Single-molecule fluorescent amplification of RNA using clampFISH probes

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Abstract:

Non-enzymatic, high-gain signal amplification methods with single-cell, single-molecule resolution are in great need. We present a new method (click-amplifying FISH; clampFISH) for the fluorescent detection of RNA that combines the specificity of oligonucleotides with bioorthogonal click chemistry in order to achieve high specificity and high-gain (>400x) signal amplification. We show that clampFISH signal enables detection with low magnification microscopy and separation of cells by RNA levels via flow cytometry. Additionally, we show that clampFISH is multiplexable, compatible with expansion microscopy, and works in tissue samples.

Main text:

Single molecule RNA fluorescence *in situ* hybridization (RNA FISH), which enables the direct detection of individual RNA molecules [1,2,3], has emerged as a powerful technique for measuring both RNA abundance and localization in single cells. Yet, while single molecule RNA FISH is simple and robust, the total signal generated by single molecule RNA FISH probes is relatively low, thus requiring high-magnification microscopy for detection. This keeps the assay relatively low throughput and precludes the ability to combine it with flow cytometry. As such, high efficiency, high gain amplification methods for single molecule RNA FISH signal could enable a host of new applications for RNA FISH.

A number of different signal amplification techniques are available for RNA FISH, but each suffers from particular limitations. Approaches such as tyramide signal amplification (TSA)[4], or enzyme ligated fluorescence (ELF)[5] utilize enzymes to catalyze the deposition of fluorescent substrates near the probes. Alternatively, enzymes can catalyze a “rolling circle” nucleic acid amplification to generate a repeating sequence that can subsequently detected using fluorescent probes[6–8]. These methods can lead to large gains in fluorescence, but can suffer from poor sensitivity because of the difficulties in getting bulky enzymes through the fixed cellular environment to the target molecule. Meanwhile, there are a number of non-enzymatic amplification methods, most notably the hybridization chain reaction[9–11] and branched DNA methods[12–14]. These methods rely only on hybridization to amplify signal by creating larger DNA scaffolds to which fluorescent probes can attach. However, these methods have an inherent tradeoff between stringency of hybridization and wash conditions for specificity and maintaining hybridization to increase signal gain. Thus, our goal was to create a non-enzymatic, exponential

amplification scheme with high sensitivity (detection efficiency), gain (signal amplification), and specificity (low background).

We first designed probes that would bind with high specificity and sensitivity; specifically, we were looking for a scheme that could allow the probes to survive repeated liquid handling in conditions stringent enough to limit nonspecific binding and prevent spurious amplification. Padlock probes are a class of circular DNA probes that achieve this by binding to the target region of complementarity via the 5' and 3' ends of the probe, with the intervening sequence not hybridized to the target.[15] Conventionally, the ends are then connected using a DNA ligase, resulting in a molecule that is physically wrapped around the target strand (**Figure 1a**). We wished to retain the benefits of padlock probes without the need for enzymes, which are bulky and thus very inefficient when used *in situ*. Therefore, we designed DNA oligonucleotide probes that contain regions of complementarity to their target RNA as well as a terminal alkyne and azide moiety at the 5' and 3' ends (click-amplifying FISH (clampFISH) probes; **Figure 1a, Supplementary Figure 1**). When the clampFISH probe hybridizes to the target RNA, the DNA:RNA hybrid brings the probe ends together in physical space. We then used a click chemistry strategy (copper(I)-catalyzed azide-alkyne cycloaddition, CuAAC[16]) to covalently link the 5'-alkyne and 3'-azide ends of the probe using small molecules rather than enzymes, effectively forming a ring around the target RNA (**Figure 1a**). The resulting probes bind efficiently to the target, as evidenced by the colocalization of clampFISH probes with smFISH probes targeting the same RNA (**Figure 1c)**.

