces in size to ~0.9× linearly. Due to the mismatch in expansion factor between the worm and the gel, the worm

1. If sample is not already in a capable or sealable tube, transfer samples into one (e.g. 2-mL tube, VWR 20170–170), because the sample will be incubated in Protein Denaturation Buffer at elevated temperatures (37 °C, 70 °C, 95 °C) in the next few steps. Without sealing the content, evaporation of water from the buffer will cause extensive precipitation of SDS, and also change the concentrations of other ingredients of the denaturation buffer.
2. Incubate sample with 1 mL of Protein Denaturation Buffer (stored at 37 °C) overnight at 37 °C. **.

Fig. 4 (continued) tissue detaches from the surrounding hydrogel, but physically remains in the hydrogel mold that was made of its own shape during the gelation step in **a**. (**c**) Specimens are processed with a denaturation treatment, in which they are incubated in a minimally-expanding protein-denaturing buffer overnight (18–24 h) at 37 °C, overnight (18–24 h) at 70 °C, and 2 h at 95 °C. Reduced calcium and NaCl concentrations are used in this buffer, compared to other non-expanding buffers designed in this paper, due to their incompatible solubility with SDS at higher concentrations. (**d**) Specimens are washed four times in a tris-buffered saline to remove SDS from the hydrogel sample. Specimens are then washed four times in tris-buffered saline with reducing calcium concentration (once with TNT Buffer + 10 mM $CaCl_2$, and then three times with TNT Buffer) to remove calcium ions from the hydrogel sample. Finally, specimens are washed with phosphate-buffer saline with reducing NaCl concentration (once with PBST-0.1% + 500 mM NaCl, twice with PBST-0.1%). (**e**) Specimens are immunostained with fluorescent antibodies against the target antigens. (**f**) Specimens are incubated with AcX at a concentration of 0.1 mg/mL in PBST-0.1% overnight at RT. This step equips proteins, including the fluorescent antibodies introduced in **e**, with a polymer-anchorable moiety. (**g**) Specimens are incubated in Non-Activated G2 Monomer Solution overnight at 4 °C, to ensure complete diffusion of the monomer solution throughout the specimen, prior to the gelation reaction. (**h**) Specimens are re-embedded into a second expandable hydrogel, by incubating the specimens in activated monomer solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, AcX-modified fluorescent antibodies are covalently anchored to the hydrogel network of the second hydrogel (orange grids). We use blue grids to represent the hydrogel network of the first, DATD-crosslinked hydrogel (i.e. the network synthesized in Panel **a**), to differentiate it from the network of the re-embedding second hydrogel. (**i**) Specimens are treated with Proteinase K at 8 U/mL, in Non-expanding Digestion Buffer overnight (18–24 h) at RT, to further reduce the mechanical strength of the original worm tissue and permit greater expansion. During this proteolytic treatment, most proteins lose antigenicity, but some of the fluorescent signals from AcX-anchored fluorescent proteins are retained. (**j**) Specimens are treated with DATD-cleaving solution for 30 min at RT, to chemically disintegrate the first hydrogel, which contains the periodate-cleavable crosslinker *N,N'*-diallyl-tartardiamide (DATD), while sparing the second hydrogel, which contains a periodate-resistant crosslinker, *N,N'*-methylene-bis-acrylamide (bis). (**k**) To visualize anatomical features, specimens can be stained with an *N*-hydroxysuccinimide ester (NHS ester) of fluorescent dye. NHS-ester staining is performed at 5 mM in PBST-0.1% overnight at RT. (**l**) Specimens are expanded with one wash in 0.1× PBS and two washes in deionized water. At this stage, the hydrogel expands by ~7.4× linearly, whereas the worm tissue expands by ~2.8× linearly, within a range from 2.5 to 3.5× (median, 2.78×; mean, 2.83×; $n = 10$ independently processed hydrogels from 2 sets of experiments [7]). After expansion, specimens are ready for imaging. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

3. Replace solution with another 1 mL of Protein Denaturation Buffer (at 37 °C). Incubate overnight at 70 °C. **.
4. Pre-warm 1 mL of Protein Denaturation Buffer to 70 °C.
5. Replace solution with 1 mL of Protein Denaturation Buffer (pre-warmed to 70 °C in **step 3**). Incubate for 2 h at 95 °C.
6. After the 95 °C incubation, incubate samples at 37 °C for 30 min. At this time, pre-warm at least 4 mL of TNC-40020 Buffer to 37 °C.
7. Wash sample three times with 1 mL of TNC-40020 (pre--warmed to 37 °C in **step 6**) for 1 h each at 37 °C. Then, incubate sample in TNC-40020 overnight at RT. **.
8. Wash sample once with TNT-10 Buffer (TNT Buffer +10 mM $CaCl_2$) for 30 min at RT.
9. Wash sample twice with TNT Buffer for 30 min each at RT. Then, incubate sample in TNT Buffer overnight or at least 6 h, at RT. The rationale here is to completely wash out remaining calcium ions from the gel, so when a phosphate-based buffer (PBS) is added in the next step to prepare for immunostaining, phosphate ions will not precipitate with calcium ions to form a nearly insoluble product, which can cause a cloudy appearance in the gel and affect image quality. **.
10. Transfer gel sample to a 24-well plate by directing pouring the content in the 2-mL tube into a well. Alternatively, use a flat-tip paint brush to transfer the gel.
11. Wash sample once with PNT-500 (1× PBS + 0.1% Triton X-100 + 500 mM NaCl) for 30 min at RT.
12. Wash sample twice with PBST-0.1% (1× PBS + 0.1% Triton X-100) for 30 min each at RT.

3.2.5 Antibody Staining

1. Incubate sample with primary antibody, at desired concentration (to determine this, the user can follow manufacturer's instruction, earlier literature, or use a default concentration of 5 μg/mL; this concentration can be later adjusted to optimize for signal to background ratios) in 500 μL of PBST-0.1% overnight at RT. **.
2. Wash sample with 1 mL of PBST-0.1% for three times, 1 h each at RT.
3. Incubate sample with secondary antibody with a fluorophore, at desired concentration (to determine this, the user can follow manufacturer's instruction, earlier literature, or use a default default concentration of 10 μg/mL; this concentration can be later adjusted to optimize for signal and background ratios) in 500 μL of PBST-0.1% overnight at RT. **.

4. Wash sample with 1 mL of PBST-0.1% for three times, 1 h each at RT.

Linear expansion factor of the worm is ~1.2× at this stage. (Hydrogel expansion factor is ~2.0×, so worms will detach from the surrounding hydrogel, but stay inside the hydrogel mold. With reasonably gentle washes, retention of worms inside the hydrogel sample is typically >95% despite of this detachment due to the expansion factor mismatch between the worm and the gel.)

3.2.6 Preliminary Imaging to Check Antibody Staining

The samples are currently at a ~1.2× expanded state. Although a round of preliminary imaging at this point is not necessary for the final imaging, we highly recommend doing this, in order to confirm that the antibody staining works as expected (e.g. positive and/or negative control conditions produce a pattern of staining consistent to past results). The fluorescent intensity at the stained sites is maximal at the current stage, and will only reduce during the following procedures. Thus, if a particular stain is not showing a clear signal at this state, there is very little possibility that it will result in a clear image after expansion.

1. Transfer sample to a container suitable for imaging, e.g. a 24-well plate (glass-bottom ones are recommended, but plastic ones are also sufficient for this preliminary imaging), if samples are not already in such a container.
2. Remove most liquid (e.g. 95–99%) from the well as possible. The liquid should be removed to the extent that no more solution that can be taken up by a P1000, and the gel does not appear to be sliding around when the plate is slightly tilted (e.g. ~20°).
3. Perform imaging. The standard imaging workflow that we recommend is to first use a low-magnification objective (e.g. 4×) to acquire a single z-plane map of the gel sample (ideally by automated acquisition and stitching of multiple evenly spaced fields of view, which is available in most imaging software), which helps to identify and locate all animals that can be imaged. Next, use the low-magnification map of the gel sample to navigate (via automated stage control, or manually) to the animals and regions of interest. Then, use a high-magnification objective (e.g. 20× or 40×) to acquire z-stacks through the regions of interest.
4. After imaging, add 1 mL of PBST-0.1% to the well, to re-immerse the sample for storage. The immunostained samples can be stored at 4 °C for up to 3 days. **.
5. Based on the imaging result (e.g. whether samples have the expected staining patterns and reasonable levels of signals), determine which samples will be processed further for the

final imaging at 2.8× linear expansion factor. Only proceed those samples through the next sections.

3.2.7 Antibody Anchoring

1. Incubate sample with AcX at 0.1 mg/mL (1:100 dilution of AcX stock) in 1 mL of PBST-0.1%, overnight at RT. **.
2. Wash sample with 1 mL of MOPST-0.1% for three times, 1 h each at RT.

3.2.8 Re-Embedding into an Expandable Second Gel

1. Flip each gel to the same side as when they were casted during the initial gelation (refer to Subheading 3.1.6 to review this concept). This ensures that the worms remain on the bottom surface of the gels, so the working distance of the objective can maximally cover the depth of the worm at the final imaging stage.
2. Incubate sample in 1 mL of Non-activated G2 Monomer Solution overnight at 4 °C. **.
3. Prepare 1 mL of Activated G2 Monomer Solution with all reagents except for APS (Epitope-preserving ExCel G2 Monomer Solution Stock + 4-HT + TEMED; do not add the activator APS yet), and thoroughly mix the solution by a vortex. Chill the solution to 4 °C on a cold block. It is important to ensure that the solution is fully chilled to 4 °C before proceeding.
4. Remove liquids from the sample.
5. Activate the monomer solution from **step 3** by adding 20 μL of 10% APS, and thoroughly mix the solution by a vortex. Start a timer right after the APS is added.
6. Add 1 mL of Activated G2 Monomer Solution to the sample. Incubate the sample at 4 °C for 30 min, on a pre-chilled cold block.
7. During incubation, construct gelling chambers (based on the same architecture as shown in Fig. 2 and the Gelation section of the standard ExCel protocol above). Use a stack of one #0 cover glass and one #1 cover glass (total height ~250 μm) for each spacer.
8. Immediately after the 30-min incubation, transfer 65 μL of the monomer solution from the well that contains the gel sample to the center of the gelling chamber, as a single droplet.
9. With a flat-tip paint brush, transfer the gel sample into the droplet of monomer solution. If the gel orientation has been confirmed in **step 1**, ensure that the hydrogel is placed in the correct orientation (i.e. with worms located at the bottom surface of the gel). Then, use a pipettor to temporarily remove the monomer solution from the gel, to ensure that the gel is completely flat and not folded. If the gel is folded, use a paint

brush to unfold the gel. Then, add the monomer solution back on top of the gel to immerse the gel.

