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RECEIVED 19 November 2025
REVISED 05 February 2026
ACCEPTED 12 February 2026
PUBLISHED 20 February 2026

CITATION
Spencer-Dene B and Miranda E (2026)
Getting the message: low-plex detection
of RNA in tissues.
Front. RNA Res. 4:1749833.
doi: 10.3389/frnar.2026.1749833

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Getting the message: low-plex detection of RNA in tissues

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The rapidly emerging field of spatial transcriptomics is transforming developmental biology, oncology, and disease pathology, mapping intricate gene expression patterns within their native tissue contexts. Traditionally, the analysis of messenger RNA (mRNA) expression has been the main focus of this research. However, the eukaryotic cell transcriptome comprises a diverse array of dynamic RNA molecules. Therefore, spatial profiling of different RNA species throughout their life cycle is essential for comprehensively elucidating RNA biology in complex tissues. Formalin-fixed paraffin-embedded (FFPE) samples are widely used in research and clinical settings optimally preserving tissue morphology and cellular details over long periods. Pathology departments have accrued vast collections of FFPE blocks, creating a rich yet under-utilized array of materials that, accompanied by clinical data, stands as a treasure trove for human biology and translational research. Nevertheless, FFPE specimens present several challenges. The RNA within these samples is prone to fragmentation during processing and may further degrade if stored improperly. Additionally, chemical modifications can occur in the RNA, leading to fragmentation or resistance to enzymatic reactions essential for sequencing. The loss of poly-A tails adds another layer of complexity, limiting the effectiveness of oligo-dT primed reverse transcription. As a result, options for spatial profiling of RNA molecules in this tissue type are restricted. Commercial assays that are now available for both manual and automated detection of single to low-plex mRNA targets in tissues, including point mutations, miRNAs and even oligonucleotides have brought these applications into more mainstream workflows, and these will be the primary focus of this review.

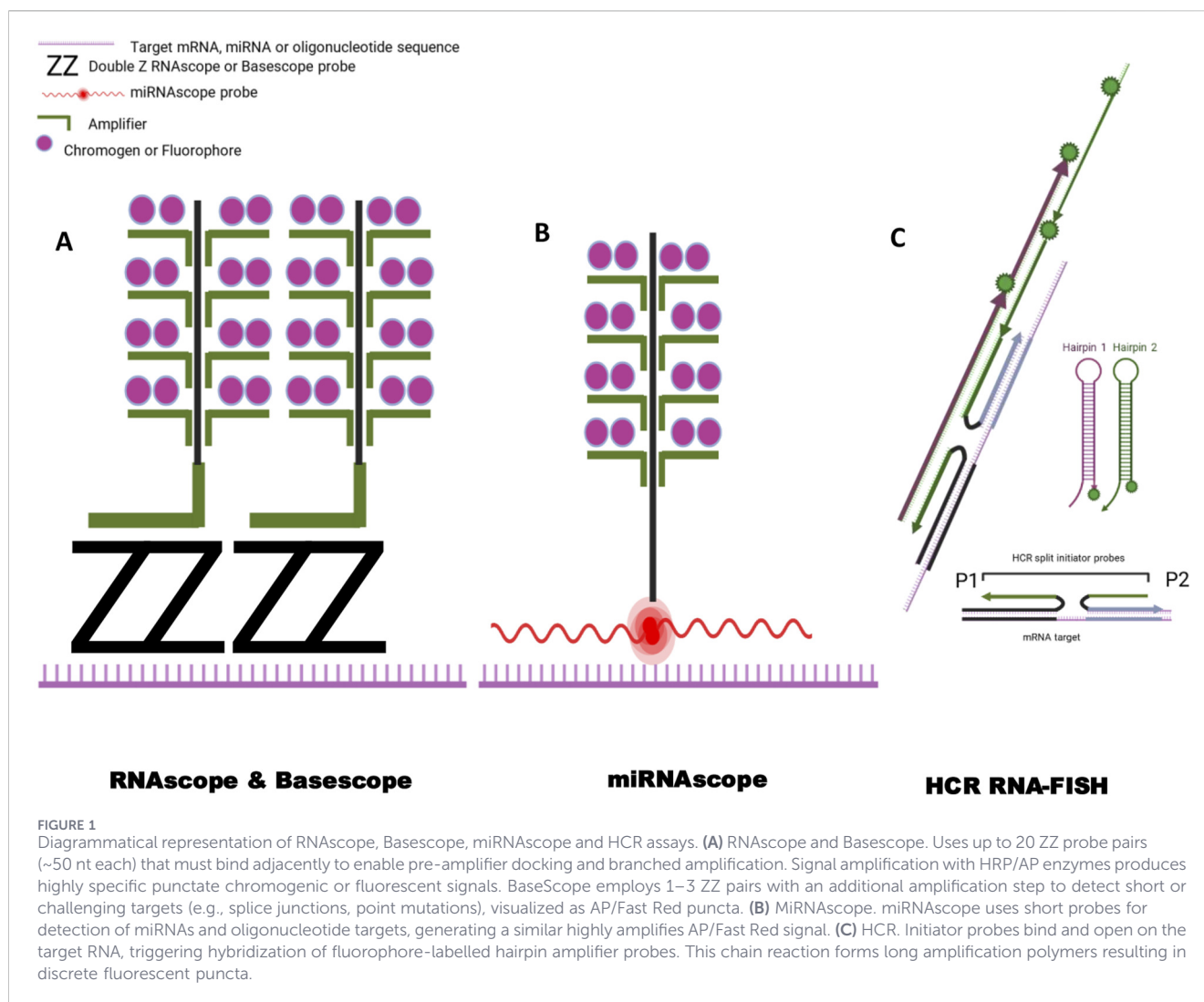
KEYWORDS

FFPE (formalin fixed paraffin embedded), *in situ* hybridization (ISH), miRNA, microRNA, oligonucleotides, tissue section