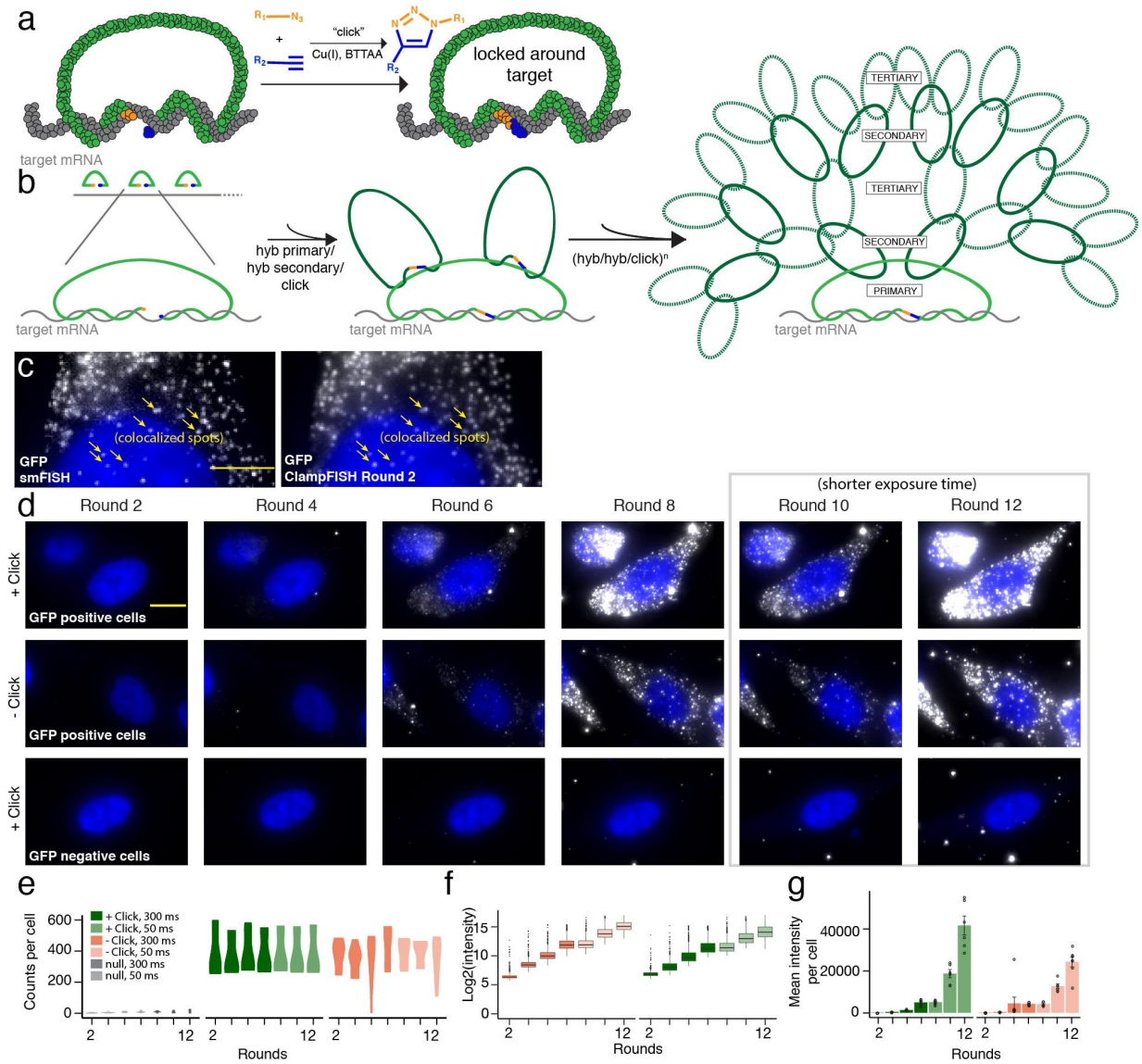

Figure 1. Design and validation of clampFISH technology (data shown is representative of 5 biological replicates). (**a**) ClampFISH probes topologically wrap around target (left) and can be ligated to connect the 5' and 3' ends of the probe using CuAAC (right). (**b**) Workflow for clampFISH: multiple, primary clampFISH probes bind to the target of interest. Secondary clampFISH probes bind 2:1 to each primary clampFISH probe, and tertiary clampFISH probes bind 2:1 to each secondary clampFISH probe. In a subsequent round, the secondary probes again bind 2:1 to the tertiary probes and so on, thus providing exponential amplification. (**c**) Colocalization of GFP mRNA smFISH (left) with GFP mRNA clampFISH round 2 (right; scale bar = 5 um) (**d**) Single cell tracking of GFP mRNA clampFISH signal on WM983b-GFP cells across 12 rounds of amplification in the presence of click ligation (top) compared to GFP mRNA clampFISH signal in the absence of click ligation (middle). Single cell tracking of the same cell line without GFP mRNA expression across rounds to assess background signal (below; scale bar = 10 um). Images are representative single-cells selected from 3 biological replicates. (**e**) mRNA counts per cells across rounds. (**f**) Log2(intensity) of click vs. no click samples across rounds. Graphs are representative of 3 biological replicates. (**g**) Mean fluorescence intensity of GFP mRNA clampFISH signal per cell on WM983b-GFP cells across 12 rounds of clampFISH. Graphs are representative of 3 biological replicates.