10. Close the chamber by carefully and slowly placing a rectangular cover glass (e.g. 22 × 50 mm) on top of the spacers, minimizing bubble formation. One way to achieve this is to use a tweezer to clip on one end of a rectangular cover glass, hold the cover glass right above the chamber by ~5 mm (in parallel to the glass slide; no contact yet), press down the un-clipped end of the rectangular cover glass until it contacts the spacer, release the clipping force so that the cover glass just rests on the lower branch of the tweezer, and then slowly lower the tweezer until the cover glass contacts the other spacer.
11. Incubate the gelling chamber at 37 °C for 2 h, in a humidified chamber (e.g. a 0.75-quart storage container, such as Amazon.com #B01M2BTKYB, with the gelling chamber sitting on top of a stack of two lids of the 6-, 24-, or 96-well plate, and with a small amount (10–20 mL) of deionized water at the bottom of the sealed container).
12. After the 2-h incubation and before proceeding to **step 13** (chamber opening), prepare the digestion solution (*see* **step 1** of the next section).
13. With a tweezer or a razor blade, remove the top cover glass and spacer and leave the gel on the glass slide. The gel can occasionally come off with the top cover glass. In that case, leave the gel on the cover glass and dispose the rest of the chamber.
14. At this point, the boundary of the re-embedded gel sample should be easily visible under sufficient room light. With a razor blade, trim away the excessive gel outside of the original gel sample.

3.2.9 Proteinase K Digestion

Based on our experience, the extent of digestion seems to be quite sensitive to the quality of Proteinase K. Use Proteinase K ordered within 2 months.

1. Prepare 1 mL of digestion solution by adding Proteinase K to Non-expanding Digestion Buffer to a final concentration of 8 U/mL (1:100 dilution).
2. Add ~50 μL of digestion solution to the trimmed gel. Let the gel sit immersed in digestion solution for 2 min at RT.
3. Add ~950 μL of digestion solution to a well in a 24-well plate. With a flat-tip paint brush to transfer the gel into the well. Incubate sample overnight (18–24 h) at room temperature. ******.
4. Wash once with 1 mL of TNT-20 Buffer (TNT Buffer + 20 mM $CaCl_2$) for 30 min at RT.

5. Wash once with 1 mL of TNT-10 Buffer (TNT Buffer + 10 mM $CaCl_2$) for 30 min at RT.
6. Wash three times with 1 mL of TNT Buffer for 1 h each at RT.
7. Wash sample once with PNT-500 (1× PBS + 0.1% Triton X-100 + 500 mM NaCl) for 30 min at RT.
8. Wash sample once with 1× PBS for 30 min at RT. **.

3.2.10 Cleave DATD-Crosslinked Gels #1

1. Transfer sample into a 6-well plate with a flat-tip paint brush.
2. Make DATD-cleaving solution from freshly prepared sodium periodate stock solution.
3. Incubate sample in 2 mL of DATD-cleaving solution (or volumes sufficient to cover the entire gel) at RT for **exactly 30 min** with gentle shaking. Longer incubation periods can degrade the structural integrity of the bis-crosslinked Gel #2, which we aim to preserve here.
4. Wash sample with 5 mL of 1× PBS for 20 min at RT with gentle shaking.
5. Repeat **step 4** five times (six washes total), and then leave in 5 mL of 1× PBS at RT overnight with gentle shaking. It is crucial to completely wash away sodium periodate from the gels, since residual amounts of periodate ion can disintegrate the bis-crosslinked Gel #2 over a time scale of ~2–24 h. **.

3.2.11 NHS-Ester Staining (Optional, for Morphology Readout)

1. Incubate sample with 5 μM of the NHS ester of a fluorescent dye in 2 mL of PBST, overnight at RT with gentle shaking. **.
2. Wash sample with 2 mL of PBST three times, 30 min each at RT.

3.2.12 Expansion and Imaging

1. To ensure that the expanded sample will fit flatly into the final imaging container, the sample needs to be trimmed into sizes that, when multiplied by ~2× in each dimension (as the sample will expand by another ~2× from the current state), will completely fit in the imaging container. For example, if the final imaging container is a glass-bottom 6-well plate (*see* **step 2**), and if sample is larger than 1 cm in any dimension, cut the sample into pieces that are each within 1 cm in any dimension. One way to achieve this is by performing two perpendicular cuts, in the shape of a plus sign, at the center of the sample, which will result in 4 adequately-sized pieces that can each be expanded and imaged separately. The cutting can be performed with a #1.5 rectangular coverslip (we do not recommend a thinner thickness, as the coverslip can break easily) directly inside the 6-well plate that currently holds the samples.
2. Transfer sample to a container suitable for imaging, e.g. a glass-bottom 6-well plate. For high-magnification objectives with

limited working distance, ensure that the gel is flipped to the same orientation as when it was casted during the initial gelation, so that the animals will be located at the bottom surface of the gel. (Refer to Subheading 3.1.6 to review this concept.)

3. Wash sample with 2 mL of 0.1× PBS for 30 min at RT.
4. Wash sample with 2 mL of deionized water for 30 min at RT.
5. Repeat **step 3** for one more time (two washes with deionized water total).
6. Remove as much liquid around the expanded gel as possible.
7. Perform imaging.
8. To store the sample, re-immerse the sample in 2 mL of 1× PBS and incubate at 4 °C after imaging. Due to the covalent linkage between the antibody and the hydrogel, performed in the "Antibody Anchoring" section above, fluorescent signal in the sample is stable for at least 1 week.

See Subheading 3.1.16 for agar immobilization protocol to prevent sample vibration or drift during imaging.

3.2.13 Example of Final Images

An example of images acquired after the epitope-preserving ExCel protocol is shown in Fig. 5.

3.3 Iterative Expansion of C. elegans (iExCel)

3.3.1 Overview

1. ~20.0× linear expansion factor.
2. Supports readout of fluorescent proteins; requires oligo-conjugated secondary antibodies (*see* Subheading 3.3.3 for the conjugation protocol; protocol duration: ~3 days).
3. Similar level of isotropy as ExCel (1.5–4.5% error over length scales between 0 and 100 μm).
4. Protocol duration, from fixation to imaging: 19 days.
5. It is highly recommended that the user first confirms successful staining and imaging of the fluorescent protein target with standard ExCel, prior to attempting the iExCel protocol on the same strain(s). This is because iExCel is both more technically demanding, requires longer processing times, and results in dimmer absolute signal (i.e. fluorescent intensity under a microscope) compared to the standard ExCel protocol. If a bright signal cannot be observed from the standard ExCel protocol for a given fluorescent protein on a given strain, the chance is very low that the signal will be successfully detected under the current iExCel protocol.

See Fig. 6 for the workflow of the iterative ExCel protocol, and Table 3 for a sample schedule. The iterative ExCel protocol described here only supports readout of fluorescent proteins, i.e. same as the standard ExCel protocol shown above. This protocol is currently not compatible with RNA readout. The sensitivity