History of mRNA detection using *in situ* hybridization (ISH)

Recent advancements have improved the sensitivity of RNA detection on tissues or whole cells, down to single molecule level (Baysoy et al., 2023; Bressan et al., 2023; Xu et al., 2023; Bai et al., 2024; Monne Rodriguez et al., 2023; Pringle et al., 1989; Wilcox, 1993). Our ability to detect RNA or DNA in tissue sections and whole mount preparations through *in situ* hybridization (ISH) using complementary Watson-Crick base pair binding of labelled probes is now very well established (Monne Rodriguez et al., 2023; Pringle et al., 1989; Wilcox, 1993), originally described more than half a century ago (Gall and Pardue, 1969).

Initially situated in individual and specialist core facility labs staffed by highly experienced technicians, ISH used to be considered a long and difficult assay to set up in the lab, especially when radio-labelled riboprobes had to be designed and synthesized in-house. Photographic emulsion-coated slides had to be stored for days or weeks before autoradiographic detection of silver grains to detect signal. As well as potential safety



concerns, these experiments resulted in relatively poor spatial resolution with noticeable background staining (Gall and Pardue, 1969).

These challenges lead to the incorporation of non-isotopic labeling of riboprobes including biotin or digoxigenin by nick translation that enabled some degree of amplification using conjugated secondary anti-hapten antibodies. The bound probe was ultimately visualized using a chromogenic or fluorescent substrate. Indeed, this also opened the potential to stain a much wider range of sample types including whole embryos, thick vibratome sections and organoids (Fleck et al., 2023; Velasco et al., 2019; Borrelli and Moor, 2020).

In the last decade, additional commercial non-isotopic ISH technologies such as RNAscope from Advanced Cell Diagnostics, ViewRNA from Affymetrix and HCR from Molecular Instruments have opened these techniques to many more labs. The relative ease of use, including probe design has made the RNAscope and related assays increasingly popular in academic as well as clinical and Pharma settings for the detection of mRNA and other RNA species in tissues, cells and whole mount preparations.

As shown in Figure 1, these commercial kits and probes use dual binding of two separate probes in close proximity (ZZ-probes) to

create a template for signal amplification using a branched DNA process. The dual probe requirement and elimination of repetitive sequence elements improve specificity, while the branched DNA hybridization process enhances sensitivity. Additionally, the reactions can be visualized as either chromogenic signals, which are suitable for standard light microscopes, or fluorescent signals. Both technologies can be used manually or with automated staining platforms (Mahmood and Mason, 2008). Hybridization signals can be quantified either manually through a modified H-Score, a semi-quantitative combined measure of the percentage of positively stained cells and the number of individual punctate signals or using image analysis tools such as HALO and Visiopharm to compare relative expression levels. Non-isotopic ISH technologies are safer and sensitive, and work well on both sections as well as whole-mount samples including fruit fly, chick and mouse embryos (Koshiba-Takeuchi, 2018).

These advanced techniques facilitate the delivery of spatial data in tissue sections and serve as excellent complements to other quantitative molecular assays such as reverse transcription quantitative real-time PCR, next-generation sequencing, and spatial transcriptomic multiplexing approaches. Increasingly, ISH on tissue sections has been utilized to orthogonally validate antibody

staining results obtained through immunohistochemistry (IHC) or to act as a substitute when validated antibodies for a target are unavailable, the target proteins are secreted, or the target is a non-coding RNA (Atout et al., 2022; Bingham et al., 2016). Furthermore, ISH has enabled the examination of less conventional species and sample types, including plants, insects, and cephalopods, especially when protein analysis via IHC is impractical or challenging due to the scarcity or high cost of developing antibodies (Solanki et al., 2020; Shao et al., 2024; Elagoz et al., 2022; Gattoni et al., 2025).

ISH is now recognized as a versatile and well-established method used in both research and clinical settings for detecting coding and non-coding RNA species as well as DNA. It provides routine diagnostic information that aids clinical decision-making. Furthermore, ISH assays are being increasingly applied to detect pathogenic viral target nucleic acid species in both the research and diagnostic settings including head and neck squamous cell carcinoma where RNAscope enabled the accurate and direct visualization of viral mRNAs encoding the E6 and E7 oncoproteins in FFPE samples detecting integrated and transcriptionally active High Risk human papillomavirus virus (HPV) in clinical samples (Bishop et al., 2012; Kravvas et al., 2025). The use of these ISH assays have demonstrated the potential for routine clinical patient screening of HPV E6/E7 mRNA in cancer samples (Musangile et al., 2021; Chen R. et al., 2023).

The same robust ISH assays have been used to detect Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which causes COVID-19 in humans, in patient tissue samples (Liu et al., 2020; Best Rocha et al., 2020; Pesti et al., 2023), as well as many other viral pathogens including HIV (Baharlou et al., 2022), Mpox (Hall et al., 2024), Ebola virus (Worwa et al., 2022) and Avian influenza (Gaide et al., 2023).

New approaches to detect RNA in tissues

Recently other commercial ISH assays have been launched for detection of single to low-plex targets and one such example is the Fluorescent *In Situ* Hybridization Chain Reaction (HCR) (Choi et al., 2018). This is an enzyme-free thermally constant amplification method dependant on pairs of fluorescently conjugated DNA hairpin probes and target specific initiator sequence (Figure 1). This method continues to evolve to improve signal to noise achieving automatic background suppression by utilizing a pair of split-initiator probes that each carry half of the HCR initiator sequence that must co-localize to generate the specific signal. This assay has been especially useful for fluorescently staining up to five targets in whole-mount vertebrate embryos and thick vibratome tissue sections, generating qualitative and quantitative data (Choi et al., 2014). This method can also be used to fluorescently detect microRNA targets alone or in combination with endogenous mRNA targets and/or proteins (Zhuang et al., 2020).