To achieve exponential amplification, we first designed a series of primary clampFISH probes to target the RNA sequence of interest. Each primary clampFISH probe contains two "landing pads" for a set of secondary, fluorescent, clampFISH probes. To these secondary probes, we hybridized a set of tertiary probes that again bound in a 2:1 ratio. In a subsequent round, the secondary probes again bind 2:1 to the tertiary probes and so on, thereby in principle doubling the signal in each round (**Figure 1b**).

To demonstrate exponential amplification using clampFISH probes, we first targeted and amplified a GFP mRNA in a human melanoma cell line (WM983b) stably expressing GFP[17] using 10 primary clampFISH probes. We used stringent hybridization conditions—specifically, a higher concentration of formamide than is traditionally used for smFISH—to limit nonspecific probe binding (**Supplementary figure 2**). As the number of rounds progressed, the intensity of the signal as measured by fluorescence microscopy increased, we observed a 3.387-fold increase (geometric average with a standard deviation interval of 2.55, 4.50) for every two rounds of amplification (**Figure 1d**), while the average number of spots per cell remained constant (393 spots per cell ± 2.478 SEM). At round 12, the mean signal per spot was 446-fold higher than in round 2 (**Figure 1e**). To demonstrate signal specificity, we performed the same clampFISH detection and amplification on the parental cell line that did not have the GFP gene. We detected very few false-positive spots in these cells (mean of 9.77 spots per cell ± 1.45 SEM), showing that the signals were specific to the target (**Figure 1d**). To assess whether the click reaction aided in the amplification process as hypothesized, we performed the same experiment in the absence of the click-ligation of the clampFISH probes. Although the number of spots detected per cell were similar (393 mean spots per cell in the clicked samples vs. 381 mean spots per cell in the non-clicked samples), we observed lower mean signal intensity (26,076 AU ± 496 SEM for non-clicked vs. 44,450 AU ± 630 SEM for clicked samples) as well as a lack of uniformity in spot intensity (Coefficient of variance at round 12 for non-clicked cells = 0.94 ± 0.013 vs. 0.69 ±0.01 for clicked cells), demonstrating that the click reaction facilitated a more uniform and higher gain amplification of primary clampFISH signal.

Owing to its low signal intensity, single molecule FISH typically requires using a microscope equipped with a high numerical aperture, high magnification objective, typically requiring oil immersion. For many applications, a low magnification air objective is preferable, both for increased throughput and for simplicity of sample handling. We reasoned that the increased RNA FISH signals that clampFISH provided had the potential to make low magnification microscopy for RNA FISH feasible (**Figure 2a, Supplementary Figure 3**). To test this, we mixed 20% WM983b cells stably expressing GFP with 80% WM983b cells and probed for GFP mRNA using clampFISH probes at round 6. Using clampFISH, the positive cells were clearly discernible at both 20X and 10X magnification, while the conventional single molecule RNA FISH signal was not (**Figure 2a, Supplementary Figure 3**).