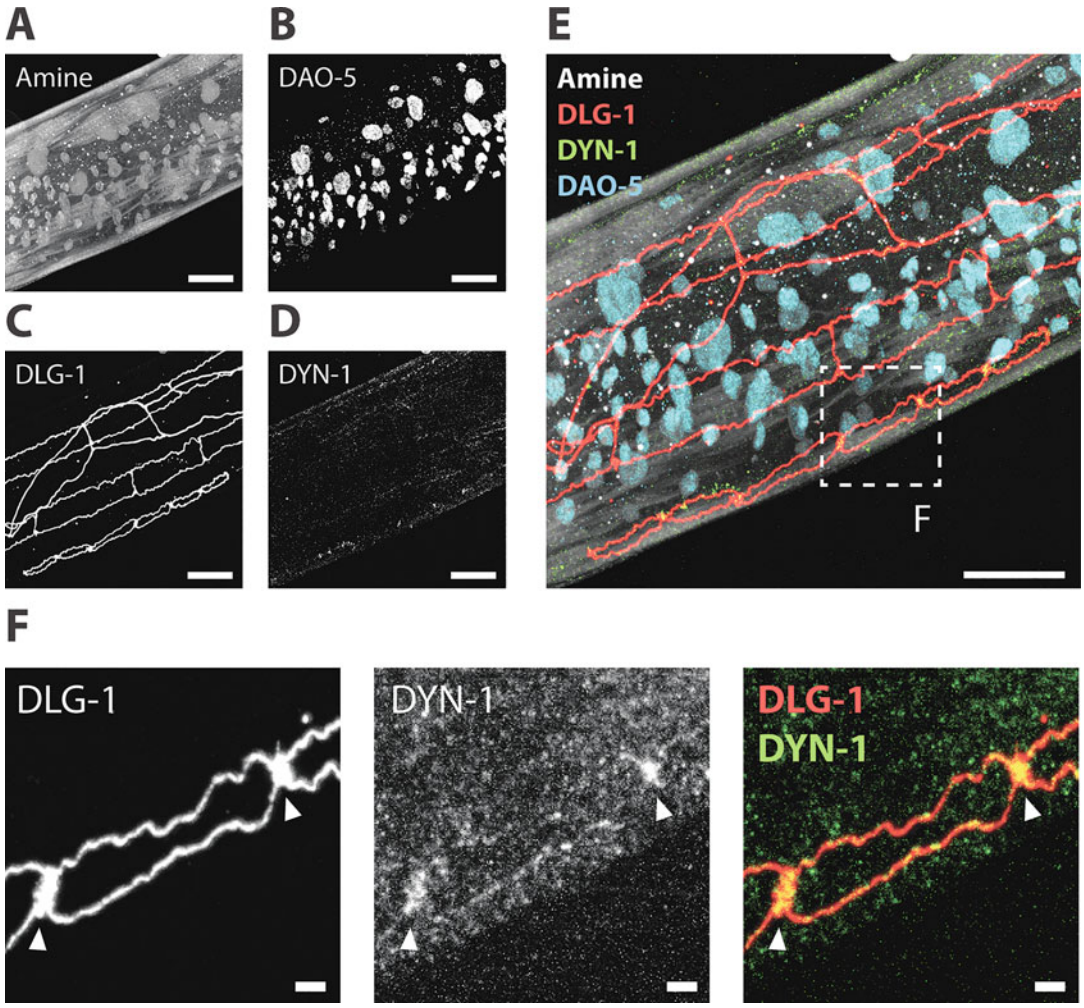

Fig. 5 Example of confocal images acquired after the epitope-preserving ExCel protocol. A representative epitope-preserving-ExCel-processed L2 hermaphrodite animal, stained with antibodies against DLG-1 (disc large; a scaffolding protein that localizes to adherens junctions), DYN-1 (dynamin; localizes to clathrin-mediated endocytic sites), DAO-5 (a nuclear protein) [18], and an NHS ester of a fluorescent dye (Alexa 405 NHS ester; against amines; for anatomical features). (**a**–**d**) Single-channel images of each staining modality, centered nearby the developing vulva (lower boxed region in **a**). (**e**) Merged composite image from combining **a**–**d**. Boxed region marks one of the six vulval progenitor cells, as delineated by the adherens junction marker DLG-1, and is shown in magnified views in Panel **f**. (**f**) Magnified view of the boxed region in **e**, as single-channel images of DLG-1 (left) or DYN-1 (middle) staining, or merged composite image between these two channels (right). Arrows, sites of contact between vulval progenitor cells, which shows co-localized signals of DLG-1 and DYN-1. Images are max-intensity projection of a confocal stack acquired through the entire animal (for Panels **a**–**e**), or only through the DLG-1 marked structure (for Panel **f**; to reduce the noise coming from planes outside of the structure of interest; i.e. the DLG-1 marked adherens junctions of the developing vulva), under a 40× objective (water immersion, with a numerical aperture of 1.15). Brightness and contrast settings: each channel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: worm, 3.2×; surrounding hydrogel, 7.9×. Scale bars: Panel **a**–**e**, 10 μm; Panel **e**, 1 μm (in biological units, i.e. post-expansion lengths are divided by the expansion factor of the worm). The pharyngeal region of a representative, standard-ExCel-processed L2 hermaphrodite animal,

to read out target signal is lower than the standard ExCel protocol (due to a greater volumetric dilution associated with the expansion process), and therefore it works best on fluorescent proteins that are strongly expressed (ideally, with clearly observable signal at the pre-expansion state, when imaged under a high NA (>~0.8) objective under typical illumination settings on a confocal microscope).

Prior to starting the iterative ExCel protocol, read through Subheading 3.3.2, and synthesize the DNA-conjugated secondary antibody (or antibodies) for the upcoming experiment, unless you have enough stock from a previous conjugation. A protocol for conjugating the DNA oligo-nucleotides to secondary antibodies is shown in Subheading 3.3.3.

To start the iterative ExCel protocol, first follow the standard ExCel protocol in Subheading 3.1 for Fixation and Cuticle Reduction (Subheading 3.1.2), Sample Allocation (Subheading 3.1.3), Protein Anchoring (Subheading 3.1.5), Gelation (Subheading 3.1.7), and Digestion (Subheading 3.1.8). Then, proceed to Subheading 3.3.4.

3.3.2 Pre-iterative ExCel Notes and Protocols

A number of oligo-nucleotides are used in this protocol. They serve several purposes: (1) acting as spatial anchors for the immunostained locations, (2) signal transfer between gels, and (3) signal amplification and fluorescent readout. Their sequences and ordering information can be found in Table 4.

It is also necessary to synthesize DNA-conjugated secondary antibody prior to starting the Iterative ExCel protocol. The procedure for this is shown in Subheading 3.3.3.

Finally, since the hydrogel sample will become ~20× larger in the *z*-dimension at the end of this protocol, if it is desired to image with a short-working-distance objective (e.g. <1 mm; most high NA magnification objectives are in this category) at the fully expanded state, it is necessary to ensure that the gel is flipped back to the same side as the Gel#1 casted state, at two time points: (1) when the gel is re-embedded into Gel #2, and (2) before the expansion process prior to the final imaging. This way, the worms (which settle to the lower ~50 μm during Gel #1 gelation, out of the full ~180-μm thickness of the gel) will remain at the bottom

Fig. 5 (continued) stained sequentially with ExFISH-HCR against the egfp mRNA, antibody against GFP, NHS ester of a fluorescent dye (Atto 647N NHS ester; against amines; for anatomical features) and DAPI (for DNA location), as schematized in Fig. 1a–i, m–q. (**a**–**d**) Single-channel images of each staining modality. (**e**) Merged composite image from combining **a**–**d**. Strain expressed tag-168p::GFP. Images are single-z-plane confocal micrographs. Brightness and contrast settings: each channel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: 3.3×. Scale bars: 10 μm. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

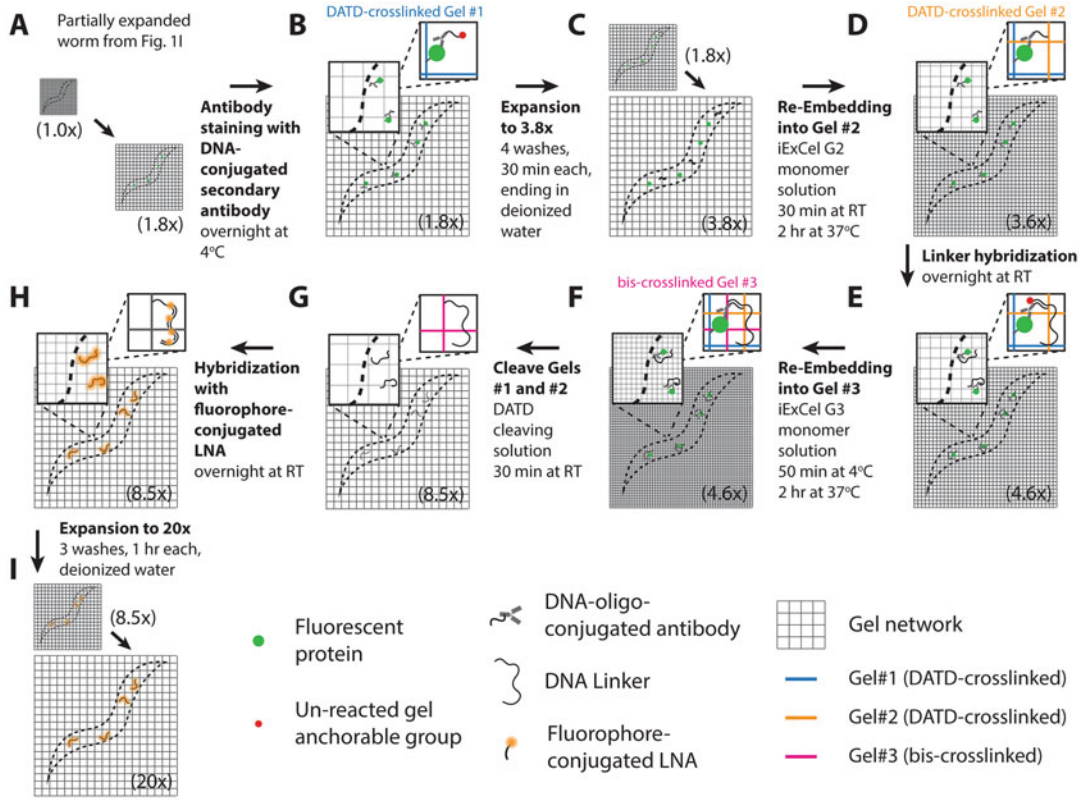

Fig. 6 Workflow for the iterative ExCel (iExCel) protocol. A method for iteratively expanding cuticle-enclosed intact *C. elegans*, for a final linear expansion factor of ~20×. Sample processing prior to Panel A is identical to the workflow for the standard ExCel protocol without ExFISH (as shown in blue arrows in Fig. 1) until, and including, the post-Proteinase-K partial expansion step (Fig. 1a–c, e–g). The linear expansion factor of the hydrogel-specimen composite is shown in parentheses. (**a–i**) Steps of the protocol, with the bold text indicating the title of the step. (**a**) Specimens are partially expanded from a linear expansion factor of 1.0× to 1.8×, with the same protocol as shown in Fig. 1i. (**b**) Specimens are immunostained first with primary antibodies against fluorescent proteins in 5×SSCT overnight at 4 °C, and then with secondary antibodies that have been conjugated to a 24-base DNA oligonucleotide, in DNA-conjugated Antibody Staining Buffer overnight at 4 °C. The DNA oligo is conjugated to the antibody at the 3′ end, and contains a gel-anchorable group at the 5′ end. (**c**) Specimens are expanded from a linear expansion factor of 1.8× to 3.8×, with the same protocol as shown in Fig. 1m. (**d**) Specimens are re-embedded into another non-expandable hydrogel ("Gel #2") to lock up its size at the expanded state, as shown in Fig. 1n, except that the monomer solution is replaced by DATD-crosslinked Re-embedding Monomer Solution, which results in a hydrogel that can be later disintegrated via crosslinker cleavage, to allow full expansion of the final expandable gel. The DATD-crosslinked re-embedding monomer solution contains a charged molecule APS. Therefore, the linear expansion factor slightly drops from 3.8× to 3.6×during this step. During hydrogel polymerization, the DNA oligo on the antibody, which contains a gel-anchorable group, is covalently anchored to the second hydrogel network (orange grids). (**e**) Specimens are incubated with a 100-base DNA oligonucleotide ("Linker"), which hybridizes to the 24-base DNA oligo on the secondary antibodies, and which contains a gel-anchorable group on its 5′ end, in iExCel hybridization buffer overnight at RT. (**f**) Specimens are re-embedded into another expandable hydrogel ("Gel #3"), by incubating the specimens in Activated iExCel G3 Monomer Solution for 30 min at 4 °C, transferring the specimens into a gelling chamber, and incubating the chamber for 2 h at 37 °C. During polymerization, the linker DNA oligo, which contains a gel-anchorable group, is covalently anchored to the hydrogel network of the third hydrogel (magenta grids). (**g**) Specimens are treated with DATD-cleaving solution

