Spatial biology *in situ* hybridization and multiplexed immunohistochemistry detection compatibility using a modified protocol

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Abstract

In the field of spatial-omics imaging, combining mRNA *in situ* hybridization (ISH) detection with multiplexed protein immunohistochemistry detection (IHC) is desirable. We present a simplified workflow in FFPE tissue sections where ISH is detected using branched ssDNA signal amplification visible by brightfield and fluorescence imaging combined with multiplexed IHC using directly labeled antibodies detected by fluorescence.

In multiple studies mRNA expression of PCNA (Proliferating Cell Nuclear Antigen) shows moderate to high nuclear positivity observed in most cancer cells, with gliomas and lymphomas being the few negative exceptions[1]. In contrast, expression of PCNA in adult non-cancerous tissue has low to no expression, with tonsil and bone marrow being the exceptions.
We first show the relationship between proliferation as measured by EdU detection, PCNA antibody and PCNA mRNA expression in the embryonic mouse model where PCNA mRNA expression is prevalent in developing tissue.
Then we demonstrate a simplified working protocol for combining ISH and IHC in FFPE human cancer tissue sections for the co-detection of PCNA mRNA together with IHC using a low plex panel antibody markers relevant to the tumor microenvironment. For IHC, we directly labeled validated unconjugated antibodies with fluorophores using amine-reactive chemistry and select the degree of labeling (DOL) and titer for optimal signal/background most useful for multiplexing on FFPE tissue. We found important protocol steps to consider for compatibility between IHC and ISH detection method. Loss of IHC protein epitope due to ISH protocol use of proteases and loss of ISH signal due to presence of RNase commonly present in IHC antibody reagents prevent optimal signal when combining IHC with ISH. Stabilizing IHC through refixation prior to ISH workflow is also required. Modifying the protocols to avoid these incompatible treatments and use of directly labeled antibodies provides useful strategies for multiplexing IHC with ISH.

Introduction

As proof of principal, we designed low-plex panels of antibodies for IHC labeling to be compatible with ISH broad fluorescence emission profile which occurs with alkaline phosphatase Fast Red and Fast Blue substrates used with mRNA ISH detection. We focused on several markers of proliferation relevant cancer models, and associated changes in protein and mRNA expression levels. The click chemistry substrate ethynyl deoxyuridine (EdU) is incorporated into nascent DNA synthesis and marks active DNA replication activity. For human models we examined Ki-67 and PCNA as surrogates for nascent DNA synthesis. PCNA mRNA is present in a majority of cancer tissue with few exceptions, so we focused on ISH detection of PCNA but also examined ERBB2 as the mRNA marker of Her2 protein expression which is often mutated and/or highly upregulated in breast cancer. Changes in protein expression in some cancer tissue are seen with the increased abundance of smooth muscle actin (SMA) indicating vascular recruitment, changes in estrogen receptor expression (ER) as well as PCNA.

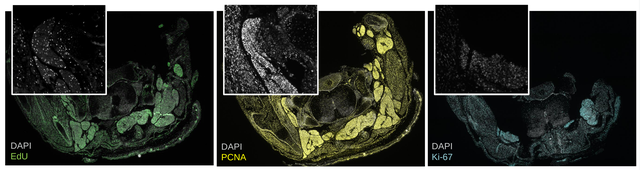


Figure 1. Transverse FFPE embryonic mouse (e16.5) sections labeled with EdU using click chemistry (left), or PCNA (center) and Ki-67 (right) detected with antibody show S-phase replication vs. G_1 proliferative potential indicated by PCNA and Ki-67 (respectively). Inserts using a 20x objective showing zoomed in regions of signal. The relative abundance of these proliferation markers reveal different aspects of proliferation, or proliferative capacity relevant to development, wound repair, and tumor progression.

Materials and methods

IHC	ISH
Deparaffinize/ rehydration	
HIER	
Endogenous peroxidase quench (H_2O_2)	
	RNase inhibitor
Block	
1° MAb incubation	
MAb fixation	
	Protease digestion
Re-fixation	
	Target probe hyb
	Pre-Amp hyb
	Amp hyb
	Label Probe hyb
	Substrate labeling
2nd Ab-dye or Ab-HRP	
Tyramide amp	
Nuclear stain	

We used FFPE embryonic mouse, and rat intestine sections, and commercially available FFPE human tonsil, colon, duodenum and invasive ductal breast carcinoma tissue sections to investigate the interactions of IHC and ISH protocols. For IHC antibodies we used Invitrogen™ ReadyLabel™ Antibody Labeling Kits to make directly labeled Alexa Fluor™ dye antibody conjugates, or we used commercially available “off the shelf” fluorescent dye conjugates which we validated for imaging. For ISH labeling we used Invitrogen™ ViewRNA™ Colorimetric Tissue Core Kit. Tissue was deparaffinized with xylene through graded ethanol rehydration prior to citrate based heat induced epitope retrieval (HIER). Sections were washed in PBS after HIER, peroxidase quenched then treated with ViewRNA Cell Plus RNase inhibitor prior to antibody incubation. Antibody diluents containing serum were treated with Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor. A diagram of the individual and combined IHC and ISH workflows is shown in Table 1. After antibody incubation a fixation step was included to minimize loss of bound antibodies during the subsequent ISH protocol that requires a protease step needed for mRNA revelation.