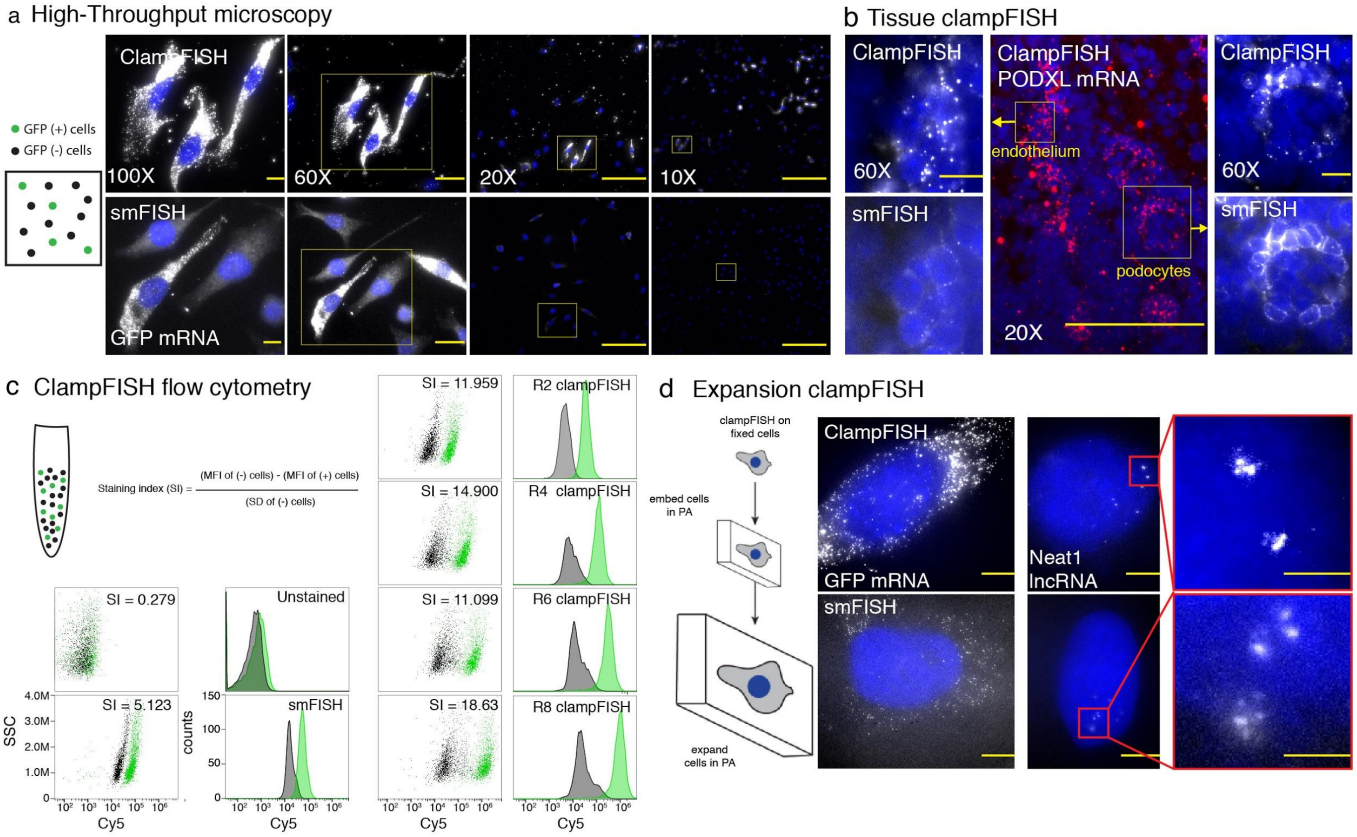

Figure 2. Applications of clampFISH amplification of RNA (images are contrasted independently). (**a**) 20% WM983b cells stably expressing GFP mixed with 80% WM983b cells and probed for GFP mRNA using clampFISH probes (top). Imaging was performed using 0.3 NA 10X, 0.5 NA 20X, 1.4 NA 60X and 1.4 NA 100X magnification objectives in the same positions and compared to smFISH of GFP mRNA using the same fluorophore (bottom; representative images of 2 biological replicates; each image is contrasted independently; scale bars are 10 um for 100X and 60X images, 5 um for 20X images and 2.5 um for 10X images). (**b**) (center) 20X image of fixed-frozen 5uM mouse kidney section stained with round 4 clampFISH probes targeting PODXL. (left) 60X image of mouth endothelium by round 4 clampFISH and by smFISH. (right) 60X image of podocyte by round 4 clampFISH and by smFISH (representative images shown of 2 biological replicates). (**c**) We applied clampFISH to a mixed population of MDA-MB 231 cells with and without GFP expression and analyzed the separation by flow cytometry across 8 rounds of amplification. Cells were gated on GFP expression and are displayed in green. (**d**) (top) Fluorescet micrographs of round 6 clampFISH targeting GFP mRNA and Neat1 lncRNA in cultured WM983b-GFP cells (bottom) fluorescent micrographs of smFISH targeting GFP mRNA and Neat1 lncRNA in cultured WM983b-GFP cells using the same dye (images representative of 2 biological replicates; scale bars are 20 um for the images, and 5 um for the inlay).