surface of the gel at the end of this protocol, ensuring maximal coverage of the worm tissue by the working distance of the objective during the final imaging. In order to provide a way to check the orientation (side) of the gel throughout the protocol, at least one of the two additional procedures is necessary at the end of the Gelation step. Refer to Subheading 3.1.6 to review these concepts.

3.3.3 Protocol for Synthesizing DNA-Conjugated Secondary Antibody for iExCel

This protocol is essentially identical to the conjugation protocol in the original ExM manuscript (available on www.expansionmicroscopy.org), except for 1 modification. For the reaction between unconjugated secondary antibody and S-HyNic, three times of the S-HyNic concentration is used in this protocol compared to the original one (6:100 dilution of the S-HyNic stock solution, instead of 2:100 dilution in the original protocol).

Prepare the DNA for Conjugation

In this part, the process can be paused after any of these steps: **steps 10**, **12**, **15**, **19**, **29**. If pausing is desired, store the reagent at 4 °C, after the specified steps are complete.

1. Order from IDT the DNA oligo "5′Ac-AA-B1-AA-3′Amine" (*see* Table 4 for sequence) at a synthesis scale of 1 μmole. If two-color readout is desired, additionally order the oligo "5′Ac-AA-A2-AA-3′Amine." Be sure to add the specified modifications at the 5′ and 3′ ends. Choose standard desalting as the purification method.

   The instruction below applies to a single tube of DNA oligo from IDT. If two-color readout is desired, perform process to both tubes of oligos.

2. Add 100 μL of deionized water to the DNA oligo (shipped dry). Vortex for 1 min to dissolve. Spin with a tabletop centrifuge for 5 s to collect solution. Transfer the solution to a separate 1.5 mL tube. Keep the original tube from IDT, as its label contains DNA dry weight information that is useful later.

3. Add 100 μL of chloroform to the tube in a chemical hood. (Chloroform is volatile; handle only in the hood.) Vortex the tube to mix, and then spin down with a tabletop centrifuge to separate the aqueous and chloroform fractions from each other. (A clear line separating the two fractions should be visible.) Use

Fig. 6 (continued) for 30 min at RT, to chemically disintegrate the first and the second hydrogels, which contain a periodate-cleavable crosslinker *N,N′*-diallyl-tartardiamide (DATD), while sparing the third hydrogel, which contains a periodate-resistant crosslinker *N,N′*- methylene-bis-acrylamide (bis). (**h**) Specimens are incubated with a fluorophore-conjugated 15-base locked nucleic acid (LNA) oligonucleotide, which hybridizes to the 100-base linker DNA oligo at four locations, in iExCel hybridization buffer overnight at RT. (**i**) Specimens are expanded to a linear expansion factor of ~20×, with three washes in deionized water. After expansion, specimens are ready for imaging. (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

Table 4
Sequences and synthesis parameters of the oligo-nucleotides used in the iExCel protocol

DNA oligo name	Purpose	Sequence	Required modification	Purification	Recommended synthesis scale
B1-B2 system (for 1-color readout)					
5′Ac-AA-B1-AA-3′Amine	Conjugation to secondary antibody	/5ACryd/AAG TTC GGA TTC TTA GGG CGT AAA/3AmMO/	5′acrydite 3′amine	Standard desalting	1 μmole
5′Ac-B1′-4xB2′	Post-Gel#2 Linker to transfer signal location between gels; amplifies signal by 4x via branched DNA scheme	/5ACryd/AT ACG CCC TAA GAA TCC GAA ATA GCA TTA CAG TCC TCA TAA TAG CAT TAC AGT CCT CAT AAT AGC ATT ACA GTC CTC ATA ATA GCA TTA CAG TCC TCA TA	5′acrydite	PAGE	1 μmole
LNA_B2-Atto647N	Post-Gel#3 final readout	T+GA +G+G+G C+T+G +TA+A +T+GC /3ATTO647NN/(+ denotes that the next base is a locked nucleic acid)	3′ Atto 647N	HPLC	1 μmole
A2-A1 system (for 2-color readout)					
5′Ac-AA-A2-AA-3′Amine	Conjugation to secondary antibody	/5ACryd/AAA GAT TGA GAT GCC TGT CAC CAA/3AmMO/	5′acrydite 3′amine	Standard desalting	1 μmole
5′Ac-A2′-4xA1′	Post-Gel#2 Linker to transfer signal location between gels; amplifies signal by 4x via branched DNA scheme	/5ACryd/GGT GAC AGG CAT CTC AA TCT ATT ACA AAG CAT CAA CGA TTA CAA AGC ATC AAC GAT TAC AAA GCA TCA ACG ATT ACA AAG CAT CAA CG	5′acrydite	PAGE	1 μmole
LNA_A1-Atto647N	Post-Gel#3 final readout	C+GT +T+GA +TG+C +T+T+T G+T+A /3ATTO565N/(+ denotes that the next base is a locked nucleic acid)	3′ Atto 565	HPLC	1 μmole

a pipette to take the top fraction (i.e. the aqueous fraction, where DNA is dissolved in) and transfer to a separate 1.5 mL tube. This process removes the impurities from the shipped oligo, as they dissolve in the chloroform.

4. Repeat **step 3** two more times (three chloroform washes total).
5. Add 10 μL of 3 M NaCl to the DNA solution. Vortex for 5 s, and spin down for 5 s.
6. Add 250 μL of ice cold 100% ethanol. White precipitates (DNA) should form immediately. Do not mix the content. Directly incubate the tube at −20 °C for 30 min.
7. Centrifuge the tube in a refrigerated centrifuge at 4 °C, for 30 min, at 18,000 × *g* (or the maximum speed of the centrifuge if lower than 18,000 × *g*). A firm white pellet (that consists of the DNA) should form after this step.
8. Remove supernatant (ethanol) from pellet. Gently add 1 mL of ice cold 70% ethanol to the inner wall of the tube, without disturbing the pellet, and then remove as much supernatant as possible.
9. Uncap the tube, and let it sit at RT for ~1 h, to briefly dry pellet.
10. After 1 h, add Buffer A to make a DNA solution at 25 μg/μL. The amount of Buffer A needed = (DNA weight/(25 μg/μL)). The DNA weight is available on the IDT data sheet, and also on the label of the shipped tube. Use a combination of vortexing and pipetting up and down to mix the content, until the pellet completely dissolves and is no longer visible.
11. To measure the DNA concentration in the tube (cannot use the information on the shipped tube, since there are losses during purification steps), mix 1 μL of the DNA oligo stock solution with 99 μL of Buffer A in a separate 1.5 mL tube.
12. Measure the absorbance at 260 nm for the 100× diluted DNA oligo solution with a Nanodrop (or equivalent spectrometer) using Buffer A as the blank solution. Use the extinction coefficient of the DNA (shown on the IDT datasheet) to convert the A260 to DNA concentration of the diluted sample. Multiply the value by 100 to obtain the concentration for the stock tube. Multiply the stock concentration by the volume inside the stock tube, to obtain the number of nanomoles of DNA in the stock tube.
13. Calculate the amount of Sulfo-S4FB solution needed:

    (μL of Sulfo-S4FB to use) = (nmol of DNA) × 349 × 40 × $15/10^6$.

    Explanation: 349 g/mol is the molecular weight of Sulfo-S4FB; (1 mg/40 μL) is the concentration of Sulfo-S4FB

solution. 15 is the target molar ratio between Sulfo-S4FB and DNA. 10^6 adjusts the unit to the correct order of magnitude.

14. Add 40 μL of anhydrous DMSO (Thermo D12345) to 1 mg of Sulfo-S4FB (TriLink S-1008). Vortex for at least 1 min, spin down, and check if the entire dried pellet has dissolved. If more than 40 μL of Sulfo-S4FB solution is needed, do this to multiple tubes and collect fully dissolved solutions into a single tube.
15. Add the calculated amount of Sulfo-S4FB to the DNA oligo stock tube. Vortex to mix, and spin down to collect the liquid. Incubate the tube at RT overnight.
16. Purify DNA from un-reacted S4FB with a Vivaspin 500 centrifugal filter with 5 kDa molecular weight cutoff (Vivaproducts VS0101). To do this, add Buffer C to the tube until the total volume is 1000 μL. Transfer 500 μL each to two centrifugal filters. Centrifuge the tube at 13,000 × *g* for 10 min. Discard the flow-through (the portion that comes out from the bottom of the filter).
17. Add Buffer C into each filter until the volume is ~500 μL. Thoroughly mix the content, by pipetting up and down ~5 times. Centrifuge the tube at 13,000 × *g* for 10 min. Discard the flow-through (the portion that comes out from the bottom of the filter).
18. Repeat **step 17** three more times (5 spins total, from **steps 16–18**).
19. Transfer the concentrate from the filter to a separate 1.5 mL tube. Calculate the amount of Buffer C to add, in order to bring the total volume to 150 μL. Add this amount of Buffer C to the centrifugal filter and pipette up and down to mix (to collect residual DNA oligo solution from the filter), and then transfer the solution to the tube. The 4FB-DNA stock solution can now be stored at 4 °C for at least 2 years.
20. Weigh out 5–10 mg of 2-hydrazinopyridine·2HCl (abbreviated as 2-HP from this point on, Millipore Sigma H17104). Dissolve the powder in ultrapure H_2O, to a concentration of 50 mg/mL.
21. Add 91 μL of the 2-HP solution to a tube containing 50 mL of 100 mM MES Buffer, pH 5.0. This 2-HP working solution remains stable for up to 30 days at 4 °C.
22. Prepare 2-HP blank solution by adding 2 μL of water to 18 μL of 2-HP working solution.
23. Prepare 4FB-DNA MSR solution by adding 2 μL of the 4FB-DNA stock (mix stock well by vortexing before use) to 18 μL of 2-HP working solution.

