SUPPLEMENTAL EXPERIMENTAL PROCEDURES

CLARITY Tissue Preparation. CLARITY tissue was prepared as described in (Tomer et al., 2014). In brief, C57/Bl6 8-12 weeks of age were anaesthetized with beuthanasia (100 mg/kg) and transcardially perfused with cold PBS, followed by cold hydrogel solution (1% or 4% acrylamide, 0.0125% bisacrylamide (for 1% acrylamide) or 0.05% bisacrylamide (for 4% acrylamide), 0.25% VA-044 initiator, 1x PBS, 4% PFA in dH_2O). Tissues were removed and post-fixed overnight at 4°C. For induction of immediate early genes, animals were injected with either saline or kainic acid (12mg/kg, i.p.) 2 hours prior to perfusion and monitored for seizure activity. For A4P0 samples, tissues were first perfused in 4% PFA, post-fixed in 4% PFA for 24h (4°C), then transferred to a PFA-free embedding solution (4% acrylamide, 0.25% VA-044 initiator, 1x PBS in dH_2O) for 48h. Conical tubes containing samples were degassed under vacuum for 10 minutes, chamber was flooded with nitrogen, oil was quickly added to the surface of the hydrogel solution and tubes were immediately capped. Gel was polymerized at 37°C for 5 hours, removed from hydrogel solution and sectioned where indicated using a vibratome (500 μm sections) or sectioning block (1, 2, or 3mm sections). Additional fixatives (EDC, PMPI, or DSS) were added, as indicated. Tissue was cleared passively in a 4%SDS/ 0.2M Boric acid (pH=8.5) clearing solution at 37 °C with gentle shaking (0.5mm, ~1 week; 1mm, 1-2 weeks; 2-3mm, ~3 weeks). Clearing solution was changed every 1-2 days. Cleared tissue was washed three times (1 hour each), plus overnight, and stored in 1x PBS with 0.3% TX-100.

RNA fixation. Fixation with EDC, PMPI, and DSS was performed after hydrogel embedding, prior to clearing. For EDC fixation, tissue sections were incubated for 30 minutes in methylimidazole buffer (0.1M, pH 8.5) and transferred to 500 μl EDC fixative solution (0.1M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 0.1M 5-Ethylthio-1H-tetrazole, in 0.1M methylimidazole buffer, pH to 8.5). For PMPI fixation, tissue sections were incubated in 500 μl of 0.1M N-[p-maleimidophenyl] isocyanate in DMSO, pH to 8.5; and for DSS fixation, tissue sections were incubated in 500 μl of 0.1M of disuccinimidyl suberate in DMSO, pH to 8.5. For all fixatives, sections were light protected and incubated overnight at 37°C, then transferred to clearing solution. For further characterization of the EDC fixative, sections were incubated in varying concentrations of the fixative (0, 0.1M, 1M, 10M) for varying durations (3h or overnight) at 37°C, then transferred to clearing solution.

Total RNA isolation and acridine orange staining. Cleared tissue was homogenized in 20 μg/ml proteinase K (100 μl total volume per 1mm tissue) and incubated on a 50°C shaker for 3h. RNA was extracted according to the standard Trizol (Invitrogen) protocol, followed by an additional acidic phenol:chloroform:isoamyl alcohol extraction (equal volume of Ph:Chl:IAA) for separation of RNA from DNA, followed by a final chloroform extraction (equal volume of Chl). The resulting aqueous layer was transferred to a new 1.5mL falcon tube, with addition of 1/10 volume of 3M sodium acetate as a carrier for precipitation, reaching a final concentration of 0.3M salt. The RNAs were then precipitated in 3 volumes of ethanol, for at least one hour at -20°C, and then recovered as a pellet by centrifuging at 12,000 RPM at 4°C. The supernatant was removed, pellet was washed in 70% ethanol, allowed to air dry, re-dissolved in 10 μl of ultrapure water, and quantified by nanodrop.

For staining of total RNA by acridine orange, tissue sections were rinsed in SC buffer (0.1M citric acid, 0.2M sodium phosphate dibasic, pH 4.0) for 10 minutes, then incubated in 1 mL of acridine orange solution (100 ug AO in 1 mL SC buffer) for three hours, then rinsed three times for 30 minutes each in SC buffer, before finally rinsing in PBS, and refractive index matching in FocusClear.