Primary tissue samples typically suffer from high background levels that contribute to a low signal-to-noise ratio using single molecule RNA FISH, and therefore require high magnification microscopy to discern positive signal from background. However, at high magnification, large structural features of the tissue are often difficult to discern. To reduce the magnification and increase the visible area while still viewing individual RNAs, we applied 4-rounds of clampFISH to mouse kidney samples and probed for PODXL mRNA, an gene that is highly expressed in podocytes[18], and observed specific expression of clampFISH signal in the appropriate regions (**Figure 2b, Supplementary Figure 4**). Interestingly, clampFISH further revealed that *PODXL* also expressed in the kidney endothelium, a signal that was only faintly visible by single molecule RNA FISH, but was clearly detected by clampFISH at low magnification (**Figure 2b**) consistent with previous findings that PODXL is expressed at low levels in the kidney endothelium[19].

Another important application that clampFISH enables is flow cytometry based measurement of RNA expression, an application for which single molecule RNA FISH typically does not produce enough signal[20,21]. We applied clampFISH to a mixed population of MDA-MB 231 cells with and without GFP expression and analyzed the cells by flow cytometry (**Figure 2c, Supplementary Figure 5**), using GFP fluorescence as an independent measure of the specificity of clampFISH signal. We observed separation of GFP positive cells by clampFISH signal with as few as 2 rounds of amplification, and observed a 2.447-fold increase in fluorescence intensity in the GFP positive population with every 2 rounds of amplification thereafter (geometric mean of fold change across rounds; **Figure 2c, Supplementary Figure 5, Supplementary Figure 6**). Notably, we observed a decreasing fold-change as we moved through the rounds (3.435-fold from rounds 2-4, 2.589-fold from rounds 4-6, and 1.648-fold from rounds 6-8).

Amplification of RNA signal can also be used in combination with a newly developed expansion microscopy technique that achieves super-resolution microscopy via the physical expansion of cells embedded in polymeric hydrogels[22,23]. Expansion microscopy is powerful in combination with single molecule RNA FISH because it can resolve the fine structure of RNAs that are in close proximity; however, the physical expansion of cells results in reduced signal intensities, at least partially due to probes dissociating under the low salt conditions required to obtain high levels of hydrogel expansion. We reasoned that the locking property of clampFISH probes would allow us to maintain signal intensity in the face of these expansion conditions. We thus performed clampFISH on GFP mRNA to round 6 followed by expansion and observed high signal intensity on all spots when the click reaction was performed, but with little signal when click was not performed (**Figure 2d, Supplementary Figure 7**). We also applied clampFISH to amplify Neat1 to round 6, a nuclearly retained long non-coding RNA, and observed higher signal intensity than with smFISH (**Figure 2d, Supplementary Figure 7**).

A key design goal for *in situ* hybridization methods is the ability to detect multiple RNA targets simultaneously. Multiplexing with clampFISH is in principle straightforward because the backbone sequence of the clampFISH probes can easily be changed, allowing one to use multiple independent amplifiers simultaneously. As a proof-of-concept, we selected 3 RNA targets with distinct expression patterns in HeLa cells: *NEAT1*, which is found in nuclear

paraspeckles of most cells; *LMNA*, which is found in the cytoplasm of all cells, and *HIST1H4E*, which expresses only in the subpopulation of cells that are in S phase. We amplified these with unique sets of non-fluorescent clampFISH probes to 7 rounds, then probed the terminal backbones with single molecule FISH probes, each labeled with different fluorophores (**Figure 3a, b**). We were able to visualize signals from the different probe sets, even using low magnification microscopy. We saw minimal bleedthrough between fluorescence channels. To prevent bleedthrough of the individual fluorescent channels (**Supplementary Figure 8**).