24. Vortex and spin down solutions made in **steps 22** and **23**. Incubate solutions at 37 °C for 60 min.
25. After incubation, spin down the tubes for 15 s to collect condensation to the bottom of the tube. Vortex for 5 s, and spin down once more for 5 s.
26. Measure the absorbance at 360 nm and 260 nm of the 4FB-DNA MSR solution on a Nanodrop (or equivalent spectrometer) using the solution made in **step 22** as the blank solution, under the UV-Vis function of the spectrometer. Read absorbance values from the 1 mm pathlength. However, if the A260 reading is much greater than 1, read from the 0.1 mm pathlength to get an accurate reading. Make sure to use A360 and A260 that came from the same path length setting (either use values that both come from the 1 mm setting or both from the 0.1 mm setting).
27. Calculate the molar substitution ratio (MSR) with the following formula:

$$\text{MSR} = \text{A360}/\text{A260} \times (\text{DNA extinction coefficient}/24,500)$$

The MSR should be close to 1.00 (0.90–1.20 is the acceptable range). If the MSR is greater than 1.20, repeat **steps 16–19** to further purify un-reacted S4FB from the 4FB-DNA stock solution, using new centrifugal filters, until the MSR is below 1.20.

28. Calculate the amount of 4FB-DNA stock solution needed for 100 μg of antibody with the following formula:

$$\begin{aligned}&(\mu\text{L of 4FB} - \text{DNA for 100 } \mu\text{g of antibody})\\ &\quad = 7.5 \times 100/150,000 \times 1000/(\text{MSR}\\ &\qquad \times [\text{4FB} - \text{DNA stock concentration in mM}])\end{aligned}$$

Explanations: 7.5 is the molar ratio between 4FB-DNA and antibody. 100 μg is the antibody mass that we are calculating for. 150,000 g/mol is the molecular weight of an IgG antibody. 1000 adjusts to the correct order of magnitude. 4FB-DNA concentration is calculated from the A260 reading from UV-Vis and the extinction coefficient of the DNA oligo—be sure to account for the 1:10 dilution performed in **step 23**, and the pathlength setting used. For example, if the A260 is measured from the 4FB-DNA MSR solution at the 0.1 mm wavelength, multiply the A260 first by a factor of 10 (accounts for 1:10 dilution), and then by a factor of 100 (accounts for the 0.1 mm pathlength used, as the IDT extinction coefficient is given in the unit of $(M \times \text{cm})^{-1}$, where 1 cm/0.1 mm = 100. Divide this post-adjusted A260 by the extinction factor, to get the 4FB-DNA stock concentration in *M*. Finally, multiple by 1000 to get the quantity in mM.

29. Record the numbers from **steps 27** and **28** for each 4FB-DNA stock solution synthesized. The analyzed 4FB-DNA stock solution is ready for conjugation with antibodies, and can be stored at 4 °C for at least 2 years.

Prepare the Antibody for Conjugation

The following protocol specifies quantities needed for a single conjugation between DNA oligo and antibody. If multiple combinations of DNA and antibody are desired, such as for the 2-color readout (e.g. anti-chicken conjugated to B1, anti-rabbit conjugated to A2), scale up by processing multiple batches in parallel. For this part, it is recommended to execute the entire protocol within a session (takes ~3–4 h) without pausing.

Buffer exchange 100 μL of the unconjugated secondary antibody into Buffer A using a spin columns with 40 kDa molecular weight cutoff. To do this, use the follow manufacturer's instructions (**steps 30–38**):

30. Remove the bottom closure (the plastic sealing material below the column) of the spin column, and loosen the cap (do not remove cap).
31. Place column into a 1.5 mL tube, and centrifuge at 1500 × *g* for 1 min.
32. Discard the flow-through and put the column back to the 1.5 mL tube.
33. Use a marker to place a mark on the side of the column where the compacted resin is slanted upward.
34. Add 300 μL of the equilibration buffer (i.e. the buffer to exchange the antibody into; in this case, use Buffer A) to the column. Place the cap back to the column (again, do not cap tightly). Centrifuge at 1500 × *g* for 1 min, with the mark facing outward from the center of the centrifuge. (This preserves the shape of the compacted resin bed across rounds of centrifugation, which yields better protein retention.).
35. Repeat **step 34** 2 more times (for a total of 3 Buffer A washes). For the third (final) wash, centrifuge at 1500 × *g* for 2 min, to completely remove buffer from the resin bed.
36. Move the column to a new 1.5 mL tube. Discard the original one.
37. Apply the antibody solution to the column. Centrifuge at 1500 × *g* for 2 min, again with the mark facing outward from the center of the centrifuge.
38. Discard the column, and save the flow-through, which is the antibody now buffer-exchanged into Buffer A.
39. Check antibody concentration in mg/mL with a Nanodrop (or equivalent spectrometer), using Buffer A as the blank

solution. Be sure to select the setting for IgG antibody (otherwise, the reading will be off by ~30%, because the mass extinction coefficient, i.e. A280 of a 10% (w/w) solution, is 13.7 for IgG, but 10.0 for general proteins). Then, dilute the antibody to the concentration of ~1.0 mg/mL with Buffer A.

40. Add 350 μL of DMSO to 1 mg of S-HyNic. Pipette up and down for 60 s, and then vortex until the dried pellet completely dissolves. This solution is stable if stored in anhydrous conditions at −20 °C, for up to 2 weeks.
41. Transfer 100 μL of the ~1.0 mg/mL antibody solution into a separate 1.5 mL tube. Add 6 μL of the S-HyNic solution. Mix thoroughly by pipetting up and down for 10 s. Incubate solution at RT for 2 h.
42. After 1 h and 50 min of incubation (i.e. 10 min before the incubation period ends), prepare a Zeba spin column using exactly the same protocol shown in **steps 30–36**, except that Buffer C should be used instead of Buffer A as the equilibration buffer, in order to exchange the antibodies into Buffer C this time.
43. After 2-h incubation, apply the S-HyNic-reacted antibody solution to the spin column, and complete the rest of the buffer exchange (same as **steps 37–38**). This S-HyNic-reacted antibody solution is not stable for more than a few hours, and needs to react with 4FB-DNA immediately (within 1 h), as instructed in **steps 44–46** in the next section.

Conjugate 4FB-DNA with S-HyNic-reacted antibody

44. Add 4FB-DNA to the S-HyNic-reacted secondary antibody, by the amount calculated in **step 28** in Part A. (If the antibody tube started from 100 μL of the ~1 mg/mL stock in Buffer A, as instructed in **step 5** in Part B, then the antibody in solution should be around 1 mg/mL × 100 μL = 100 μg. The amount calculated in **step 28** was for 100 μg of antibody.)
45. Measure the total volume of the antibody-DNA mixture. Divide the total volume by 9, and then add this amount of 10× Turbolink Catalyst Buffer to the reaction tube.
46. Mix thoroughly by pipetting up and down for 10 s. Incubate the tube at RT overnight.
47. Purify un-reacted 4FB-DNA from the DNA-conjugated antibodies, by using 0.5 mL centrifugal filters with 100 kDa molecular weight. To do this, transfer all content of the reaction tube into the spin column.
48. Add 1× PBS to the column until volume is at 500 μL. Thoroughly mix the content, by pipetting up and down ~5 times.

Centrifuge the filter for 5 min at 14,000 × *g*. Discard the flow-through.

49. Repeat **step 48** two more times, for a total of 3 spins.
50. Transfer the concentrate from the filter to a separate 1.5 mL tube. Calculate the amount of 1× PBS needed to bring the volume to the starting volume at the ~1 mg/mL stage (100 μL). Add this amount of 1× PBS to the filter to collect residual DNA-conjugated antibodies, pipette up and down to mix, and then transfer the entire content into the 1.5 mL tube. The DNA-conjugated antibody is now ready for use, and is stable at 4 °C for at least 1 year.

3.3.4 Antibody Staining (for Fluorescent Protein Readout)

1. Pre-incubate sample in 1 mL of 5× SSCT for 30 min at RT.
2. Incubate sample with primary antibody against fluorescent protein, at desired concentration (a recommended default concentration is 10 μg/mL; this concentration can be later adjusted to optimize for signal to background ratios) in 500 μL of 5× SSCT overnight at 4 °C. ******.
3. Wash sample with 1 mL of 5× SSCT three times, 1 h each at RT.
4. Incubate sample with secondary antibody conjugated to an acrydite-bearing DNA oligo (synthesis instruction is shown in the earlier section "Synthesis of DNA-conjugated secondary antibody"), at desired concentration (a recommended default concentration is 10 μg/mL; this concentration can be later adjusted to optimize for signal to background ratios) in 500 μL of DNA-conjugated Antibody Staining Buffer overnight at 4 °C. ******.
5. Wash sample with 1 mL of 5× SSCT for three times, 1 h each at RT.