Probe Design. For riboprobes, (Figure 2B,C), cDNA templates for *somatostatin* (Probe #1, Accession#BC010770, 280-429) or *parvalbumin* (Probe #2, Accession#BC027424, 203-352) were generated by Genscript. Vectors were linearized and reverse transcribed using T7 RNA polymerase and DIG-labeled dNTPs (Roche), and purified by phenol chloroform extraction. smFISH probes (Figure 4J-L) were designed and synthesized by Biosearch Technologies (Petaluma, CA). DNA 50mer initiator-labeled oligonucleotide probes (Figure 3-5) were either purchased from Molecular Instruments (Pasadena, CA; *Parvalbumin*, *Tac1*, *Th*, 10 probes each), or designed using OligoWiz software (Wernersson et al., 2007) and synthesized by Integrated DNA Technologies (Figure 4 and 5, *somatostatin*, *NPY*, *VIP*, *Tac2*, *Malat1*, *Npas4*, *Arc*, 4-6 probes each). LNA probes were synthesized by Exiqon (Figure 5).

Probe and Antibody Diffusion. For experiments comparing RNA and DNA probe diffusion (Figure 2B,C), coronal sections of cleared tissue (2mm) were incubated in 50% formamide, 5x SSC for 3 hours at 55°C in 0.5ml eppendorf tubes. These conditions were used because they produced successful *in situ* hybridization with both DNA and RNA

probes in parallel experiments. Tubes were cooled to 4°C for 15 min to allow for non-specific binding of probe in order to better immobilize probe for PFA fixation. Sections were postfixed overnight in 4% PFA, washed in PBST, embedded in 2% agarose, and re-sectioned (200μm) on a vibratome. Cross-sections of the center of tissue were selected for staining and transferred to PBST containing anti-DIG antibody conjugated to HRP (1:1000) for 1 hour at room temperature. Sectioning tissue in this way allowed us to eliminate the contribution of inhomogeneities in antibody or TSA diffusing by performing these steps directly on the newly exposed tissue surface. Antibody was washed (3x15 minutes) and tissue was transferred to TSA (1:200) for 5 minutes, washed in PBST, and mounted in PBS. The surface of each cross section was imaged by confocal microscopy, and 2-3 RIOs containing the tissue edge from each section were quantified, taking care to select regions of homogeneous tissue lacking major fiber tracts.

For experiments comparing DNA oligonucleotide diffusion at different time points (Figure 2E), tissue was incubated in 40% formamide and 2x SSC at 37°C for 30, 60 or 180 minutes. Temperature and formamide concentration were reduced to reflect the conditions optimized for DNA hybridization and preservation of endogenous YFP fluorescence used in subsequent experiments.

For experiments measuring antibody diffusion, tissue was incubated in 50mer DIG-labeled oligonucleotides overnight in 40% formamide and 2xSSC (probe distribution is mostly uniform at this point). The tissue was cooled to 4C for 15 minutes to immobilize probe and crosslinked in 4% PFA for one hour at room temperature. The tissue was then incubated with anti-DIG Fab fragment antibody coupled to HRP (1:1000) in PBST for the corresponding time and tissue was further processed as above for re-sectioning and TSA amplification.

***In situ* hybridization**. For Oligo(dT) *in situ* hybridization (Figure 1), cleared tissue was equilibrated in hybridization solution (15% deionized formamide, 2x SSC, 10% dextran sulfate) for 1 hour. Tissue was hybridized in the same solution containing oligo(dT) probe (50nM, 50 base deoxy thymine probe conjugated to Cy5) overnight at 37°C. Stringency washes: 3 x 1 hour (15% formamide, 2x SSC) and 2 x1 hour (2x SSC). For Propidium Iodide stain, tissue was incubated in PropI/RNase solution for 1 hour and then washed with 2xSSC 3x1 hour. Sections were transferred to FocusClear for 4 hours prior to imaging.