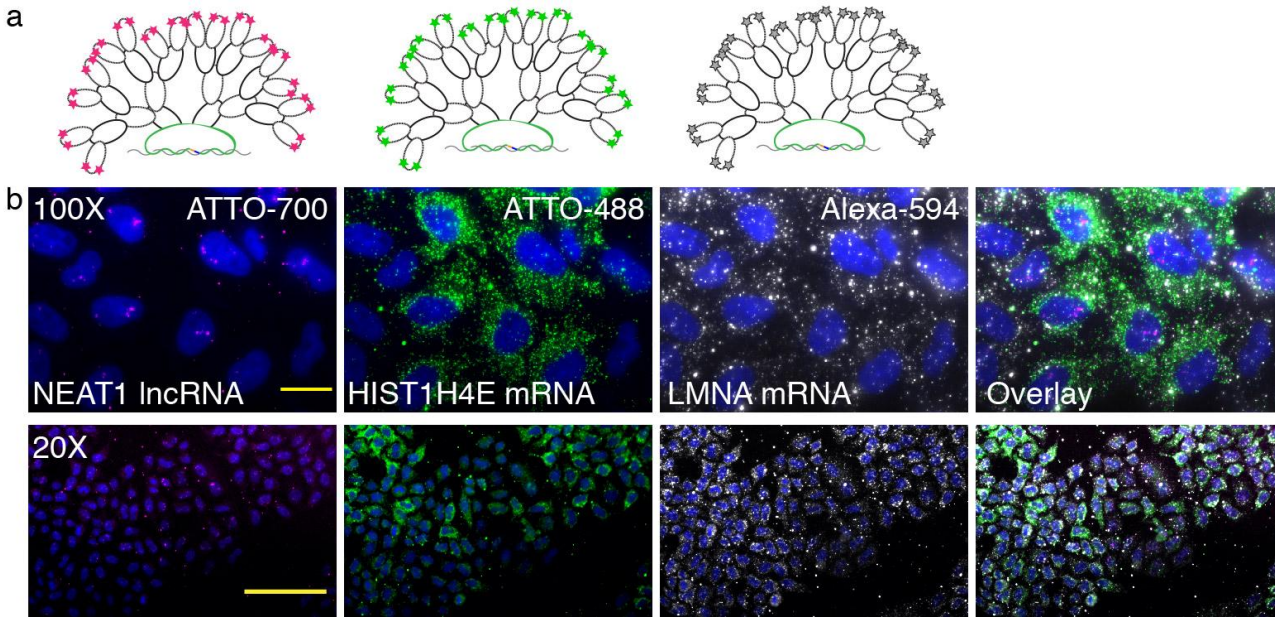

Figure 3. Multiplexing 3 RNA targets on HeLa cells. (**a**) Schematic diagram of probe hybridization scheme. Unique clampFISH probe sets are designed for each target, and probed at the final round with a single molecule RNA FISH probe labeled with a unique fluorophore (represented with ☆). (**b**) Fluorescent micrographs of individual probe channels: (from left) NEAT1 lncRNA labeled with ATTO700, HIST1H4E mRNA labeled with ATTO 488, and LMNA mRNA labeled with Alexa 594 and an overlay on the far right. (top) 100X magnification with 20 um scale bars, (bottom) 20X magnification with 20 um scale bars. Images are representative of 2 biological replicates.

We present here are new scheme for the fluorescent, non-enzymatic amplification of fluorescent RNA signal *in situ*. The resulting procedure, clampFISH, enables the probing and visualization of individual RNAs on cells and tissues using low powered microscopy and is compatible with expansion microscopy. Additionally, clampFISH amplification may be used to separate cells based on their RNA expression using flow cytometry. Interestingly, the combination of probe hybridization and click chemistry moieties on the ends of the primary clampFISH probes behave as a proximity ligation wherein the click reaction will occur if and only if the two arms are hybridized adjacent to each other (**Supplementary Figure 9**). These data suggest that clampFISH may find uses in specifically probing other difficult-to-image RNA subsets such as splicing junctions, short alternatively spliced variants, or edited RNAs.

Materials and Methods

Cell culture. We cultured WM983b cells and WM983b-GFP-NLS cells (a human metastatic melanoma cell line from the lab of Meenhard Herlyn) in tumor specialized media containing 2% FBS. The WM983b-GFP-NLS contains EGFP fused to a nuclear localization signal driven by a cytomegalovirus promoter that we stably transfected into the parental cell line.