New assays designed to fluorescently detect up to 12 mRNA targets in FFPE sections and 48 targets (in cryosections) have also been developed, including the Hplex assay from ACD, that requires iterative rounds of 4-plex staining imaging and gentle stripping of the bound probes (Hashikawa et al., 2020; Goodman et al., 2023). In addition, co-detection of proteins has now been facilitated using a protease-free pre-hybridization step *in lieu* of the original protease

digestion step that preserves target protein epitopes, enabling IHC or immunofluorescence co-detection.

Spatial transcriptomics is a rapidly growing field encompassing many technological innovations allowing scientists to investigate the spatial distribution of thousands of mRNA transcripts within individual cells and tissues in FFPE tissue sections (Jain and Eadon, 2024; Sibai et al., 2025). This extremely rich gene expression data is quantitative and can be coupled with cellular segmentation to help confirm cell lineage and cell boundaries to add increased granularity to the data. Broadly, these can be divided into two categories, those based on sequencing (e.g., Visium and GeoMX) and those that are more image-based (e.g., Xenium, CosMX and MERFISH). The sequencing-based assays have enabled whole transcriptome mapping and identification of splice variants (Chen T. Y. et al., 2023; Li et al., 2025; Nishi et al., 2025). The data obtained from sequencing-based assays from most of these methods present as individual spots within in a grid format. However, one disadvantage is that due to the size of these spots which normally overlay several cells, these technologies fail to provide true single cell resolution.

These platforms employ dozens of primary probes that bind to specific RNA targets in order to achieve high sensitivity, however, these fluorescent methods are not able to detect small RNAs, nor oligonucleotides.

Existing sequencing-based techniques including Visium require the acquisition of polyadenylated transcripts, lacking the sensitivity to detect many species of non-A-tailed RNAs, such as microRNAs and newly transcribed RNAs. Newer single-cell RNA-sequencing protocols, Smart-Seq-Total and VASA-seq, have modified the enzymatic polyadenylation process to allow plate-based and microfluidic-based single-cell total RNA-sequencing, respectively, enabling the spatial profiling of different RNA species in complex tissues (McKellar et al., 2023).

These powerful techniques generate a huge amount of data; however, they require expensive equipment and need highly specialized staff to run these experiments to fully utilize the data they generate.

Here we have focussed on lower plex approaches for detecting mRNA, miRNA and oligonucleotides in tissues that do not require such large capital expenditure and experience to set up and run., the higher plex assays used as part of spatial transcriptomic approaches have been reviewed elsewhere (Xia et al., 2019; McKellar et al., 2023).

Detection of short targets including short interfering RNA, antisense oligonucleotides, and miRNAs in tissues

Whilst the ability to detect relatively long mRNA transcripts has been well established (Wang et al., 2012; Zhang et al., 2024; Hu et al., 2025), more recently assays have been developed that enable the spatial profiling of much shorter RNA species including microRNAs (miRNA) and unlabelled oligonucleotide drugs in single and low-plex options in both frozen and paraffin tissue samples (Robles-Remacho et al., 2025). These approaches include miRNAscope

TABLE 1 Comparison of FFPE compatible branched DNA-based ISH assays.

Assay	Target type	Advantages	Disadvantages
RNAscope and ViewRNA	mRNA	Well established, industry leading assay, automated, chromogenic or fluorescent, quantifiable, works protease free. Specific probe design. Manual or automated	Relatively expensive compared to IHC, normally, requires at least 300 nt of binding and good quality undegraded mRNA to elicit a good signal
HCR	mRNA	Enzyme-free method using probe pairs and hairpin DNAs to detect single or up to 10 mRNA targets in sections or whole mount preparations. Manual or automated. Potential for longer amplification chains, increasing signal amplification, particularly beneficial for visualizing low-abundance RNA transcripts	Can produce background non-specific staining unrelated to the target RNA. Potentially due to non-specific hybridization of the HCR probes or amplifiers to off-target RNA molecules Complex probe design
Basescape	mRNA	Detects shorter target mRNAs 50–300 nt long, including degraded mRNA, exon junctions/splice variants and point mutations. Quantifiable signal. Manual or automated	Only available as a chromogenic assay, maximum number of targets detectable is 2
miRNAscope	miRNA, siRNA, shRNA and oligos	Can only detect a single target 17–50 nt long. Using the protease-free option can co-detect proteins by IHC. Chromogenic. Semi-quantifiable for miRNA targets. Manual or automated	Minimum target size is 17 nt. Not ideal for optically cleared tissues
RNAscope plus smRNA-RNA	mRNA plus miRNA, siRNA, shRNA and oligos	Enables the simultaneous fluorescent detection of short RNA and oligo targets together with 3 mRNAs in the same tissue section at single-cell and subcellular resolution protease free, enabling co-detection of proteins by IHC. mRNA targets are quantifiable; miRNAs are semi-quantifiable. Manual or automated. Fluorescent assay: Need to mitigate tissue autofluorescence	Minimum short target size is 17 nt. Not ideal for optically cleared tissues

(Wang et al., 2012; Spencer-Dene et al., 2023), HCR (Choi et al., 2018), NanoSIMS (King et al., 2024; He et al., 2021), Mass Spectrometry Imaging (van der Vloet et al., 2025) and CARS approaches (Spencer-Dene et al., 2023; Shi et al., 2023).

miRNAs comprise a set of small, non-coding RNA species typically 18–22 bp long that negatively regulate gene expression post transcriptionally. They play roles in many diseases including cancer and are also involved during development. Previously these could not be detected in tissue sections using standard *in situ* hybridization techniques but there are now several non-isotopic approaches that can be used including miRNAscope (Figure 1) and HCR (Jorgensen et al., 2010; Zedan et al., 2017; Nielsen et al., 2021). Other methods including CLAMP-FISH and SABER-FISH have not yet been reported for short targets, however this may be achievable in future (Higo et al., 2023).