TABLE 1 Steps for performing compatible IHC + ISH protocols are highlighted in blue. Wash steps not listed. Steps common to both protocols are marked in gray with white script

Results

IHC labeling

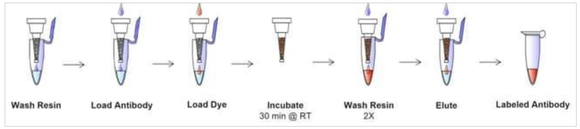


Figure 2. Workflow for labeling unconjugated antibodies with ReadyLabel Antibody Labeling kit.

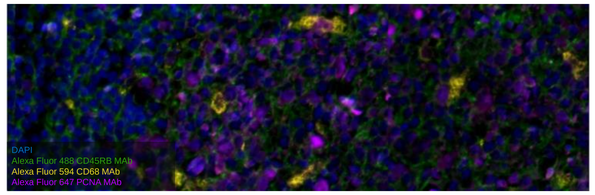
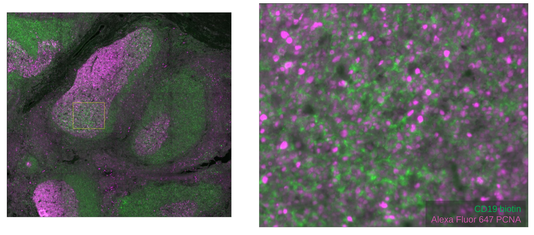
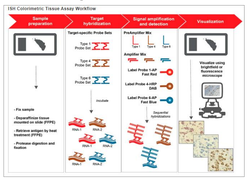


Figure 3. ReadyLabel antibodies demonstrating multiplexing of 3-plex + DAPI panel (above) labeling FFPE tonsil. 2-plex panel (below) detected with Alexa Fluor 647 succinimidyl ester labeling of PCNA combined with commercially available biotinylated CD19 antibody, detected with Alexa Fluor™ 488 Tyramide SuperBoost™ Kit



ISH labeling



ISH detection of mRNA on tissue uses an oligo DNA target probe which is hybridized to the complimentary region of two adjacent sequences of the mRNA which creates a two target probes Z-structure that enables three rounds of hybridization using ssDNA pre-amp, amp, and a label probe with alkaline phosphatase (AP) or horseradish peroxidase (HRP). Fast Red and Fast Blue are AP substrates used for both brightfield and fluorescence imaging. DAB is an HRP substrate used only for brightfield imaging.

Figure 4. ISH signal amplification strategy using AP or HRP substrates in ViewRNA colorimetric kits (left)

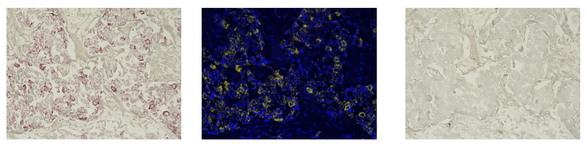


Figure 5. ViewRNA Tissue Colorimetric Fast Red module used to detect PCNA mRNA in invasive ductal breast carcinoma tissue section. Bright field image (left) with corresponding RFP fluorescence (middle) and No Target Control (right)

Results

IHC + ISH labeling

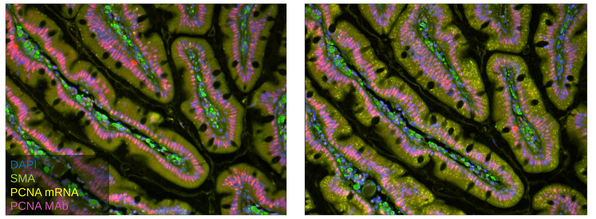


Figure 6. FFPE human colon IHC labeling using Alexa Fluor 488 SMA + Alexa Fluor 647 PCNA antibody conjugates followed by ISH labeling of PCNA mRNA with ViewRNA. No protease treatment for ISH results in weak mRNA signal (left). Fixation following IHC labeling, protease treatment, and re-fixation results in improved mRNA signal of PCNA (right).