Clamp probe design and synthesis. Clamp probes are 150 nt long (15mer left RNA binding arm, 10 nt left adapter, 100mer backbone, 10 nt right adapter, 15mer right RNA binding arm). RNAs are targeted by probe sets containing one or more Clamp probes, each targeting a 30 nt region of RNA (2 adjacent 15mer binding arms). We chose binding regions with approximately 40% GC content as well as minimal repetitive regions. We designed backbones so as to minimize secondary structure (using mFold; http://unafold.rna.albany.edu/?q=mfold). We ordered modified DNA oligonucleotides from Integrated DNA Technologies (IDT) as standard DNA oligonucleotides with modifications (5'-phosphate on the backbone, 3′-azide and 5'-phosphate for the right arm and 5′-hexynyl for the left arm). Strands were resuspended in nuclease free water, at a working stock concentration of 400 uM. The left arm (30 uM), backbone (20 uM) and right arm (30 uM) are brought together using adapter probes (30 uM each) and heated to 70C for 3 min prior to being enzymatically ligated using 600 U of T7 DNA ligase for a minimum of 1 hour at room temperature. Following ligation, the probes were purified using Monarch purification columns (New England Biolabs) and eluted in 4X the starting volume to make the working dilution. For a schematic protocol and probe sequences, see **Supplementary figure 1 and Supplementary table 1.**

ClampFISH procedure on cultured cells. We grew cells on glass coverslides until ~70% confluent. We washed the cells twice with 1X PBS, then fixed for 10 minutes with 4% formaldehyde/1X PBS at room temperature. We aspirated off the formaldehyde, and rinsed twice with 1X PBS prior to adding 70% ethanol for storage at 4°C. We incubated our cells for at least 4 hours at 37°C in hybridization buffer (10% dextran sulfate, 2X SSC, 20% formamide) and 1 ul of the working dilution of the primary ClampFISH probe. We performed two washes in wash buffer (2X SSC, 10% formamide), each consisting of a 30-min incubation at 37°C. We then incubated the cells for at least 2 hours at 37°C in hybridization buffer (10% dextran sulfate, 2X SSC, 20% formamide) and 1 ul of the working dilution of the secondary ClampFISH probe and repeated the washes. After the second wash, we performed the 'click' reaction. A solution containing 75 μM $CuSO_4 \cdot 5H2O$ premixed with 150 μM BTTAA ligand[16] and 2.5 mM sodium ascorbate (made fresh and added to solution immediately before use) in 2X SSC was added to the samples, and these were then incubated for 30 min at 37°C. The samples were then rinsed briefly with wash buffer, then we continued cycling this protocol, alternating between secondary and tertiary ClampFISH probes until reaching the desired level of amplification. After the final wash, we rinsed once with 2X SCC/DAPI and once with anti-fade buffer (10 mM Tris (pH 8.0), 2X SSC, 1% w/v glucose). Finally, we mounted the sample for

imaging in an anti-fade buffer with catalase and glucose oxidase (Raj et al. 2008) to prevent photobleaching.

ClampFISH for flow cytometry. ClampFISH for flow cytometry was performed as described above however the cells were kept in suspension. Wash buffer and 2X SSC were supplemented with 0.25% Triton-X, and the clampFISH hybridization buffer was supplemented with the following blocking reagents: 1μg/μl yeast tRNA (Invitrogen P/N 54016), 0.02% w/v Bovine Serum Albumin, 100ng/μl sonicated salmon sperm DNA (Agilent, 201190-81).

ClampFISH for expansion microscopy. Acryloyl-X, SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester, here abbreviated AcX; Thermo-Fisher) was resuspended in anhydrous DMSO at a concentration of 10 mg/mL, aliquoted and stored frozen in a desiccated environment. Label-IT ® Amine Modifying Reagent (Mirus Bio, LLC) was resuspended in the provided Mirus Reconstitution Solution at 1mg/ml and stored frozen in a desiccated environment. To prepare LabelX, 10 μL of AcX (10 mg/mL) was reacted with 100 μL of Label-IT ® Amine Modifying Reagent (1 mg/mL) overnight at room temperature with shaking. LabelX was subsequently stored frozen (−20 °C) in a desiccated environment until use.
Fixed cells were washed twice with 1× PBS and incubated with LabelX diluted to 0.002 mg/mL in MOPS buffer (20 mM MOPS pH 7.7) at 37 °C for 6 hours followed by two washes with 1× PBS.

