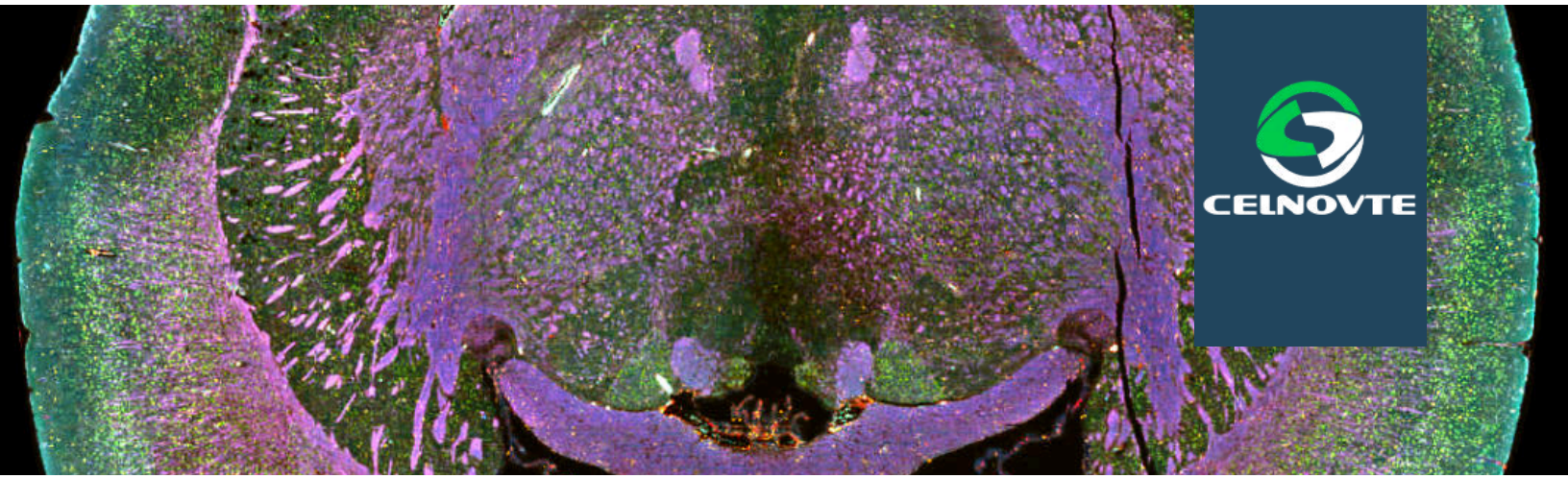

Multiplex Immunofluorescence IHC User Guide

Tips & Tricks to Improve Your mIHC Staining

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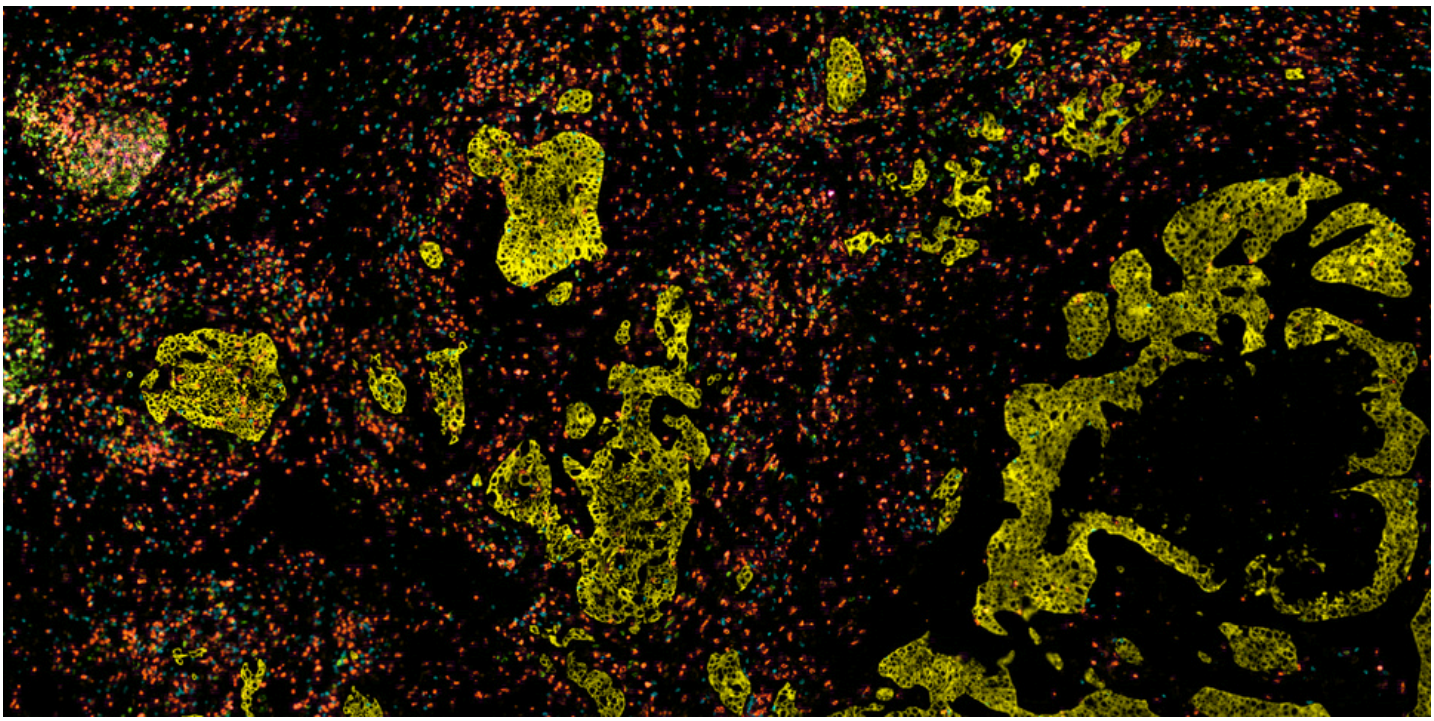
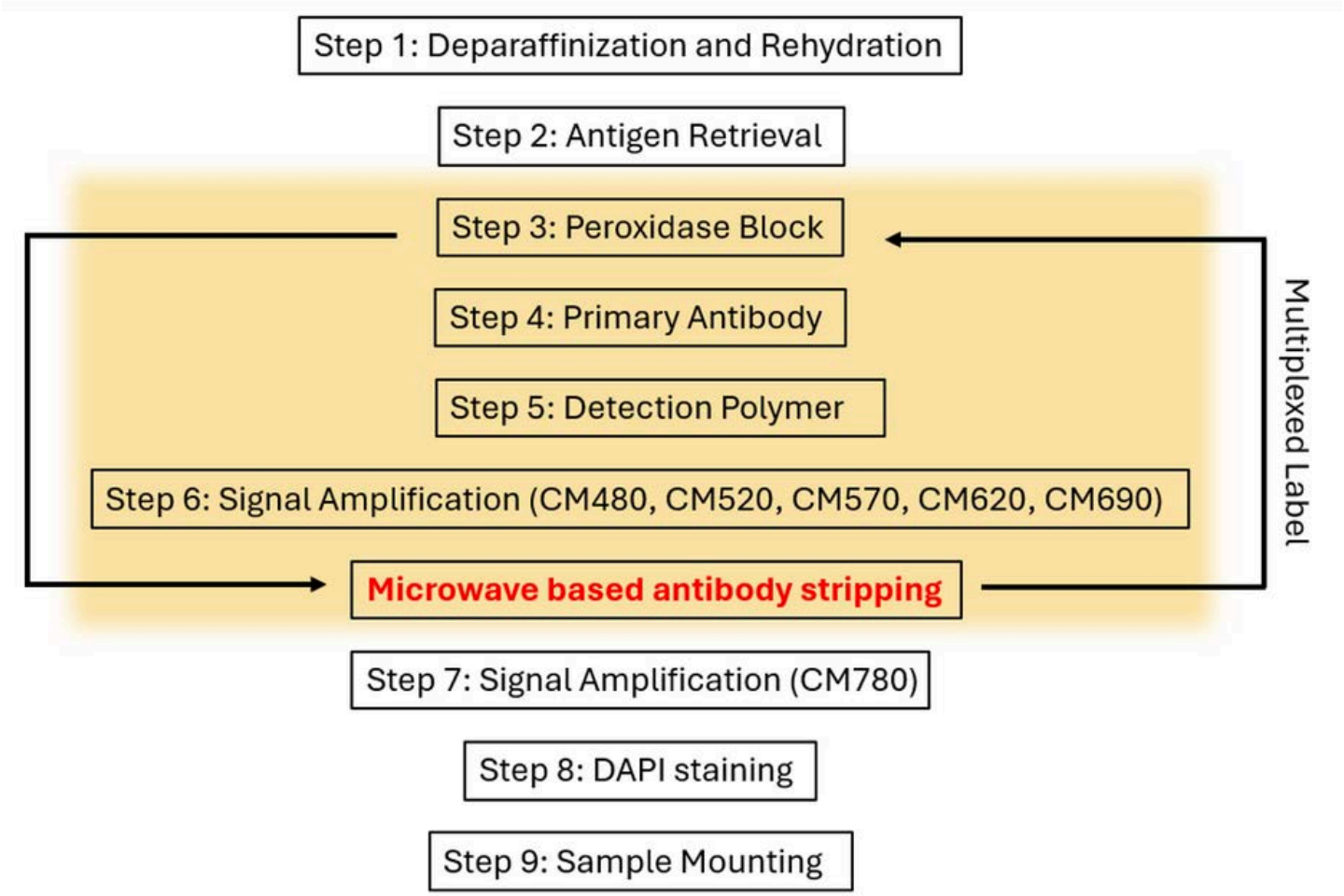
Introduction



Multiplex ImmunoHistoChemistry (mIHC) is a cutting-edge technique that enables simultaneous detection of multiple biomarkers within a single tissue section. It provides researchers with deeper insights into complex biological systems, especially in fields such as cancer research and immunology. To take full advantage of this powerful method, it is crucial to optimize staining and mounting strategies to ensure maximal signal preservation and the best overall image quality.