Shorter 50–150 bp mRNA targets, including point mutations and splice variants in sub-optimally fixed and preserved tissues including human brain bank samples, can best be detected by a modified version of the RNAscope assay called BaseScope. While RNAscope uses paired 18–25 base “ZZ” probes that hybridize to the target sequence, BaseScope utilizes shorter probe sets of 1–3 ZZ pairs, and an extra signal amplification step which significantly increases sensitivity without generating excessive background staining. This chromogenic assay is developed chromogenically with Fast Red and is now also available as a chromogenic duplex assay (Rifai et al., 2023; Gregory et al., 2020; Baker et al., 2017). A summary of the most common branched DNA methods for detecting mRNA, miRNA and oligonucleotides in tissue sections is shown in Table 1.

Oligonucleotides are short single or double stranded RNA or DNA molecules that are increasingly being used as therapeutic modalities to treat both rare and common

diseases. Once these short stretches of chemically modified nucleotides arrive at their target organ or cell type, they bind to their specific target in a complementary manner altering the transcriptional fate of that mRNA target, for example, catalysing its rapid degradation by RNase H1 thus reducing the downstream translation of that target protein that may be involved in a pathological process.

Recent advances in the detection of single and double stranded oligonucleotides in tissues designed to improve sensitivity and spatial resolution include the use of chloroalkane-conjugated oligonucleotides (Deprey et al., 2022), or novel highly sensitive imaging techniques including nanoscale secondary ion mass spectrometry (NanoSIMS) to detect radio-labelled antisense oligonucleotides (ASOs) in tissue (He et al., 2021). These labelled oligos could be readily discerned in specific cellular compartments and organelles including within the endolysosomal vesicles in hepatocytes and non-hepatocyte populations including the Kupffer cells and the liver sinusoidal endothelial cells. There are downsides to these powerful approaches, such as the need to use radiolabelled probes and the NanoSIMS hardware itself is highly complex and expensive.

In addition, commercial mouse monoclonal antibodies have now been generated against phosphorothioate groups that are one of the most common modifications engineered into oligo backbone chemistries to protect against endonuclease digestion. These have been shown to detect unlabelled single stranded ASOs in FFPE and frozen tissue sections by IHC (Kordasiewicz et al., 2012; Hung et al., 2013) and immunofluorescence (Brown et al., 2022; Ly et al., 2025), but unlike the miRNAscope and RNAscope Plus smRNA-RNA ISH assays shown in Table 1, are not specific for individual oligonucleotide sequences.

Frozen ISH

Whilst the use of FFPE samples for ISH detection offers many advantages, especially when starting material is particularly scarce, alternative sample types including fresh frozen and paraformaldehyde-fixed frozen samples have been used very successfully with ISH. In recent years, single-molecule fluorescent ISH approaches have been successfully used to detect multiple transcripts on fresh frozen skin and brain samples to complement larger transcriptomic and cell atlas studies (Gopee et al., 2024; Bayraktar et al., 2020). However, some aspect of the sample preparation such as perfusion fixation and sucrose cryoprotection can lead to adverse shrinkage and poor signal (Asp et al., 2006).

More recent innovations to try to achieve improved tissue clearing, greater imaging depth and more consistent and reproducible three dimensional labelling of mRNA transcripts in larger samples incorporating fluorescent ISH HCR have been used in fresh frozen mouse and rat brain as well as post-mortem human brain samples to detect neuronal transcripts (Kumar et al., 2021).

Discussion

The growing success of modern ISH based assays and their increasing versatility in both the research and clinical diagnostic sectors (Monne Rodriguez et al., 2023; Pringle et al., 1989; Wilcox, 1993) has hugely significant economic considerations as the technology becomes more sophisticated and continues to grow. The ISH market size has been valued at \$1.9 billion in 2025 and is predicted to rise significantly to \$5.2 billion by 2035 (In Situ Hybridization Market | Global Market Analysis Report - 2035).

The ability to analyse gene expression and spatial localization within tissues has opened new avenues for understanding complex biological processes and developing novel therapeutic strategies (Higo et al., 2023). The move towards ever increasing higher plex spatial transcriptomic approaches incorporating automated platforms from whole tissues down to sub-cellular spatial resolution have dramatically improved our ability to resolve entire transcriptomes on tissue sections. There are now dozens of different methods available using different approaches for detection of many thousands of transcripts and whole genomes (Moffitt et al., 2022; Goltsev et al., 2018; Orjalo and Johansson, 2016; Kishi et al., 2019; Xia et al., 2019; Ren et al., 2024), and these technologies are continuing to improve. However, many of these assays are not yet able to detect smaller RNA species such as miRNAs nor oligonucleotides, suggesting that the lower plex approaches still have significant utility in the field.

Some challenges remain to be resolved. Whilst mRNA transcripts can be easily quantified as punctate signals corresponding to individual transcripts using image analysis tools, absolute quantification of unlabelled oligonucleotide concentration in tissues at single cell resolution is currently a major challenge as the oligo is primarily concentrated within the

endolysosomal compartment. Use of image analysis tools can try to solve this through fluorescent or chromogenic intensity measurements to complement bioanalytical whole tissue bulk oligo pharmacokinetic quantification approaches.