Monomer solution (1x PBS, 2 M NaCl, 8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, 0.15% (w/w) N,N'-methylenebisacrylamide) was mixed, frozen in aliquots, and thawed before use. Prior to embedding, monomer solution was cooled to 4°C to prevent premature gelation. Concentrated stocks (10% w/w) of ammonium persulfate (APS) initiator and tetramethylethylenediamine (TEMED) accelerator were added to the monomer solution up to 0.2% (w/w) each. 100uL of gel solution specimens were added to each well of a Lab Tek 8 chambered coverslip and transferred to a humidified 37° C incubator for two hours.
Proteinase K (New England Biolabs) was diluted 1:100 to 8 units/mL in digestion buffer (50 mM Tris (pH :sunglasses:, 1 mM EDTA, 0.5% Triton X-100, 0.8 M guanidine HCl) and applied directly to gels in at least ten times volume excess. The gels were then incubated in digestion buffer for at least 12 hours. Gels were then incubated with wash buffer (10% formamide, 2× SSC) for 2 hours at room temperature and hybridized with RNA FISH probes in hybridization buffer (10% formamide, 10% dextran sulfate, 2× SSC) overnight at 37 °C. Following hybridization, samples were washed twice with wash buffer, 30 minutes per wash, and washed 4 times with water, 1 hr per wash, for expansion. Samples were imaged in water with 0.1ug/mL DAPI.

clampFISH for mouse tissues. Dissected tissues were embeded in OCT, then flash frozen using liquid nitrogen. 5μ tissue sections were cut at -20°C and mounted on charged slides. Slides were washed briefly in PBS, then immersed in 4% paraformaldehyde for 10 min at room temperature. Following fixation, the slides were transferred to 70% ethanol for permeabilization

for at least 12 hours, or for long-term storage. To begin clampFISH procedure, slides were transferred to wash buffer for 3 minutes to equilibrate, then 500ul of 8% SDS was added to the top of the flat slide for 1 minutes for tissue clearing. Samples were transferred to wash buffer, and normal clampFISH procedure was used.

Imaging. We imaged each samples on a Nikon Ti-E inverted fluorescence microscope a cooled CCD camera (Andor iKon 934). For 100× imaging, we acquired z-stacks (0.3 μm spacing between stacks) of stained cells. The filter sets we used were 31000v2 (Chroma), 41028 (Chroma), SP102v1 (Chroma),17 SP104v2 (Chroma) and SP105 (Chroma) for DAPI, Atto 488, Cy3, Atto 647N/Cy5 and Atto 700, respectively. A custom filter set was used for Alexa 594 (Omega). We varied exposure times depending on the dyes and degree of amplification used. Typically, ClampFISH imaging was done at a 300 ms exposure and smFISH was done at 2-3 s exposure.

Image analysis. We first segmented and thresholded images using a custom Matlab software suite (downloadable at https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/Home). Segmentation of cells was done manually by drawing a boundary around non-overlapping cells. The software then fits each spot to a two-dimensional Gaussian profile specifically on the Z-plane on which it occurs in order to ascertain subpixel-resolution spot locations. Colocalization took place in two stages: In the first stage, guide spots searched for the nearest-neighbor SNP probes within a 2.5-pixel (360-nm) window. We ascertained the median displacement vector field for each match and subsequently used it to correct for chromatic aberrations. After this correction, we used a more stringent 1.5-pixel (195-nm) radius to make the final determination of colocalization. In order to test random colocalization due to spots occurring randomly by chance, we took our images and shifted the guide channel by adding 5 pixels (1.3 μm) to the X and Y coordinates and then performing colocalization analysis.

Reproducible analyses. Scripts for all analyses presented in this paper, including all data extraction, processing, and graphing steps are freely accessible at the following url: https://www.dropbox.com/sh/b2mv4o9wmzcicqv/AABARZsKtD1TQKMseoG_LnyWa?dl=0

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