This guide is designed to help researchers easily adopt our mIHC product. We present a side-by-side comparison with the Akoya Opal system, demonstrating that a seamless transition to our platform requires minimal adjustments. Additionally, our product offers superior signal-to-noise ratio, clearer and more reliable results. By following the best practices outlined in this guide, researchers can maximize the effectiveness of their multiplex staining experiments and achieve optimal imaging outcomes.

CNT Workflow Schematic



Step-by-Step Protocol Comparison with Akoya Opal System

1.1 Deparaffinization and Hydration

CNT	Akoya
Immerse paraffin embedded tissue slides in fresh Xylene (2x10 min)	
Remove excess liquid from the slides and incubate in anhydrous ethanol (2x5 min)	
Remove excess liquid from the slides and incubate in 95% ethanol for 5 min.	
Remove excess liquid from the slides and incubate in 85% ethanol for 5 min.	
Rinse slides in tap water (3x3 min).	

1.2 Antigen Retrieval

CNT	Akoya
Bring antigen retrieval buffer to boiling in a high pressure cooker, and cook samples under high pressure for 3min (Alternatively, cook samples under high heat in a microwave oven for 3 min-5 min – first bring antigen retrieval buffer to boiling, then set microwave oven to low heat and heat samples for 15-20 min, make sure boiling does not occur during the low heat process), let samples cool to room temperature (RT) and incubate samples in pure water (2x3 min), then incubate samples in wash buffer for 3min. During the antigen retrieval process, make sure samples are always covered by liquid.	

1.3 Addition of Peroxidase Blocker

CNT	Akoya
After antigen retrieval, rinse samples in pure water (3x3 min), circle the region of tissue with a hydrophobic pen, rinse samples with wash buffer for 3 min. Remove excess wash buffer and apply	

100 µL of hydrogen peroxidase blocking buffer for a 10 min incubation at room temperature. After

1.4 Addition of Primary Antibody

CNT	Akoya
Remove excess wash buffer, apply 100 µL of primary antibody, incubate under RT for 60 min. Rinse samples with wash buffer (3x3 min).	

1.5 Addition of Enzyme-Labeled Polymer

CNT	Akoya
Remove excess liquid, add 100 µL of enzyme conjugated polymer and incubate for 30 min at RT. Rinse samples with wash buffer (3x3 min).	

1.6 Fluorescence Development

1.6.1 480 Development

CNT	Akoya
TSA dye CM480 is diluted in amplification diluent at 1:100, apply 100 µL to the region of tissue, incubate for 10 min at RT, rinse with wash buffer (3x3 min). Boil antibody stripping buffer in a pressure cooker and cook samples under pressure for 3 min. (Alternatively, microwave samples in antibody stripper buffer for 3-5min until boiling and heat for another 15-20 min under low heat. Let samples cool to RT and wash with pure water for 2x3 min and then incubate in wash buffer for 3 min. DO NOT let samples dry.	Reconstitute Opal Polaris 480 reagent in 75 µL of DMSO and further dilute in 1X Plus Amplification Diluent at 1:100 ratio to make the Opal Polaris 480 Working Solution. Apply 100 µl of Opal Polaris 480 Working Solution to the region of tissue and incubate for 10 min at RT. Rinse samples with TBST at RT for 3x2 min.

1.6.2 520 Development

CNT	Akoya
TSA dye CM520 is diluted in amplification diluent at 1:100, apply 100 µL to the region of tissue, incubate for 10 min at RT, rinse with wash buffer (3x3 min).	Reconstitute Opal Polaris 520 reagent in 75 µL of DMSO and further dilute in 1X Plus Amplification Diluent at 1:100 ratio to make the Opal Polaris 520 Working Solution. Apply 100 µl of Opal Polaris 520 Working Solution to the region of tissue and incubate for 10 min at RT. Rinse samples with TBST at RT for 3x2 min.

1.6.3 570 Development

CNT	Akoya
TSA dye CM570 is diluted in amplification diluent at 1:100, apply 100 µL to the region of tissue, incubate for 10 min at RT, rinse with wash buffer (3x3 min).	Reconstitute Opal Polaris 570 reagent in 75 µL of DMSO and further dilute in 1X Plus Amplification Diluent at 1:100 ratio to make the Opal Polaris 570 Working Solution. Apply 100 µl of Opal Polaris 570 Working Solution to the region of tissue and incubate for 10 min at RT. Rinse samples with TBST at RT for 3x2 min.

1.6.4 620 Development

CNT	Akoya
TSA dye CM620 is diluted in amplification diluent at 1:100, apply 100 µL to the region of tissue, incubate for 10 min at RT, rinse with wash buffer (3x3 min).	Reconstitute Opal Polaris 620 reagent in 75 µL of DMSO and further dilute in 1X Plus Amplification Diluent at 1:100 ratio to make the Opal Polaris 620 Working Solution. Apply 100 µl of Opal Polaris 620 Working Solution to the region of tissue and incubate for 10 min at RT. Rinse samples with TBST at RT for 3x2 min.