For *in situ* hybridization with DIG-labeled 50mer DNA oligonucleotides (Figure 3), cleared tissue was equilibrated in hybridization solution (50% deionized formamide, 5x SSC, 0.5mg/ml yeast tRNA, 10% dextran sulfate) for 1 hour. Tissue was hybridized in the same buffer containing 50nM DIG-labeled DNA probe to target in addition to 10nM N50 oligo to reduce nonspecific binding overnight at 55 °C. Stringency washes: 3 x 1 hour (50% formamide, 5x SSC), 2 x 1 hour 5x SSC. Tissue was transferred to PBST (0.3% TX-100 in 1x PBS) and washed twice, 1 hour each. Sections were incubated with anti-DIG antibody conjugated to HRP (Roche, 1:500 dilution) for 2d per mm tissue thickness, washed overnight in PBST. TSA amplification reaction was performed at 1:50 dilution in commercial buffer for 30 minutes, washed in PBST and transferred to FocusClear for imaging.

For microRNA *in situ* hybridization with DIG-labeled LNA probes, cleared tissue was rinsed in PBST (0.3% TX-100 in 1x PBS) for at least one night, then incubated in hybridization solution overnight (1 mL of hybridization solution consisting of 50% deionized formamide, 5x SSC, 0.5 mg/ml yeast tRNA, and 12.5 nM probe labeled on both ends with DIG). Probe hybridization was performed at a temperature that is 20 degrees below the Tm of the probe (usually between 45 to 55 °C, avoiding incubation temperatures above 55 °C). We noticed good signal to noise even at temperatures as low as 37 °C). Stringency washes: 2x 1 hour (5x SSC), then 1x 1 hour (1x SSC). Signal amplification with TSA and refractive index matching with FocusClear were performed as described above.

For *in situ* hybridization with initiator-tagged 50mer DNA oligonucleotides (Figure 3-6), cleared tissue was equilibrated in hybridization solution for one hour (40% deionized formamide, 2x SSC, 0.5mg/ml yeast tRNA, 10% dextran sulfate). Tissue was hybridized in the same buffer containing 0.5-4nM initiator-labeled probe overnight at 37°C (See Supplemental Table 1 for probe concentrations). Stringency washes: 3x 1 hour (40% formamide, 2x SSC) with an additional overnight wash for sections thicker than 1 mm). Tissue was then transferred to 5x SSCT (5x SSC, 0.1% Tween 20) and washed twice, 1 hour each. Sections were pre-incubated in amplification solution for 30 min (5x SSC, 10% dextran sulfate, 0.1% Tween 20). 3 μM stock hairpin solutions were separately diluted in 20x SSC (for a final concentration of 2.25 μM hairpin in 5x SSC) and heated to 90°C for 90 seconds, then cooled bench top for 30 min. Cooled hairpins were transferred to amplification buffer (120-240 nM final concentration) and tissue

was incubated in amplification buffer with hairpins for 1-2 days at room temperature. Tissue was washed in 5x SSCT 5x1 hour (overnight for sections over 1 mm) and transferred to FocusClear for 4 hours prior to imaging.

An exception to the protocol above is the HCR experiment in Figure 3D, in which some hybridizations were performed in 50% formamide, 5xSSC, 0.5mg/ml yeast tRNA, 10% dextran sulfate, at 55C and stringencies in 50% formamide and 5xSSC to directly compare with their TSA counterparts. There was no significant difference between HCR hybridizations for *somatostatin* performed under the two conditions (other probe sets were not tested at the 50% formamide condition).

For *in situ* hybridization with smFISH probes bearing the initiator sequences on the 5' end, procedure was the same as above, but with a hybridization solution containing 10% formamide, 2x SSC,10% dextran sulfate and 5nM N20 oligo; stringency washes were with 10% formamide in 2x SSC.

Human Tissue. Human tissue was obtained from 2 surgical patients. Both samples are putative healthy tissue from surgical corridors of temporal lobe resections for epilepsy treatment. For the first patient (46 y.o. female), tissue was collected and transferred to PFA <2 hours after removal, fixed overnight in PFA, transferred to 1% hydrogel solution for 2 days at 4°C, polymerized for 5 hours at 37 °C, and cleared for 5 weeks in 4% SDS. The second sample (18 y.o. male) was removed and placed in oxygenated solution for 2 hours in the presence of AP5, CNQX, Gabazine, then transferred to 1% hydrogel solution for 2 days at 4°C, polymerized for 5 hours at 37 °C, fixed overnight in EDC at 37°C, and cleared for 5 weeks in 4% SDS at 37°C.