Despite limitations and challenges, these persistently evolving techniques will lead to an expanding and intriguing field of novel applications with a substantial impact on a wide range of scientific fields, from developmental and tissue maintenance to tumour and aging biology.

Author contributions

BS-D: Writing – original draft, Writing – review and editing.
EM: Writing – original draft, Writing – review and editing.

Funding

The author(s) declared that financial support was not received for this work and/or its publication.

Acknowledgements

We would like to formally acknowledge all members of GSK Non-Clinical Histology team for their discussions and support.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

- Asp, J., Abramsson, A., and Betsholtz, C. (2006). Nonradioactive *in situ* hybridization on frozen sections and whole mounts. *Methods Mol. Biol.* 326, 89–102. doi:10.1385/1-59745-007-389
- Atout, S., Shurrab, S., and Loveridge, C. (2022). Evaluation of the suitability of RNAscope as a technique to measure gene expression in clinical diagnostics: a systematic review. *Mol. Diagn Ther.* 26 (1), 19–37. doi:10.1007/s40291-021-00570-2
- Baharlou, H., Canete, N., Vine, E. E., Hu, K., Yuan, D., Sandgren, K. J., et al. (2022). An *in situ* analysis pipeline for initial host-pathogen interactions reveals signatures of human colorectal HIV transmission. *Cell. Rep.* 40 (12), 111385. doi:10.1016/j.celrep.2022.111385
- Bai, Z., Zhang, D., Gao, Y., Tao, B., Zhang, D., Bao, S., et al. (2024). Spatially exploring RNA biology in archival formalin-fixed paraffin-embedded tissues. *Cell.* 187 (23), 6760–6779 e24. doi:10.1016/j.cell.2024.09.001
- Baker, A. M., Huang, W., Wang, X. M. M., Jansen, M., Ma, X. J., Kim, J., et al. (2017). Robust RNA-based *in situ* mutation detection delineates colorectal cancer subclonal evolution. *Nat. Commun.* 8 (1), 1998. doi:10.1038/s41467-017-02295-5
- Bayraktar, O. A., Bartels, T., Holmqvist, S., Kleshchevnikov, V., Martirosyan, A., Polioudakis, D., et al. (2020). Astrocyte layers in the mammalian cerebral cortex revealed by a single-cell *in situ* transcriptomic map. *Nat. Neurosci.* 23 (4), p500–p509. doi:10.1038/s41593-020-0602-1
- Baysoy, A., Bai, Z., Satija, R., and Fan, R. (2023). The technological landscape and applications of single-cell multi-omics. *Nat. Rev. Mol. Cell. Biol.* 24 (10), 695–713. doi:10.1038/s41580-023-00615-w
- Best Rocha, A., Stroberg, E., Barton, L. M., Duval, E. J., Mukhopadhyay, S., Yarid, N., et al. (2020). Detection of SARS-CoV-2 in formalin-fixed paraffin-embedded tissue sections using commercially available reagents. *Lab. Invest.* 100 (11), 1485–1489. doi:10.1038/s41374-020-0464-x
- Bingham, V., Ong, C. W., James, J., Maxwell, P., Waugh, D., Salto-Tellez, M., et al. (2016). PTEN mRNA detection by chromogenic, RNA *in situ* technologies: a reliable alternative to PTEN immunohistochemistry. *Hum. Pathol.* 47 (1), 95–103. doi:10.1016/j.humpath.2015.09.009
- Bishop, J. A., Ma, X. J., Wang, H., Luo, Y., Illei, P. B., Begum, S., et al. (2012). Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA *in situ* hybridization method. *Am. J. Surg. Pathol.* 36 (12), 1874–1882. doi:10.1097/PAS.0b013e318265fb2b
- Borrelli, C., and Moor, A. E. (2020). Single-molecule RNA FISH in whole-mount organoids. *Methods Mol. Biol.* 2171, 237–247. doi:10.1007/978-1-0716-0747-3_15
- Bressan, D., Battistoni, G., and Hannon, G. J. (2023). The dawn of spatial omics. *Science* 381 (6657), eabq4964. doi:10.1126/science.abq4964
- Brown, K. M., Nair, J. K., Janas, M. M., Anglero-Rodriguez, Y. I., Dang, L. T. H., Peng, H., et al. (2022). Expanding RNAi therapeutics to extrahepatic tissues with lipophilic conjugates. *Nat. Biotechnol.* 40 (10), 1500–1508. doi:10.1038/s41587-022-01334-x