1.6.5 690 Development

CNT	Akoya
TSA dye CM690 is diluted in amplification diluent at 1:100, apply 100 µL to the region of tissue, incubate for 10min at RT, rinse with wash buffer (3x3 min).	Reconstitute Opal Polaris 690 reagent in 75 µL of DMSO and further dilute in 1X Plus Amplification Diluent at 1:100 ratio to make the Opal Polaris 690 Working Solution. Apply 100 µl of Opal Polaris 690 Working Solution to the region of tissue and incubate for 10 min at RT. Rinse samples with TBST at RT for 3x2 min.

1.6.6 780 Development

CNT	Akoya
Dilute CM780 dye in PBS buffer at 1:10 ratio to make CM780 working solution; Dilute TSA-biotin in amplification diluent at 1:100 ratio to make TSA-biotin working solution. Remove excess liquid from samples and apply 100 µl of TSA-biotin working solution onto the slide (make sure samples are covered by liquid). Incubate samples at RT for 5-10 minutes, and wash samples with wash buffer (3x3 min). Apply 100 µl of CM780 working solution onto the slide (make sure samples are covered by liquid). Incubate samples at RT for 60 minutes, and wash samples with wash buffer (3x3 min).	Reconstitute Opal TSA-DIG reagent in 75 µL of DMSO and further dilute in 1X Plus Amplification Diluent at 1:100 ratio to make the TSA-DIG Working Solution. Dissolve Opal 780 Reagent in 300 µL of ddH2O and further dilute in Blocking/Ab Diluent at 1:25 ratio to make Opal Polaris 780 Working Solution. Remove excess wash buffer and apply 100 µL of Opal TSA-DIG Working Solution and incubate at RT for 10 min. Wash samples in TBST for 3x2 min at RT. Place samples in AR buffer and cook in microwave for 45 seconds at 100% power (may require optimization). Microwave for an additional 15 min at 20% power and allow samples to cool down at RT for 15-30 min (Do not let samples dry out during the process). Rinse samples in pure water and then TBST. Remove excess liquid and apply 100 µl of Opal Polaris 780 Working Solution onto the slide and incubate at RT for 1 hour. Rinse slides for 3x2 min in TBST at RT.

1.7 DAPI Staining

CNT	Akoya
Remove excess liquid, apply 100 μl of prepared DAPI staining solution to the sample area on the slide, and incubate at RT for 10 minutes. Rinse samples with ddH2O for 3x3 min.	

1.8 Mounting

CNT	Akoya
Apply anti-fade fluorescence mounting medium to the slide, then use nail polish to secure the coverslip.	

Performance Results: Signal-to-Noise Ratio Comparison

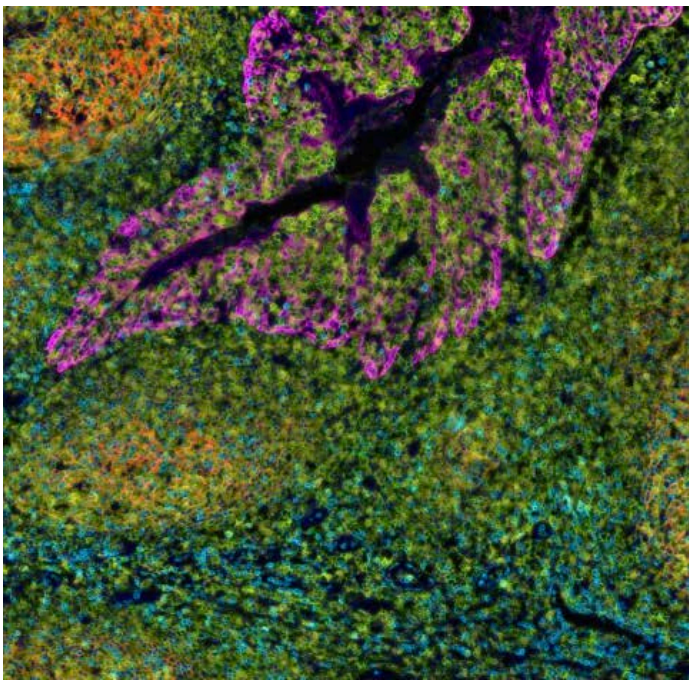
Comparison of Multiplexed Labeling

Vendor	Reagent	Catalog	Comment
Akoya	Opal Polaris - 7 Color Manual IHC Kit	NEL861001KT	/
CNT	7-Plex Multiplexed IHC Kit	FM2007	/