Confocal Microscopy. All images were taken on a Leica SP5 confocal microscope with a 10x/0.4 objective (WD: 2.2 mm) or 20x/0.75 objective (WD: 0.66 mm) at 488 nm (FITC), 514 nm, 543 nm, or 633 nm excitation. Tissue shrinks once equilibrated to FocusClear for imaging, and all scale bars represent the imaged volume, which is approximately 50% of original tissue volume. Tissue sections were sandwiched between a glass slide and coverslip, using sticky tack as a spacer, and the chamber was filled with refractive index matching solution (FocusClear), as previously described in (Chung et al., 2013).

Image Analysis. For comparisons between HCR and TSA amplification (Figure 3), images were acquired at 10 μm intervals and analyzed using Fiji software. Individual xy planes were locally thresholded using a mid-gray filter and particles were included only if they were larger than 75 μm^2 with a circularity of 0.4 (where circularity = $4\pi(\text{area}/\text{perimeter}^2)$) in order to eliminate axons and dendrites in Thy1-YFP cell detection. For these images, signal to background ratios were determined by calculating the ratio of mean fluorescence intensity of all cells detected in each xy plane to the mean intensity of the rest of the image. For Sst-TFP (Figure 3F), this may underestimate the signal to background ratio, since TFP is cytosolic and some dendrites will be included in the background measurement.

For three-dimensional volumetric rendering (Figures 4-6), confocal images were acquired at 5 μm or 10 μm intervals and analyzed using Imaris software (BitPlane). For tiled images, tiles were assembled by the Leica acquisition software (SP5) before exporting to Imaris. To analyze the number of cells detected in these volumes (Figure S4), we used Imaris cell detection algorithms (spots) to identify and quantify cell number. For comparisons of HCR to directly labeled fluorophores (Figure S4E), cell detection was performed in the control HCR channel (Alexa514), and then fluorescence intensity of both channels was measured in the resulting cell boundaries. Using 50 hand-annotated ROIs containing only background, mean background fluorescence was calculated and subtracted from the data set for each channel. For comparisons to RNA-seq data (Figure S4H), all samples from a given experiment were processed in parallel. A low gain condition was chosen which did not saturate in the highest expressing transcript, *Malat1*. All images were acquired first at low gain, and then low expressing transcripts were imaged again at a higher gain to facilitate cell identification. After identifying the cells, all subsequent calculations used the low gain images. For comparing fluorophore and hairpins, volumes were captured and cells segmented using Imaris software (spots). To compare fluorophores more directly, the mean intensity value from 50 hand-annotated ROIs corresponding to known background was subtracted from each condition to normalize for differences in tissue autofluorescence at each wavelength.

EDC-CLARITY PROTOCOL, related to Experimental Procedures
CLARITY Tissue Preparation for *In Situ* Hybridization
Passive tissue clearing is performed as described in Tomer et al. Nature, 2014. In brief:

1. Perfuse animal with cold PBS, then cold CLARITY hydrogel solution:

Hydrogel Solution

Chemical	Volume in 400ml	Final Concentration
Acrylamide (40%)	10 mL	1% final conc
Bis-acrylamide (2%)	2.5 mL	0.00125 % final conc
VA-044 Initiator	1 g	0.25% final conc
10X PBS	40 mL	1X
16% PFA	100 mL	4%
d H_2O	247.5 mL	-

2. Postfix brain in 20ml of hydrogel solution at 4°C overnight.

3. Degas solution under vacuum to remove dissolved oxygen, which inhibits polymerization. This can be done by degassing, flooding the chamber with nitrogen, then quickly capping the tube. *We have found that degassing under vacuum and then covering the hydrogel solution with a thin layer of sunflower oil (to slow any oxygen in the headspace from dissolving into the solution) has been helpful.*

4. Incubate 5 hours at 37°C

5. Section tissue, if applicable.

6. Transfer tissue to methylimidizole buffer (80µl methylimidizole in 10ml water) for 15 minutes.

7. Incubate tissue in EDC solution at 37°C o/n. This compound acts as a fixative for 5' terminal phosphates (Pena et al., 2009; Tymianski et al., 1997) . This fixative is particularly helpful in preserving and detecting small RNAs, but also increases retention of mRNAs. To note: EDC fixation will increase clearing time by few days.