- Chen, R., Zhang, R., Zhang, M., Liu, S., Xie, M., Yang, Z., et al. (2023). CIN grades possessing different HPV RNA location patterns and RNAscope is helpful tool for distinguishing squamous intraepithelial lesions in difficult cervical cases. *Diagn Pathol.* 18 (1), 23. doi:10.1186/s13000-023-01308-w
- Chen, T. Y., You, L., Hardillo, J. A. U., and Chien, M. P. (2023). Spatial transcriptomic technologies. *Cells* 12 (16), 2042. doi:10.3390/cells12162042
- Choi, H. M., Beck, V. A., and Pierce, N. A. (2014). Next-generation *in situ* hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano* 8 (5), 4284–4294. doi:10.1021/nn405717p
- Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., et al. (2018). Third-generation *in situ* hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* 145 (12). doi:10.1242/dev.165753
- Deprey, K., Batistatou, N., Debets, M. F., Godfrey, J., VanderWall, K. B., Miles, R. R., et al. (2022). Quantitative measurement of cytosolic and nuclear penetration of oligonucleotide therapeutics. *ACS Chem. Biol.* 17 (2), 348–360. doi:10.1021/acscchembio.1c00830
- Elagoz, A. M., Styfals, R., Maccuro, S., Masin, L., Moons, L., and Seuntjens, E. (2022). Optimization of whole mount RNA multiplexed *in situ* hybridization chain reaction with immunohistochemistry, clearing and imaging to visualize octopus embryonic neurogenesis. *Front. Physiol.* 13, 882413. doi:10.3389/fphys.2022.882413
- Fleck, J. S., Jansen, S. M. J., Wollny, D., Zenk, F., Seimiya, M., Jain, A., et al. (2023). Inferring and perturbing cell fate regulomes in human brain organoids. *Nature* 621 (7978), 365–372. doi:10.1038/s41586-022-05279-8
- Gaide, N., Crispo, M., Jbenyeni, A., Bleuart, C., Delverdier, M., Vergne, T., et al. (2023). Validation of an RNAscope assay for the detection of avian influenza A virus. *J. Vet. Diagn. Invest.* 35 (5), 500–506. doi:10.1177/10406387231182385
- Gall, J. G., and Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. U. S. A.* 63 (2), 378–383. doi:10.1073/pnas.63.2.378
- Gattoni, G., Keitley, D., Sawle, A., and Benito-Gutiérrez, E. (2025). An ancient apical patterning system sets the position of the forebrain in chordates. *Sci. Adv.* 11 (4), eadq4731. doi:10.1126/sciadv.adq4731
- Goltsev, Y., Samusik, N., Kennedy-Darling, J., Bhat, S., Hale, M., Vazquez, G., et al. (2018). Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. *Cell.* 174 (4), 968–981 e15. doi:10.1016/j.cell.2018.07.010
- Goodman, R. L., Moore, A. M., Onslow, K., Hileman, S. M., Hardy, S. L., Bowdridge, E. C., et al. (2023). Lesions of KNDy and Kiss1R neurons in the arcuate nucleus produce different effects on LH pulse patterns in female sheep. *Endocrinology* 164 (11), bqad148. doi:10.1210/endo/bqad148
- Gopee, N. H., Winheim, E., Olabi, B., Admane, C., Foster, A. R., Huang, N., et al. (2024). A prenatal skin atlas reveals immune regulation of human skin morphogenesis. *Nature* 635 (8039), 679–689. doi:10.1038/s41586-024-08002-x
- Gregory, J. M., Livesey, M. R., McDade, K., Selvaraj, B. T., Barton, S. K., Chandran, S., et al. (2020). Dysregulation of AMPA receptor subunit expression in sporadic ALS post-mortem brain. *J. Pathol.* 250 (1), 67–78. doi:10.1002/path.5351
- Hall, J. M., Lyons, C. E., Li, J., Martinez-Romero, G., Hayes, T., Cook, A., et al. (2024). Mpx infection of stromal cells and macrophages of macaque with endometriosis. *Sci. Rep.* 14 (1), 21947. doi:10.1038/s41598-024-73012-8
- Hashikawa, Y., Hashikawa, K., Rossi, M. A., Basiri, M. L., Liu, Y., Johnston, N. L., et al. (2020). Transcriptional and spatial resolution of cell types in the mammalian habenula. *Neuron* 106 (5), 743–758 e5. doi:10.1016/j.neuron.2020.03.011
- He, C., Migawa, M. T., Chen, K., Weston, T. A., Tanowitz, M., Song, W., et al. (2021). High-resolution visualization and quantification of nucleic acid-based therapeutics in cells and tissues using nanoscale secondary ion mass spectrometry (NanoSIMS). *Nucleic Acids Res.* 49 (1), 1–14. doi:10.1093/nar/gkaa1112