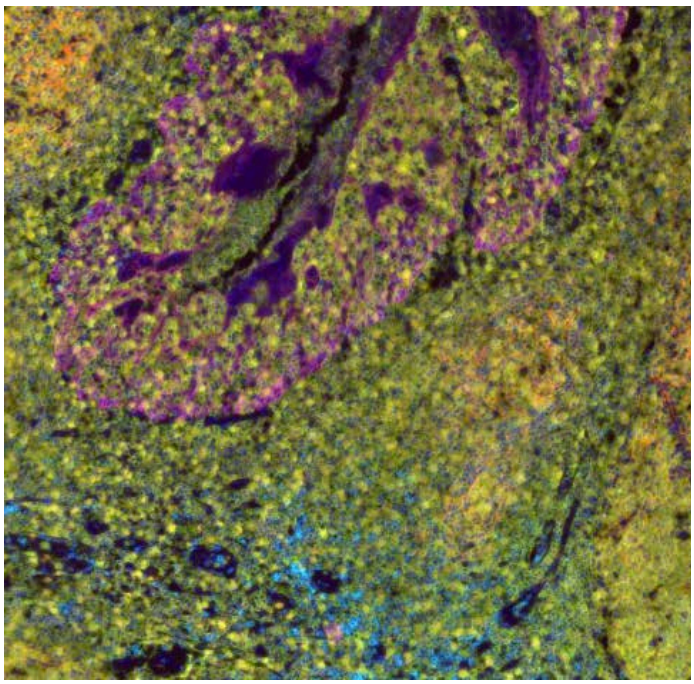
6 Targets 7 Color (Tonsil, 480-CD3, 520-CD20, 5780-CD21, 620-CD23, 690-CK, 780-Ki67)

6 Targets: Overall, CNT yields better definition than Akoya

CNT

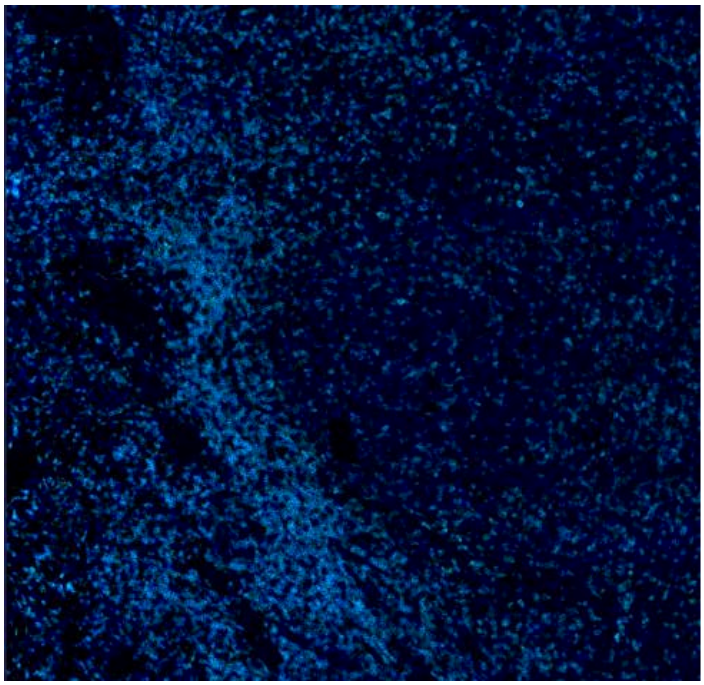


Akoya

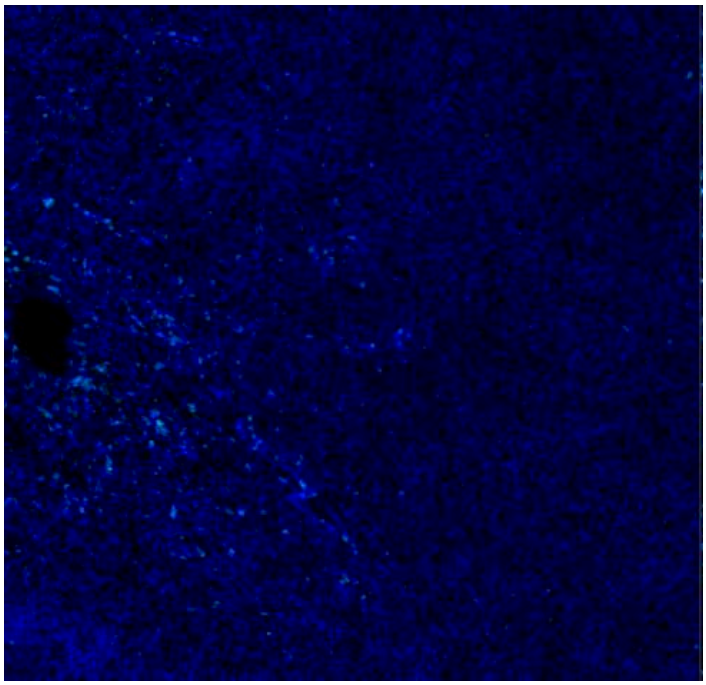


480 Channel: Akoya produces weaker signal and less defined localization. Frequency of positivity is also lower in Akoya results.