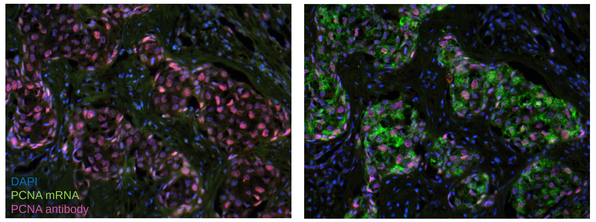
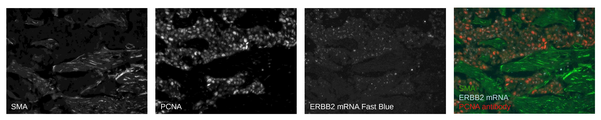


Figure 7. FFPE invasive ductal breast carcinoma sections showing the effect of fixation and protease treatment on IHC and ISH signal retention. Tissue labeled with Alexa Fluor 647 PCNA antibody conjugate and PCNA mRNA detection using ViewRNA. Without protease treatment, IHC antibody labeling is bright while mRNA signal is very dim (left panel). Pretreating IHC reagents with RNase inhibitors and refixation after IHC antibody labeling prior to ISH protease treatment partially abrogates loss of IHC signal while enabling mRNA detection. (right panel). Protease treatment without fixation following antibody labeling results in bright ISH labeling but dim antibody labeling (not shown). Images are gain and exposure matched.

Building an IHC + ISH panel compatible with ViewRNA mRNA detection reagents



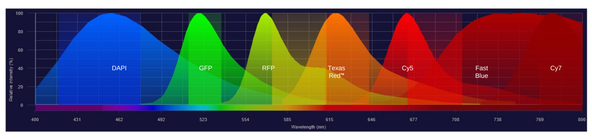


Figure 8. Multiplex panel of invasive ductal breast carcinoma labeled with DAPI (not shown), Alexa Fluor 488 SMA and Alexa Fluor 647 PCNA antibody conjugates combined with ERBB2 mRNA labeling with Fast Blue (upper panel). Thermo Fisher Scientific fluorescence SpectraViewer showing spectra of Fast Blue emission fluorescence used for ViewRNA mRNA detection combined with fluorophore emission spectra commonly use for IHC detection (lower figure). Fast Blue, a blue colorimetric alkaline phosphatase substrate, used for ViewRNA detection of mRNA also has a fluorescence signal in the Cy5.5 and Cy7 channels which can be used to multiplexed with other spectrally compatible fluorophores from IHC labeling.

Conclusions

- We observed simultaneous fluorescence detection from both mRNA detection when using ViewRNA Colorimetric Tissue kits and off the shelf fluorescent dye-antibody conjugates. Antibody panels of low-plex can be designed to work with the broad fluorescent emissions of ISH colorimetric substrates Fast Red and Fast Blue. Fast Red emission spans the RFP-Texas Red channel, while Fast Blue emission spans the Cy5.5 to Cy7 channel. Fast Blue can be detected in the Cy7 emission channel while leaving the Cy5 channel usable for bright antibody labeling with minimal bleed through.
- IHC and ISH protocols each have incompatible steps that reduce the signal of the other. Protease digestion needed for ISH detection causes loss of epitopes and/or degradation of antibodies bound during the IHC step. Antibody incubation in serum-based blocking solutions introduces RNases resulting in degradation of mRNA and loss of ISH signal. We successfully use RNase inhibitors and refixation in the combined protocol to moderate the loss of signals.
- Combining IHC and ISH we observed a loss of antibody signal of ~5x as measured by increase exposure time during imaging. For bright signals can be acceptable. Refixation increased auto-fluorescence of the shorter wavelength channels. Strategic antibody panel design and autofluorescence subtraction can manage the higher background.
- Protocol recommendations are:
- ISH detection improves when tissue sections are first treated with RNase inhibitors such as ViewCell Plus RNase Inhibitor following HIER treatment
- Antibody diluent should be RNase free or treated with RNase inhibitors such as RNaseOUT Recombinant Ribonuclease Inhibitor or similar
- Use IHC validated antibody-dye conjugates labeled prior to ISH detection seemed most compatible with the combined protocol and resulting in not more than ~ 5x increase in exposure time.
  - Use “off the shelf” (commercially available) antibodies
  - DIY using unconjugated MAb with ReadyLabel Antibody Labeling Kits
  - DIY or custom amine labeling of unconjugated MAbs with NHS ester modified dyes
- IHC antibody labeling after ISH detection, resulted in much dimmer antibody detection.
- IHC detection with a 2nd antibody-dye, streptavidin dye, or 2nd antibody-enzyme conjugate gave weak signals and still requires further optimization to be compatible with ISH detection

References

1. https://www.cancer.gov/ccg/research/genome-sequencing/tcga
2. Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer Acta Histochem. 2016 Jun;118(5):544-52DOI: 10.1016/j.acthis.2016.05.002

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