3.3.5 Expansion and Re-Embedding into Non-Expanding Gel #2

Same as in the standard ExCel protocol, in sub-section "*Part A: Maintain expansion factor of the gelled sample with re-embedding*" of the section "ExFISH-HCR", except for 2 points:

1. Prior to the re-embedding protocol, flip the gels to the same side as when it was casted in Gel#1 gelation, to ensure that the worms are at the bottom of the gel. (Refer to Subheading 3.1.6 to review this concept.)
2. For re-embedding, replace the Re-embedding monomer solution with iExCel G2 monomer solution.

After Gel#2 gelation, it is recommended to trim the gels, to avoid samples too large to handle and image at the fully expanded final state. Trimming can be facilitated by observing where worms are located inside the gel, with naked eye, or on an upright

microscope, if it is desired to not dissect certain animals. Ideally, gels are trimmed into less than ~6 mm on each side, and have at least several worms (≥3) or regions of interest in the trimmed gel. Also, not all trimmed gels need to proceed with the protocol (which might be laborious to handle), and can be saved at this point, in 5× SSC at 4 °C for at least 2 months. For the first several attempts of this protocol, it is recommended to pick at most ~6 trimmed gels to proceed.

3.3.6 Linker Hybridization (to Amplify and Transfer Signals from Gel#1 to Gel#3)

1. Transfer trimmed gels into a 24-well plate, if not already there.
2. Pre-incubate each trimmed gel in 1 mL of iExCel hybridization buffer for 30 min at RT.
3. Incubate gel with Post-Gel#2 acrydite-bearing linkers (refer to the second entry of the readout systems shown in Table 4) corresponding to the oligo sequence on the secondary antibody applied in the section "Antibody Staining," at the concentration of 100 nM, in 1 mL of iExCel hybridization buffer overnight at RT with gentle shaking. ******.
4. Wash gel with 1 mL of iExCel hybridization buffer four times, for 1 h each for the first three washes and overnight for the final wash, at RT with gentle shaking. ******.
5. Wash gel with 1 mL of 1× PBS three times, 1 h each at RT.

3.3.7 Re-Embedding into Expanding Gel #3

1. Flip each trimmed gel to the same side as when they were casted during Gel#1 and #2 gelation. This ensures that the worms remain on the bottom surface of the gels, so the working distance of the objective can maximally cover the depth of the worm at the final imaging stage. (Refer to Subheading 3.1.6 to review this concept.)
2. Prepare 1 mL of Activated iExCel G3 Monomer Solution with all reagents except for APS (iExCel G3 Monomer Solution Stock + 4-HT + TEMED; do not add the activator APS yet), and thoroughly mix the solution by a vortex. Chill the solution to 4 °C on a cold block. It is important to ensure that the solution is fully chilled to 4 °C before proceeding.
3. Remove liquids from the sample.
4. Activate the monomer solution from **step 3** by adding 20 μL of 10% APS, and thoroughly mix the solution by a vortex. Start a timer right after the APS is added.
5. Add 1 mL of Activated iExCel G3 Monomer Solution to worms. Incubate sample at 4 °C for 30 min, on a pre-chilled cold block.
6. During the incubation, construct a gelling chamber with the same architecture as in Gel#1 (**step 6** in Subheading 3.1.7) but with 3× thicker spacer (i.e. the same thickness as used in Gel

#2; e.g. if a #1.5 coverslip was used for Gel #1, use a stack of 3 here for each spacer). Do not close the chamber with the top coverslip yet.

7. Immediately after 30 min of incubation, transfer 105 μL of the monomer solution from the well to the center of the gelling chamber. Remove bubbles if any. Then, transfer the gel from the 24-well plate into the monomer solution droplet on the gelling chamber, using a flat-tip paint brush. Be sure that the gel is on the side where the worms are at the bottom surface of the gel, as confirmed in **step 1** (i.e. if the gel is already correctly sided in the 24-well plate, do not flip the gel during this transfer.). Slowly place a rectangular top cover slip on the gel, to enclose the gelling chamber in the same style as performed in previous rounds of gelation, avoiding any bubbles. If the distance between the gel to the border of the gelation solution is less than ~2 mm in any direction, add more monomer solution to the chamber until the solution border is sufficiently away from the gel. Alternatively, fill the entire chamber with monomer solution. (As explained previously, ambient oxygen inhibits gelation, and so the gel right at the solution-air interface is not well-formed.)
8. Incubate the gelling chamber at 37 °C for 2 h, in a humidified chamber.
9. Remove top cover glass. There should be a visible interface around the re-embedded gel. Trim away the excessive gel around the re-embedded gel.
10. Transfer trimmed gels into a 6-well plate. Wash three times with 5× SSC, 10 min each. **.

3.3.8 Cleave DATD-Crosslinked Gels #1 and #2

1. Make DATD-cleaving solution from sodium periodate powder.
2. Incubate trimmed gels in 3 mL of DATD-cleaving solution (or volumes sufficient to cover the entire gel) at RT for 30 min with gentle shaking.
3. Wash gels with 5 mL of 1× PBS at RT for 15 min with gentle shaking.
4. Repeat **step 3** seven times (eight washes total), and then leave in 5 mL of 1× PBS at RT overnight with gentle shaking. It is crucial to completely wash away sodium periodate from the gels, since residual amounts of periodate ion will also disintegrate Gel #3 over a time scale of ~2–24 h. **.

3.3.9 LNA Hybridization (to Attach Fluorophores to the Stained Locations for Final Readout)

1. Pre-incubate each trimmed gel in 1 mL of iExCel hybridization buffer for 30 min at RT.
2. Incubate gel with fluorophore-conjugated LNA oligos corresponding to the DNA linker applied in the section

"Linker Hybridization," at the concentration of 100 nM, in 1 mL of iExCel hybridization buffer overnight at RT with gentle shaking. **.

3. Wash gel with 1 mL of iExCel hybridization buffer four times, for 1 h each for the first three washes and overnight for the final wash, at RT with gentle shaking. **.
4. Wash gel with 1 mL of 5× SSC three times, 1 h each at RT.

To ensure that the sample (currently at ~8.5× expansion factor) can be fully expanded (to ~20× expansion factor) in the upcoming section, and still stay flat and fully contained inside the container, it might be necessary to trim the hydrogel sample at this point. First, identify the dimensions of the container where the expanded sample will be imaged in. Determine the maximum gel size allowed at the current stage, by evaluating (container size)/(20×/8.5×). For a 6-well plate, the maximum gel size at the current stage is ~9 mm × ~9 mm. Trim the samples into sizes below the maximum gel size, by first transferring the sample into a plastic 6-well plate, or on a glass slide, with a paintbrush. Then, use a #1.5 or thicker cover glass, or a razor blade, to cut the gel into smaller portions; perform this on an upright fluorescent microscope, to confirm the locations of animals, if it is desired to not dissect certain animals. Not all trimmed portions need to be expanded and imaged within the current round, and can be stored at this point, in 5× SSC at 4 °C for at least 2 months.

3.3.10 Expansion and Imaging

1. Transfer sample to a container suitable for imaging, e.g. a glass-bottom 6-well plate. For high-magnification objectives with limited working distance, ensure that the gel is flipped to the same side as when it was first casted in Gel #1, so that the animals will be at the bottom of the gel. (Refer to Subheading 3.1.6 to review this concept.)
2. Wash sample with 5 mL of deionized water three times, for 1 h each at RT.
3. Remove as much liquid around the expanded gel as possible.
4. Perform imaging.

See Subheading 3.1.16 for agar immobilization protocol to prevent sample vibration or drift during imaging.

3.3.11 Example of Final Images

An example of confocal images acquired after the iterative ExCel protocol is shown in Fig. 7. In this example, a transgenic L3 hermaphrodite animal expressing *tag-168p::GFP* is processed by the protocol for detecting the localization of GFP molecules. For the purpose of demonstrating the resolution after each round of expansion, we included additional steps, which are not incorporated into the routine protocol shown above (in Fig. 6),