CNT

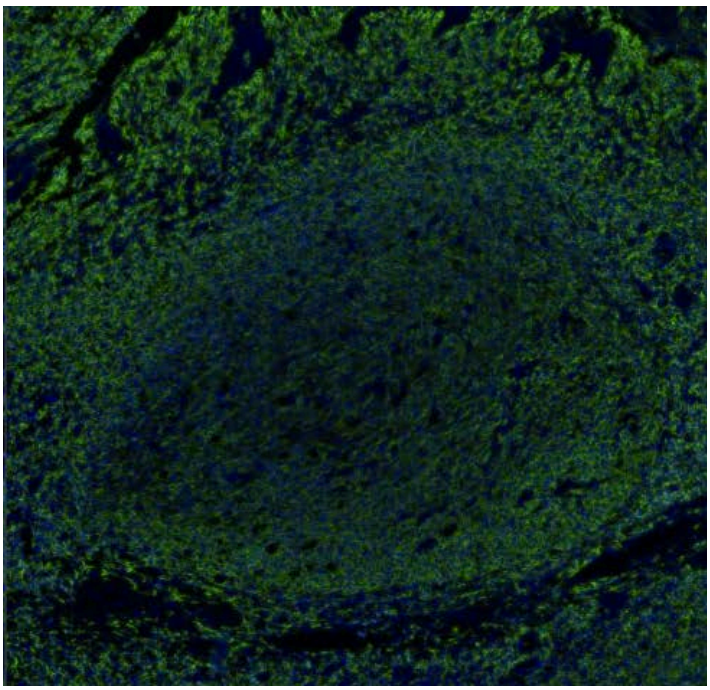


Akoya

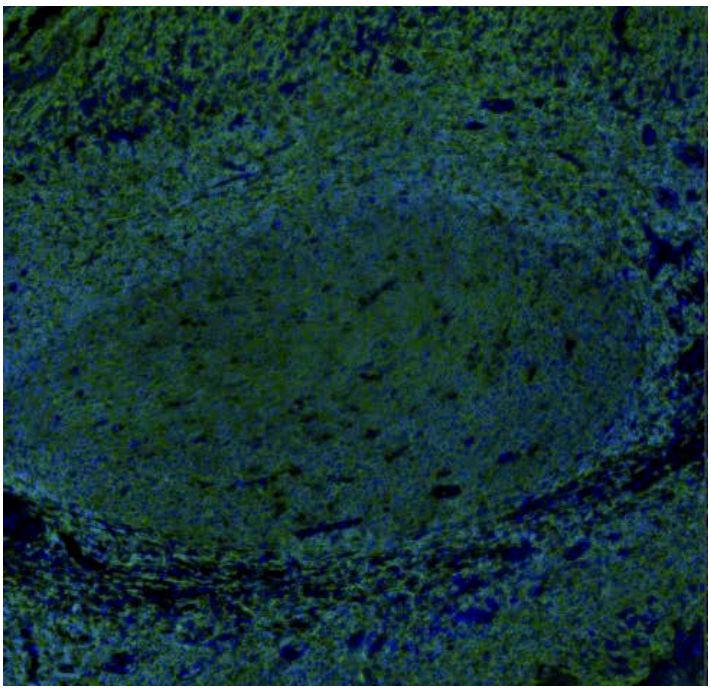


520 Channel: CNT is brighter with better localization

CNT

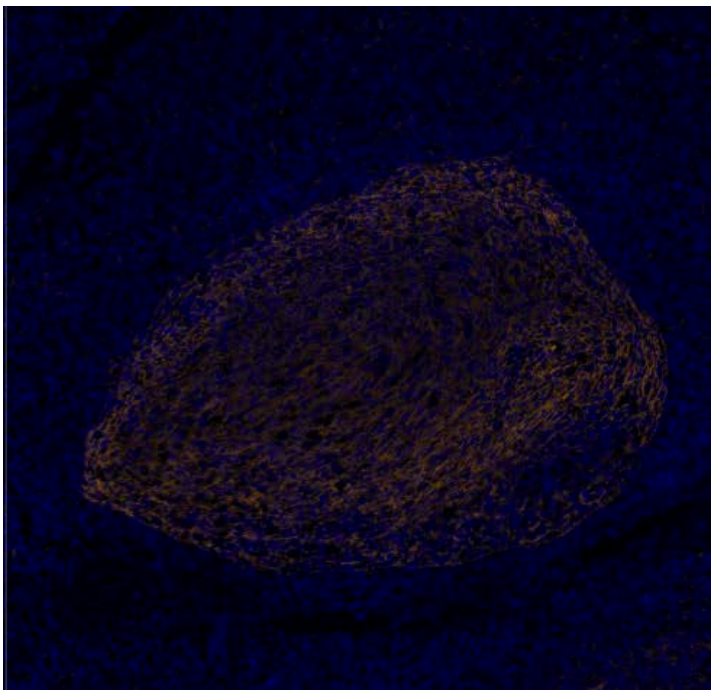


Akoya



570 Channel: CNT is brighter with more defined localization.

CNT