- Higo, H., Ishii, H., and Ozawa, H. (2023). Recent advances in high sensitivity *in situ* hybridization and costs and benefits to consider when employing these methods. *Acta Histochem. Cytochem.* 56 (3), p. 49–54. doi:10.1267/ahc.23-00024
- Hu, C., Zhang, X., Zhu, T., Hou, Y., Shi, Y., Sun, J., et al. (2025). High-sensitivity BK virus detection system using viewRNA *in situ* hybridization. *Diagn. Microbiol. Infect. Dis.* 112 (2), 116790. doi:10.1016/j.diagmicrobio.2025.116790
- Hung, G., Xiao, X., Peralta, R., Bhattacharjee, G., Murray, S., Norris, D., et al. (2013). Characterization of target mRNA reduction through *in situ* RNA hybridization in multiple organ systems following systemic antisense treatment in animals. *Nucleic Acid. Ther.* 23 (6), 369–378. doi:10.1089/nat.2013.0443
- Jain, S., and Eadon, M. T. (2024). Spatial transcriptomics in health and disease. *Nat. Rev. Nephrol.* 20 (10), 659–671. doi:10.1038/s41581-024-00841-1
- Jorgensen, S., Baker, A., Møller, S., and Nielsen, B. S. (2010). Robust one-day *in situ* hybridization protocol for detection of microRNAs in paraffin samples using LNA probes. *Methods* 52 (4), 375–381. doi:10.1016/j.jmeth.2010.07.002
- King, J. J., Chen, K., Evans, C. W., Norret, M., Almasri, R., Pavlos, N. J., et al. (2024). High-resolution visualisation of antisense oligonucleotide release from polymers in cells. *Chem. Sci.* 15 (38), 15690–15697. doi:10.1039/d3sc06773d
- Kishi, J. Y., Lapan, S. W., Beliveau, B. J., West, E. R., Zhu, A., Sasaki, H. M., et al. (2019). SABER amplifies FISH: enhanced multiplexed imaging of RNA and DNA in cells and tissues. *Nat. Methods* 16 (6), 533–544. doi:10.1038/s41592-019-0404-0
- Kordasiewicz, H. B., Stanek, L. M., Wancewicz, E. V., Mazur, C., McAlonis, M. M., Pytel, K. A., et al. (2012). Sustained therapeutic reversal of huntington's disease by transient repression of huntingtin synthesis. *Neuron* 74 (6), 1031–1044. doi:10.1016/j.neuron.2012.05.009
- Koshiba-Takeuchi, K. (2018). Whole-mount and section *in situ* hybridization in mouse embryos for detecting mRNA expression and localization. *Methods Mol. Biol.* 1752, 123–131. doi:10.1007/978-1-4939-7714-7_12
- Kravvas, G., Xie, B., Ganier, C., van den Munckhof, H., van den Munckhof, E., de Koning, M., et al. (2025). A direct comparative analysis of HPV DNA with single-molecule RNA and p16(INK4a) protein expression in lichen sclerosus: implications for diagnostics and pathogenesis. *JID Innov.* 5 (4), 100367. doi:10.1016/j.jidi.2025.100367
- Kumar, V., Krolewski, D. M., Hebda-Bauer, E. K., Parsegian, A., Martin, B., Foltz, M., et al. (2021). Optimization and evaluation of fluorescence *in situ* hybridization chain reaction in cleared fresh-frozen brain tissues. *Brain Struct. Funct.* 226 (2), 481–499. doi:10.1007/s00429-020-02194-4
- Lim, H. J., Wang, Y., Buzdin, A., and Li, X. (2025). A practical guide for choosing an optimal spatial transcriptomics technology from seven major commercially available options. *BMC Genomics* 26 (1), 47. doi:10.1186/s12864-025-11235-3
- Liu, J., Babka, A. M., Kearney, B. J., Radoshitzky, S. R., Kuhn, J. H., and Zeng, X. (2020). Molecular detection of SARS-CoV-2 in formalin-fixed, paraffin-embedded specimens. *JCI Insight* 5 (12). doi:10.1172/jci.insight.139042
- Ly, M., Diaz-Garcia, S., Roscoe, N., Ushach, I., Hong, Z., França, M., et al. (2025). Multiplexed siRNA immunoassay unveils spatial and quantitative dimensions of siRNA function, abundance, and localization *in vitro* and *in vivo*. *Toxicol. Pathol.* 53 (6), 536–547. doi:10.1177/0192623241311539
- Mahmood, R., and Mason, I. (2008). *In-situ* hybridization of radioactive riboprobes to RNA in tissue sections. *Methods Mol. Biol.* 461, 675–686. doi:10.1007/978-1-60327-483-8_45
- McKellar, D. W., Mantri, M., Hinchman, M. M., Parker, J. S. L., Sethupathy, P., Cosgrove, B. D., et al. (2023). Spatial mapping of the total transcriptome by *in situ* polyadenylation. *Nat. Biotechnol.* 41 (4), 513–520. doi:10.1038/s41587-022-01517-6