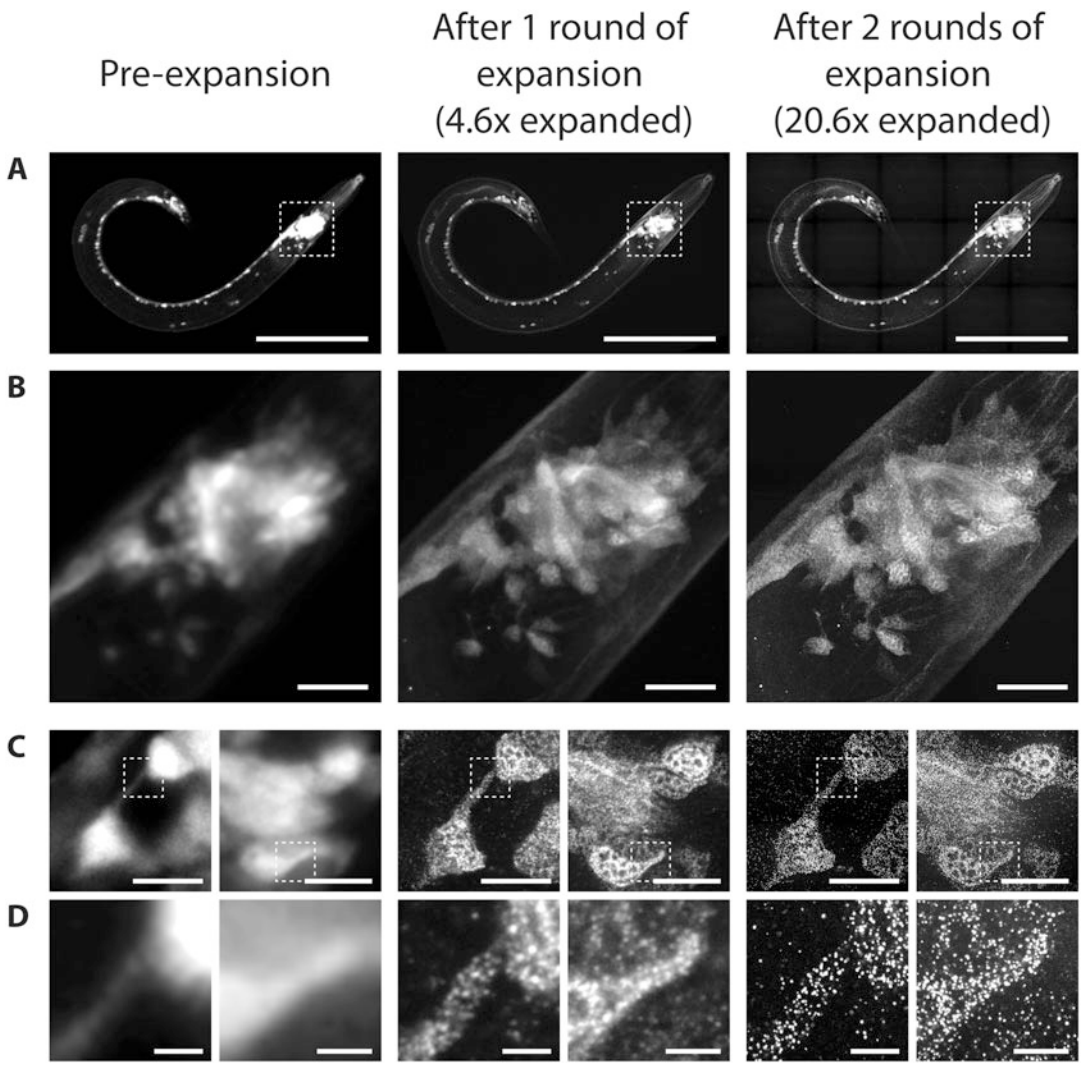

Fig. 7 Example of confocal images acquired after the iterative ExCel protocol. A representative iExCel-processed L3 hermaphrodite animal at various stages along the iExCel protocol. These stages include: (left column) right after first hydrogel embedding, (middle column) after re-embedding into the second hydrogel, and (right column) after full expansion with the third hydrogel. The strain used had pan-neuronal cytosolic expression of GFP, under tag-168p::GFP. Pre-expansion images were acquired from native GFP fluorescence. Post-1-round-expansion images were acquired after linker hybridization and before re-embedding into the third gel (as in Fig. 6e), accompanied by the following additional steps (not included in the routine protocol shown in Fig. 6, because we performed this intermediate readout only for the purpose of method validation): specimens were incubated with a fluorophore-conjugated 15-base DNA oligo that hybridizes to the 100-base linker, imaged, incubated in de-hybridization buffer (80% formamide, 0.1% Triton X-100) at 37 °C for 6 h to remove the fluorophore-conjugated-DNA-bound linker, and re-hybridized with a fresh set of linker, using the same hybridization protocol shown in Fig. 6e. This linker refreshment ensures that the linkers have completely unoccupied binding sites for the downstream LNA hybridization. Post-2-round-expansion images were acquired after full iExCel protocol (as in Fig. 6i). (**a**–**d**) The animal at various optical and digital magnifications. (**a**) Entire worm. White dotted box marks the pharyngeal region of the worm, which is shown in greater magnification in **b**. (**b**) Pharyngeal region of the worm, as marked by the white dotted box in **a**. (**c**) Two regions within the pharyngeal region of the worm, as shown in **b**. Corresponding regions were not marked in **b**,

to enable detection of GFP after the first round of expansion [7]. Briefly, these steps include incubation of the linker-hybridized samples (at the state of Fig. 6e) with a fluorescent-dye-conjugated 15-based DNA oligo, which hybridizes to the linker, and which fluorescently report the stained positions at the post-1-round-expansion state (with an expansion factor of 4.6×). Additional details of this non-routine process can be found in the original publication [7]. Afterwards, the sample is de-hybridized in a de-hybridization buffer (80% formamide +0.1% Triton X-100), to remove the bound linker and fluorescent oligo molecules. The sample is then re-hybridized with the linker, and proceed through the rest of the routine protocol (as in Fig. 6d–i), to yield an expansion factor of 20.6×. All images were acquired under confocal microscopy under a 40× objective (water immersion, with a numerical aperture of 1.15).

4 Notes

1. The quality of sodium acrylate can differ substantially from vendor to vendor, and even from batch to batch from the same vendor. Their differences include the following:
   (a) Solubility: fully dissolved at the stock concentration 33% (w/w) [high quality], or having insoluble, string-like precipitates [poor quality].
   (b) Color of the 33%(w/w) stock solution: close to clear [high quality], or moderately yellow [poor quality].

Fig. 7 (continued) because the single-confocal-plane images shown in **c** do not clearly register to regions in **b**, which is a maximum-intensity projection acquired through the entire thickness of the animal. White dotted box marks subcellular features that are shown in greater magnification in **d**. (**d**) Subcellular features of neurons, such as a neuronal process (right) and a portion of the neuronal soma (left), as marked by the white dotted box in **c**. Objective used: (**a**, **b**) 10×, NA 0.50; (**c**, **d**) 40×, NA 1.15. Image depth: (**a**, **b**) max-intensity projections of confocal stacks acquired through the entire thickness of the animal. (**c**, **d**) single z-position confocal images, except for post-2-round-expansion images (right column), which are max-intensity projections of 2 consecutive images within the confocal stack, because the expansion-mediated improvement in the axial resolution causes each z-plane image to capture a reduced tissue thickness. Thus, a combination of features captured across two consecutive z-planes was required to register to all perceptible, z-distributed features in the pre-expansion and the post-1-round-expansion images. Brightness and contrast settings: each panel was first set by the automatic adjustment function in Fiji, and then manually adjusted (raising the minimum-intensity threshold and lowering the maximum-intensity threshold) to improve contrast. Linear expansion factor: post-1-round expansion, 4.6×; post-2-round expansion, 20.6×. Scale bars: (**a**) 100 μm; (**b**) 10 μm; (**c**) 5 μm; (**d**) 1 μm (in biological units, i.e. post-expansion lengths are divided by the expansion factor of the worm). (Figure is modified from Yu et al. 2020 [7], with permission granted by the Creative Commons Attribution license)

(c) Timing of hydrogel formation at 4 °C, using the recipe of "Activated Monomer Solution" in Materials: >60 min [high quality], 30–60 min [moderate quality], <30 min [poor quality].

We screened through sodium acrylates from various vendors, and compiled the solubility of their lots (see results in the ExCel protocol on expansionmicroscopy.org). We recommend the following practice for ExCel users:

- Order sodium acrylate from multiple vendors. Dissolve ~5 g of each powder to make a 33% (w/w) stock solution. Reject powders that do not fully dissolve after ~1–2 min of vortexing. Among the stock solutions in which the sodium acrylate fully dissolves, select the one that appears the least yellow (the most colorless).
- Immediately re-order multiple bottles of sodium acrylate from the same vendor, with the same lot number. Store the quality-screened lots of sodium acrylate powder at −20 °C in anhydrous conditions, which should remain stable for at least 1–2 years.
- Never store 33% (w/w) stock solutions of sodium acrylate. Always use freshly made stock to produce a large batch of the Monomer Solution Stock (we typically make ~50 mL per batch), which can be in turn stored at −20 °C as 1 mL aliquots for 6 months, as the reduced final concentration of sodium acrylate (7.5%(w/w)) is more stable for storage.
- Prior to using a newly made batch of Monomer Solution Stock, do a pilot run of gelation with the stock without any worm samples. To do this, follow the recipe "Activated Monomer Solution," mix the content well, place the solution at 4 °C, and incubate for 45 min. Immediately after 45 min of incubation, use a pipettor to determine whether the solution is still completely liquid or whether hydrogel has partially formed. If the hydrogel has partially formed, repeat the test with gradually reduced (e.g. by 10 min each round) incubation times, until the timepoint where the solution remains completely liquid at 4 °C is determined. Apply this timing for the 4 °C incubation, during the actual gelation step for the worm samples. This procedure avoids the situation where a new batch of Monomer Solution Stock gels too fast (due to variations in the sodium acrylate quality), such that a precious worm sample is trapped inside the pre-maturely formed hydrogel, which would render the worm sample no longer usable.

2. PFA Fixative: Do not store; make this solution from brand new ampules of 16% paraformaldehyde, within 30 min before

usage. After the solution is made, keep solution on ice or at 4 °C until usage.

3. BTB: Do not store; make this solution from 2-mercaptoethanol within 30 min before usage; store opened bottles of 2-mercaptoethanol at 4 °C for <6 months.
4. AcX Stock: Store as ~50-μL aliquots, in a desiccated environment (e.g. in a capped 50-mL tube that contains ~10 mL of Drierite desiccant particles), at −20 °C, for <6 months.
5. LabelX Stock: Store either as aliquots or as a single tube, in a desiccated environment (e.g. in a capped 50-mL tube that contains ~10 mL of Drierite dessicant particles), at −20 °C, for <3 months.
6. Monomer Solution Stock: Store as 1-mL single-use aliquots to avoid multiple freeze-thaw cycles at −20 °C for <6 months; stock solutions of sodium acrylate, acrylamide and DATD crosslinker should be prepared freshly from powder, within ~3 h before all the monomer solution stock ingredients are mixed, aliquoted into 1-mL portions, and stored at −20 °C.
7. Non-activated Monomer Solution: Make at RT immediately before usage. Do not store.
8. Activated Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT, TEMED and APS can be stored as ~100–200-μL aliquots at −20 °C for <3 months; aliquots of these stock solutions can withstand freeze-thaw cycles at least three times.
9. Non-expanding Digestion Buffer: Add Proteinase K right before use; store the buffer itself (i.e. without Proteinase K) at RT for up to 2 years.
10. We recommend the following practices for receiving and storing antibodies: Upon receipt of an antibody, follow the storage condition specified by the manufacturer. In general, antibodies specified to be stored only at 4 °C (e.g. secondary antibodies from Thermo Fisher Scientific) can be stored accordingly without aliquoting. All other antibodies should be aliquoted into volumes that can be used up within a single experiment, or within a 4 °C storage period of ~1–2 weeks, and then frozen at the specified temperature (−20 °C or −80 °C). Avoid freeze-thaw cycles. Thaw frozen aliquots of antibodies at 4 °C, for at least 3 h prior to usage. A thawed aliquot can be stored at 4 °C for <2 weeks.
11. The following final concentrations are recommended (for the first experiment; after observing the signal to background properties associated with a particular concentration, the user can adjust this parameter to optimize for image quality). For standard ExCel, 10 μg/mL is used for staining fluorescent

proteins that are known to localize to large (e.g. >1-μm scale), non-punctate, continuous structures, such as FP-filled cytosol of cells, FP-labeled cytoskeleton, etc.; 2 μg/mL is used for staining targets that are known to be smaller (e.g. <500-nm scale), sparse, and/or can appear as punctate, such as synapses or vesicles. This lower concentration is recommended, because some antibodies (e.g. Abcam ab13970, an anti-GFP that the authors commonly used) can result in non-specific background puncta in the worm tissue, and can obscure the FP-tagged structure of interest; the reduced concentration significantly reduces the amount of this non-specific puncta. For epitope-preserving ExCel, 5 μg/mL is used for all targets. For iterative ExCel, 10 μg/mL is used for staining all fluorescent proteins.