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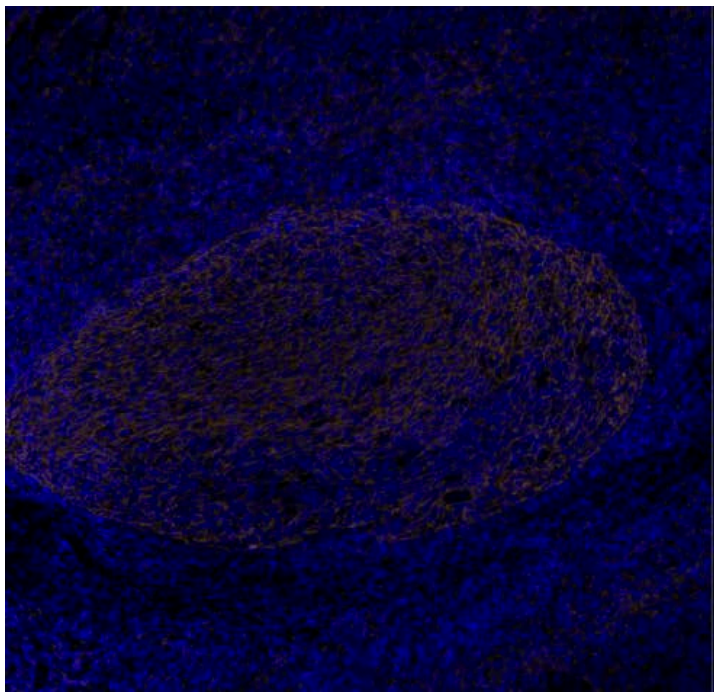
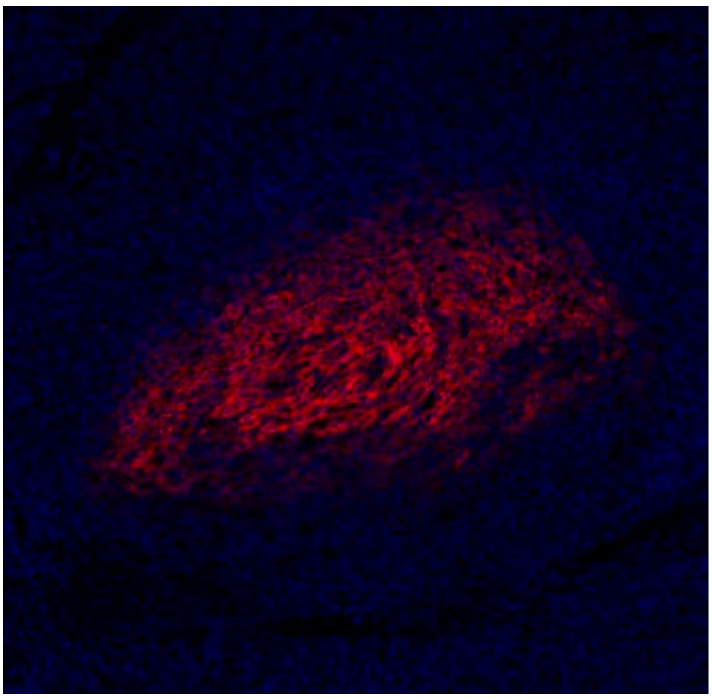


620 Channel: CNT is brighter with better defined localization.

CNT



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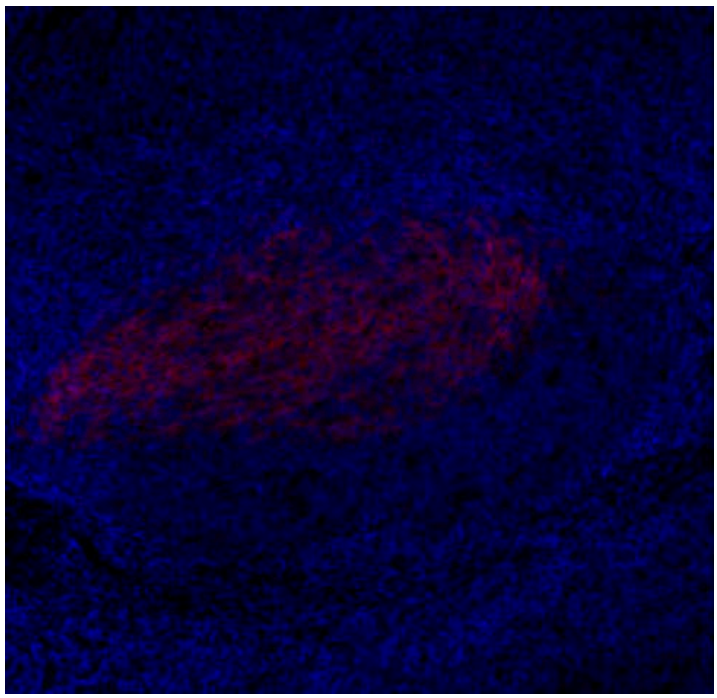
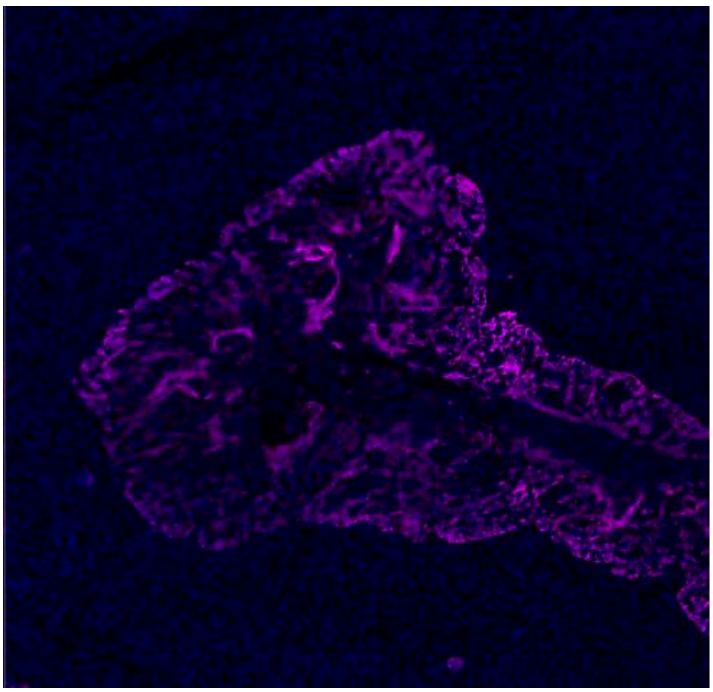