- Moffitt, J. R., Lundberg, E., and Heyn, H. (2022). The emerging landscape of spatial profiling technologies. *Nat. Rev. Genet.* 23 (12), 741–759. doi:10.1038/s41576-022-00515-3
- Monne Rodriguez, J. M., Frisk, A.-L., Kreutzer, R., Lemarchand, T., Lezmi, S., and Saravanan, C. (2023). European society of toxicologic pathology (pathology 2.0 molecular pathology special interest group): review of *in situ* hybridization techniques for drug research and development. *Toxicol. Pathol.* 51 (3), 92–111. doi:10.1177/01926233231178282
- Musangile, F. Y., Matsuzaki, I., Okodo, M., Shirasaki, A., Mikasa, Y., Iwamoto, R., et al. (2021). Detection of HPV infection in urothelial carcinoma using RNAscope: clinicopathological characterization. *Cancer Med.* 10 (16), 5534–5544. doi:10.1002/cam4.4091
- Nielsen, B. S., Larsen, J., Hoffding, J., Nhat, S. L., Madsen, N. H., Møller, T., et al. (2021). Detection of lncRNA by LNA-based *in situ* hybridization in paraffin-embedded cancer cell spheroids. *Methods Mol. Biol.* 2348, 123–137. doi:10.1007/978-1-0716-1581-2_8
- Nishi, K., Yoshimoto, S., Tanaka, T., Kimura, S., Tsunoda, T., Watanabe, A., et al. (2025). Spatial transcriptomics of the epipharynx in long COVID identifies SARS-CoV-2 signalling pathways and the therapeutic potential of epipharyngeal abrasive therapy. *Sci. Rep.* 15 (1), 8618. doi:10.1038/s41598-025-92908-7
- Orjalo, A. V., Jr., and Johansson, H. E. (2016). Stellaris(R) RNA fluorescence *in situ* hybridization for the simultaneous detection of immature and mature long noncoding RNAs in adherent cells. *Methods Mol. Biol.* 1402, 119–134. doi:10.1007/978-1-4939-3378-5_10
- Pesti, A., Danics, K., Glasz, T., Várkonyi, T., Barbai, T., Reszegi, A., et al. (2023). Liver alterations and detection of SARS-CoV-2 RNA and proteins in COVID-19 autopsies. *Geroscience* 45 (2), 1015–1031. doi:10.1007/s11357-022-00700-6
- Pringle, J. H., Primrose, L., Kind, C. N., Talbot, I. C., and Lauder, I. (1989). *In situ* hybridization demonstration of poly-adenylated RNA sequences in formalin-fixed paraffin sections using a biotinylated oligonucleotide poly d(T) probe. *J. Pathol.* 158 (4), 279–286. doi:10.1002/path.1711580403
- Ren, J., Luo, S., Shi, H., and Wang, X. (2024). Spatial omics advances for *in situ* RNA biology. *Mol. Cell.* 84 (19), 3737–3757. doi:10.1016/j.molcel.2024.08.002
- Rifai, O. M., O’Shaughnessy, J., Dando, O. R., Munro, A. F., Sewell, M. D. E., Abrahams, S., et al. (2023). Distinct neuroinflammatory signatures exist across genetic and sporadic amyotrophic lateral sclerosis cohorts. *Brain* 146 (12), 5124–5138. doi:10.1093/brain/awad243
- Robles-Remacho, A., Zou, Y., Grillo, M., and Nilsson, M. (2025). Spatially resolved microRNA expression in tissues: technologies, challenges, and opportunities. *Trends Genet.* 41 (12), 1131–1143. doi:10.1016/j.tig.2025.06.005
- Shao, Z., Hu, J., Jandura, A., Wilk, R., Jachimowicz, M., Ma, L., et al. (2024). Spatially revealed roles for lncRNAs in drosophila spermatogenesis, Y chromosome function and evolution. *Nat. Commun.* 15 (1), 3806. doi:10.1038/s41467-024-47346-w
- Shi, J., Bera, K., Mukherjee, P., Alex, A., Chaney, E. J., Spencer-Dene, B., et al. (2023). Weakly supervised identification and localization of drug fingerprints based on label-free hyperspectral CARS microscopy. *Anal. Chem.* 95 (29), 10957–10965. doi:10.1021/acs.analchem.3c00979
- Sibai, M., Cervilla, S., Grases, D., Musulen, E., Lazcano, R., Mo, C. K., et al. (2025). The spatial landscape of cancer hallmarks reveals patterns of tumor ecological dynamics and drug sensitivity. *Cell. Rep.* 44 (2), 115229. doi:10.1016/j.celrep.2024.115229
- Solanki, S., Ameen, G., Zhao, J., Flaten, J., Borowicz, P., and Brueggeman, R. S. (2020). Visualization of spatial gene expression in plants by modified RNAscope fluorescent *in situ* hybridization. *Plant Methods* 16, 71. doi:10.1186/s13007-020-00614-4
- Spencer-Dene, B., Mukherjee, P., Alex, A., Bera, K., Tseng, W. J., Shi, J., et al. (2023). Localization of unlabeled bepirovirsen antisense oligonucleotide in murine tissues using *in situ* hybridization and CARS imaging. *RNA* 29 (10), 1575–1590. doi:10.1261/rna.079699.123
- van der Vloet, L., Hilaire, P. B. S., Bouillod, C., Isin, E. M., Heeren, R. M. A., and Vandenbosch, M. (2025). How can MSI enhance our understanding of ASO distribution? *Drug Discov. Today* 30 (1), 104275. doi:10.1016/j.drudis.2024.104275
- Velasco, S., Kedaigle, A. J., Simmons, S. K., Nash, A., Rocha, M., Quadrato, G., et al. (2019). Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature* 570 (7762), 523–527. doi:10.1038/s41586-019-1289-x
- Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., et al. (2012). RNAscope: a novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn.* 14 (1), 22–29. doi:10.1016/j.jmoldx.2011.08.002
- Wilcox, J. N. (1993). Fundamental principles of *in situ* hybridization. *J. Histochem Cytochem* 41 (12), 1725–1733. doi:10.1177/41.12.8245419
- Worwa, G., Cooper, T. K., Yeh, S., Shantha, J. G., Hirschak, A. M. W., Klim, S. E., et al. (2022). Persistent intraocular ebola virus RNA is associated with severe uveitis in a convalescent rhesus monkey. *Commun. Biol.* 5 (1), 1204. doi:10.1038/s42003-022-04158-2
- Xia, C., Babcock, H. P., Moffitt, J. R., and Zhuang, X. (2019). Multiplexed detection of RNA using MERFISH and branched DNA amplification. *Sci. Rep.* 9 (1), 7721. doi:10.1038/s41598-019-43943-8
- Xu, Z., Zhang, T., Chen, H., Zhu, Y., Lv, Y., Zhang, S., et al. (2023). High-throughput single nucleus total RNA sequencing of formalin-fixed paraffin-embedded tissues by snRandom-seq. *Nat. Commun.* 14 (1), 2734. doi:10.1038/s41467-023-38409-5
- Zedan, A. H., Blavnsfeldt, S. G., Hansen, T. F., Nielsen, B. S., Marcussen, N., Pleckaitis, M., et al. (2017). Heterogeneity of miRNA expression in localized prostate cancer with clinicopathological correlations. *PLoS One* 12 (6), e0179113. doi:10.1371/journal.pone.0179113
- Zhang, X., Lu, W., Feng, Y., Zhang, Z., and Yuan, Z. (2024). A chromogenic *in situ* hybridization (CISH) assay for detection of HBV RNA, DNA, and cccDNA in liver tissue. *Methods Mol. Biol.* 2837, 137–148. doi:10.1007/978-1-0716-4027-2_12
- Zhuang, P., Zhang, H., Welchko, R. M., Thompson, R. C., Xu, S., and Turner, D. L. (2020). Combined microRNA and mRNA detection in mammalian retinas by *in situ* hybridization chain reaction. *Sci. Rep.* 10 (1), 351. doi:10.1038/s41598-019-57194-0