EDC Fixative Solution

Chemical	Mass in 10ml	Final Concentration
EDC	0.19g	0.1M
ETT	0.13g	0.1M
Methylimidizole Buffer (80µl Methylimidizole in 10 ml in H_2O	80 µl	

pH to 8.5 with NaOH. pH is critical; EDC will precipitate if pH is too low, or if phosphate buffer is inadequately washed out.

8. Move post-fixed sections to clearing solution. Passive clearing in 4%SDS/ 0.2M Boric acid (pH=8.5) clearing solution at 37°C until clear. Switch out solutions every day for at least first few days, then every other day should suffice.

Clearing Solution

Chemical	Mass in 1L	Final Concentration
Sodium tetraborate	40.24	0.2M
SDS	40g	4%
H_2O	1L	-

pH to 8.5 with NaOH.

9. After clearing, wash 3x in PBST (PBS + 0.3% Triton), 1 hour each, at RT and once overnight.

***In situ* hybridization in CLARITY – TSA Amplification**

The following protocol is for DIG-labeled riboprobes and end-labeled DNA or LNA oligonucleotides. If you already have these types of probes working for your targets of interest, this approach may be easiest to implement, as long as the volumes are small, since antibody penetration into EDC-CLARITY tissue is slower than nucleic acid diffusion.

1. Quench with 1%H_2O_2 in PBST at RT overnight.

2. Wash 3 x in PBST (30min - 1h ea) at RT.

3. Pre-incubate in hybridization solution 1 hour.

4. Hybridize probe in hybridization solution overnight.

   Hybridization should be done at temperatures 10-20deg below Tm of the selected probes; individual optimization might be necessary. We have had success with 50-mer DNA probes, 20-mer LNA probes, and hydrolyzed riboprobes spanning 100-1000bp. For 50mer DNA probes, we selected sequences that minimize secondary structure and cross hybridization, and have similar Tms by using OligoWiz (Wernersson et al., 2007). For LNA probes, we used Exiqon probe selection algorithms. Individual hybridization conditions are listed in Supplementary Table 1 and online at
   http://wiki.claritytechniques.org/index.php/ISH
   and
   http://clarityresourcecenter.org

   Hybridization Solution

Chemical	Volume in 10ml	Final Concentration
Deionized Formamide	5ml	50%
20x SSC	2.5ml	5x
Yeast tRNA (10mg/ml)	0.5ml	0.5mg/ml
dH_2O	2ml	-

   *For 50mer DNA oligonucleotide probes, we included an N50 oligo to reduce non-specific hybridization.

5. Wash twice in 5 x SSC (1h ea), and once in 0.5x SSC (1h) at hybridization temperature.

6. Rinse in PBST at 37°C (30min).

7. Anti-DIG-POD Fab fragment antibody (Roche) in PBST 37°C (1:2000) o/n.

8. Wash with PBST 3 x (60 min ea) RT, plus overnight.

9. TSA reaction (Perkin Elmer, TSA Plus Fluorescein).

   Dilute fluorescein 1:50 and incubate tissue section for 30 minutes.

10. Wash with PBST, 3 x 60 min ea. at RT.

11. Focus Clear (4h-o/n), ready for mounting/imaging.

***In situ* hybridization in CLARITY – HCR Amplification**

To be able to multiplex RNA targets more effectively, and make staining more uniform, we adapted the hybridization chain reaction (HCR) amplification for use in CLARITY tissue (Choi et al., 2010, Choi et al., 2014). This method allows for simultaneous and orthogonal detection of several RNA targets. Importantly, the components are DNA-based and are all under ~150bases, which allows for more even diffusion and a more uniform staining. Although this amplification technique has reduced background and improved tissue penetration, it relies heavily on a good set of DNA oligonucleotide probes. We either purchased probe sets directly from Molecular Instruments (Caltech) or we have used OligoWiz software to design our own. We use probes approximately 50 nucleotides long and started with sets of 3-5 probes, each containing initiator sequences on both 5' and 3' ends. Sequences were chosen to generate non-overlapping probes with low secondary structure and cross-hybridization, and that have similar Tms. If signal was weak, we increased the number of probes to 10 per target. If background is high in initial screening, we have found that testing individual probes to remove those contributing to non-specific staining is very

helpful. Probe concentration may need to be modified for each probe set. Once a good probe set is identified, we perform the EDC-CLARITY in the following way:

1. Incubate in probe hybridization buffer without probe for 1 hour.

2. Transfer to hybridization solution with probe. Hybridize overnight (37°C unless otherwise noted).

Hybridization– 50mer DNA oligonucleotides

Chemical	Volume in 10ml	Final Concentration
Deionized Formamide	4ml	40%
20x SSC	1ml	2x
Yeast tRNA (mg/ml)	0.5ml	0.5mg/ml
Dextran Sulfate (50%)	2ml	10%
dH_2O	2.5ml	-

Probe concentration varies with target and may need optimization. Concentrations used in this paper are indicated in Supplemental Table 1.

Hybridization– smFISH 20mer probe sets

Chemical	Volume in 10ml	Final Concentration
Deionized Formamide	1ml	10%
20x SSC	1ml	2x
Dextran Sulfate (50%)	2ml	10%
dH_2O	6ml	-

**Biosearch Technologies(Petaluma, CA) has designed the 20mer probe sets described in this study, which include 30-50 probes per mRNA target.*

Probe concentration varies with target and may need optimization. Concentrations used in this paper are indicated in Supplemental Table 1 and online at
http://wiki.claritytechniques.org/index.php/ISH
and
http://clarityresourcecenter.org

3. Perform 3x1hour stringency washes at hybridization temperature with the solution listed below. If tissue section is > 1mm, an overnight stringency may be necessary.

50mer Oligonucleotide Stringency Solution

Chemical	Volume in 10ml	Final Concentration
Deionized Formamide	4ml	40%
20x SSC	1ml	2x
dH_2O	5ml	-

20mer smFISH Stringency Solution

Chemical	Volume in 10ml	Final Concentration
Deionized Formamide	1ml	10%
20x SSC	1ml	2x
dH_2O	8ml	-

Perform additional 2x 1 hour wash with 5xSSCT at room temperature.

5xSSCT

Chemical	Volume in 40ml	Final Concentration
20x SSC	10	5x
Tween20 (10%)	400µl	0.1%
dH2O	29.6ml	-

4. Pre-incubate in amplification buffer, 1 hour.

Amplification Buffer

Chemical	Volume in 40ml	Final Concentration
20x SSC	10	5x
Tween20 (10%)	400µl	0.1%
Dextran Sulfate (50%)	8ml	10%
dH2O	21.6ml	-

5. Snap Cool Hairpins:

   For 300µl of amplification buffer (120 nM):

   12µl of 3µM Hairpin 1 + 4 µl of 20xSSC in PCR tube
   12µlof 3µM Hairpin 2 + 4µl of 20xSSC in PCR tube
   Heat both tubes to 95^{o}C for 90 seconds, cool to room temperature 30 minutes.
   Add both hairpins to 300µl amplification buffer in Eppendorf tube.
   For B1 hairpins with Alexa 647, we use 120nM. For B2- Alexa 543 and B5- Alexa 514 hairpins we use 240nM.
   Transfer CLARITY tissue to hairpin solution, incubate overnight at room temperature. For tissue >2mm thick, it may helpful to incubate for 2 days.
6. Wash with 5 x 1 hr with 5xSSCT at RT, plus one wash overnight if >1mm thickness.

Transfer to refractive index matching solution; wait until transparent (1-4 hours). Signal is stable in FocusClear 1-2 days. Signal is stable for longer periods in ScaleA2, RIMS, or Glycerol, but sample transparency suffers, so may be suitable for <1mm sections, but not for larger volumes. Larger volumes can be challenging to make transparent again during refractive index matching. During hybridization and stringency, the tissue shrinks considerably. For tissue >2mm thick, we've found it helpful to re-expand the tissue before refractive index matching. For the 3mm section in Supplemental Movie 4, we transferred the tissue from 5xSSCT (after hairpin amplification) back to clearing solution (4% SDS in 0.2M borate buffer, 37 ^{o}C) overnight, then wash three times with 0.2M borate buffer to remove SDS. The expanded tissue equilibrates to RI matching in FocusClear more quickly and more thoroughly.