12. Refer to expansionmicroscopy.org for a list of antibodies against fluorescent proteins, which the authors have validated and repeatedly used.
13. Re-embedding Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT and TEMED can be stored as ~100–200-μL aliquots at −20 °C for <3 months; aliquots of these stock solutions can withstand freeze-thaw cycles at least three times.
14. Probe Set Stock Solution: Store in manufacturer's packaging at −20 °C for ≤5 years.
    (a) Order from Molecular Instruments (https://www.molecularinstruments.com/) with the following instruction and parameters.
    (b) On the ordering page, input the sequence of your mRNA target. For endogenous mRNA targets, you can find the sequence under the "Sequences" tag in the page of its corresponding gene on Wormbase (https://wormbase.org/) and/or from RefSeq (https://www.ncbi.nlm.nih.gov/refseq/).
    (c) Select the probe set scale (used at 2 pmol per sample) based on the projected number of samples that will be stained with this probe.
    (d) Select the probe set size (this is the number of 52-bp regions on the input mRNA sequence that will be detected by the hybridization; larger number of probes improves specificity, but also the cost; if budget-limited, a reasonable strategy is to use the minimal probe set size of 20 probes for screening across large numbers of mRNA targets, and the higher probe set sizes for getting the highest-quality images for a fewer number of selected targets).

(e) Select the HCR initiator (B1-B5) associated with the probe, such that all the targets that you plan to simultaneously visualize within a single sample (by eventually attaching them with different colors of fluorophores) receive a different initiator sequence.

15. HCR Amplifier Stock Solution: Store in manufacturer's packaging at −20 °C for ≤5 years.
    (a) Order from Molecular Instruments (https://www.molecularinstruments.com/) with the following instruction and parameters.
    (b) Check which fluorophores are compatible with your microscope, and assign fluorophores to the HCR initiator sequences that you will use (i.e. the HCR initiators that you selected to associate with the HCR v3.0 Probe Set Stock Solution; see final step in the ordering information for the previous reagent). If multiple fluorophores are available, we recommend Alexa 488 and 546, which are particularly photostable amongst the provided list of fluorophores.
    (c) Select the amplifier scale (used at 7.5 pmol per sample).
16. Probe Hybridization Buffer: Store in manufacturer's packaging at −20 °C for ≤6 months. Order an appropriate volume (used at 1 mL per sample) from Molecular Instruments (https://www.molecularinstruments.com/).
17. Probe Wash Buffer: Store in manufacturer's packaging at −20 °C for ≤2 years. Order an appropriate volume (used at 4 mL per sample) from Molecular Instruments (https://www.molecularinstruments.com/).
18. Probe Amplification Buffer: Store in manufacturer's packaging at 4 °C for ≤2 years. Order an appropriate volume (used at 1 mL per sample) from Molecular Instruments (https://www.molecularinstruments.com/).
19. Collagenase VII Stock Solution: Store as 500-μL single-use aliquots to avoid multiple freeze-thaw cycles at −20 °C for <6 months; do not re-use aliquots after thawing.
    (a) Refer to expansionmicroscopy.org for the recommended vendor, as enzyme quality and product purity can vary substantially across vendors. In addition, collagenase VII products can exhibit a noticeable level of batch to batch variability; to control for its quality over multiple experiments (which is helpful in case the protocol needs to be debugged), it is recommended to order a single large batch with at least 7.5 kU, instead of ordering multiple smaller batches for each individual experiment.

20. Protein Denaturation Buffer: Add reagents in the order indicated, to prevent SDS precipitation; store at 37 °C for <1 month; if precipitation occurs during storage at 37 °C (which could be due to slight variations in incubation temperature, and/or increase in reagent concentration due to buffer evaporation, because the formulation is nearly saturating for SDS at 37 °C), the buffer is re-usable by first fully dissolving the SDS back into solution via heating the solution to 70–95 °C until the solution is clear, and then storing the solution at a temperature slightly greater than 37 °C, e.g. 40–45 °C, at which there should not be SDS precipitates at the steady state.
21. Epitope-preserving ExCel G2 Monomer Solution Stock: Store as 1-mL single-use aliquots (to avoid multiple freeze-thaw cycles) at −20 °C for <6 months; stock solutions of sodium acrylate, acrylamide and Bis crosslinker should be prepared freshly from powder, within ~3 h before all the epitope-preserving ExCel G2 monomer solution stock ingredients are mixed, aliquoted into 1-mL portions, and stored at −20 °C.
22. Non-activated G2 Monomer Solution: Make at RT immediately before usage (do not store).
23. Activated G2 Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT, TEMED, and APS can be stored as ~100–200-μL aliquots at −20 °C for <3 months; aliquots of these stock solutions can withstand freeze–thaw cycles at least three times.
24. DATD-cleaving solution: Make at RT immediately before usage (do not store); make the 500 mM sodium meta-periodate solution from powder right before the mixing of all solution ingredients; do not use if the sodium meta-periodate powder has been dissolved for more than 3 h. We note that the 500 mM stock solutions could either look completely clear or moderately cloudy, depending on the quality of the sodium meta-periodate powder. Even in the case of cloudy stock solution, good outcomes could be obtained by ensuring that the stock solution is well mixed (via vortexing) before its addition into the final cleaving solution, as it eventually dissolves to reach a final concentration of 20 mM.
25. DNA-conjugated Antibody Staining Buffer: Store at 4 °C for <6 months; store the stock solutions of 20 mg/mL baker's yeast tRNA and 100% normal donkey serum as 1-mL single-use aliquots at −20 °C for up to 2 years.
26. iExCel G2 Monomer Solution: The components without TEMED and APS can be pre-mixed and can be stored for up to 3 months at 4 °C.

27. iExCel G3 Monomer Solution Stock: Store as 1-mL single-use aliquots (to avoid multiple freeze-thaw cycles) at −20 °C for <6 months; stock solutions of sodium acrylate, acrylamide, and Bis crosslinker should be prepared freshly from powder, within ~3 h before all the iExCel G3 Monomer Solution Stock ingredients are mixed, aliquoted into 1-mL portions, and stored at −20 °C.
28. Activated iExCel G3 Monomer Solution: Make at RT immediately before usage (do not store); stock solutions of 4-HT, TEMED, and APS can be stored as ~100–200-μL aliquots at −20 °C for <3 months; aliquots of these stock solutions can withstand freeze-thaw cycles at least three times.
29. Determine the primary antibody that will be used against the target fluorescent protein. A list of primary antibodies that we have successfully used, against GFP, mCherry, and tagRFP, are available on www.expansionmicroscopy.org. Then, order unconjugated whole IgG secondary antibody to match the host species type of the primary antibody.
30. RNA molecules, which are the targets of ExFISH-HCR readout in the standard ExCel protocol, are highly sensitive to degradation by naturally occurring RNAses found in biological debris (skin, hair) and microorganisms (bacteria, fungi), both which can contaminate specimens. RNAse-contaminated samples can in turn result in reduced fluorescent intensity and counts of ExFISH-HCR puncta. We recommend applying the following procedures at all times, to prepare the reagents for, and to execute, any ExFISH-HCR experiments (i.e. standard ExCel protocol with RNA readout):
    (a) Use RNAse decontamination solutions (e.g. RNAse Zap; Thermo Fisher Scientific #AM9780) to frequently clean bench and work surfaces, and any tool that comes into close proximity to or direct contact with the samples, e.g. pipettors and paintbrushes. To decontaminate a paintbrush, first spray the decontamination solution on the tip of the paintbrush, until the tip is completely wet. Then, rinse the paintbrush with deionized water, to remove the decontamination solution.
    (b) Always use filtered pipette tips, for making RNAse-free solutions, and for handling hydrogel samples.
    (c) Minimize the duration in which containers that carry RNAse-free solutions and hydrogel samples are open (i.e. without a lid). Always place the lids back right after handling. Small biological debris, e.g. dust, hair, flaked skin, and tiny droplets of bodily fluids expelled during speaking or sneezing, can all contain RNAses, so it is

beneficial to minimize the chance that they fall onto the solutions or samples.

(d) Exchange gloves frequently; constantly track the surfaces that the gloves have come into contact (e.g. door of a refrigerator, light switches of the room, screen of a PCR machine); recognize ones that are not RNAse-free, and could thus transmit RNAse into your sample through gloves.

(e) Use Ultrapure water (e.g. Thermo Fisher Scientific #10977015) for making any solution that will be used in an ExFISH-HCR experiment, including the fixative solution.

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