690 Channel: CNT is brighter with better localization

CNT



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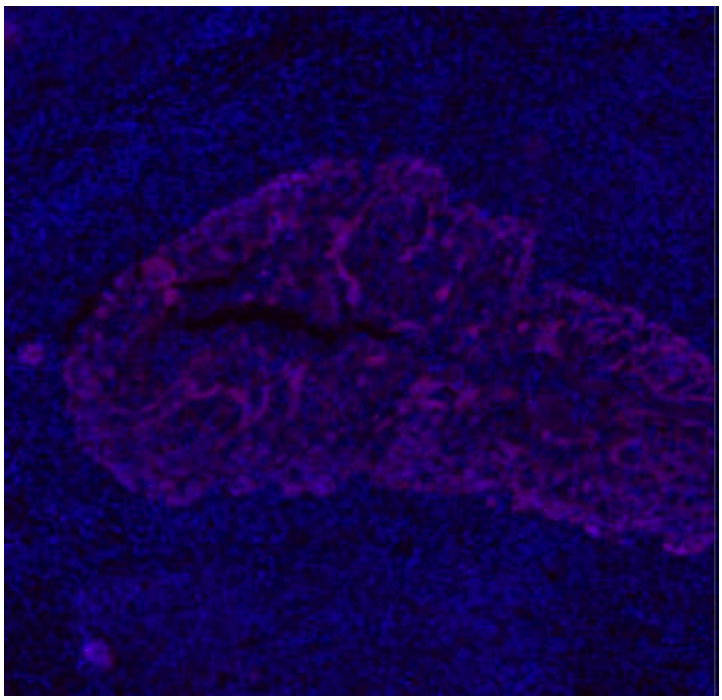
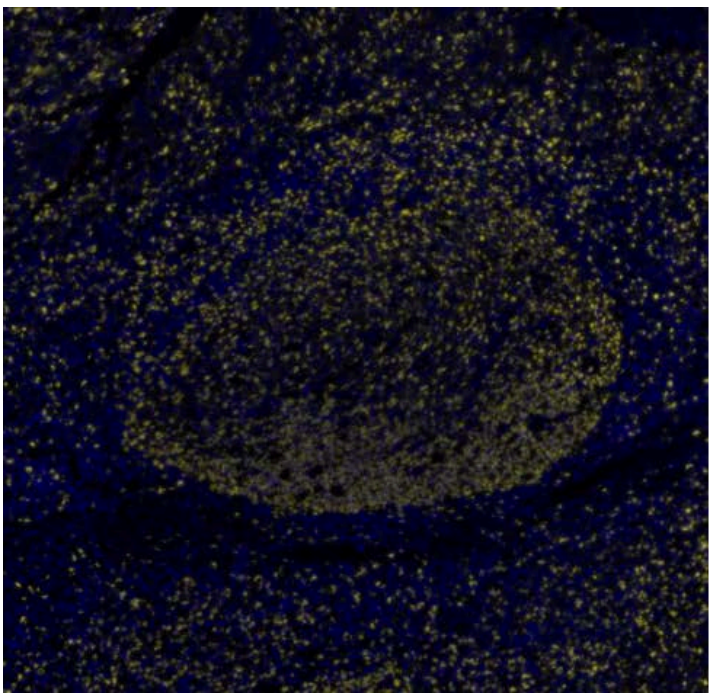


780 Channel: CNT produces weaker signal than Akoya. Both methods yield similar localization quality.

CNT



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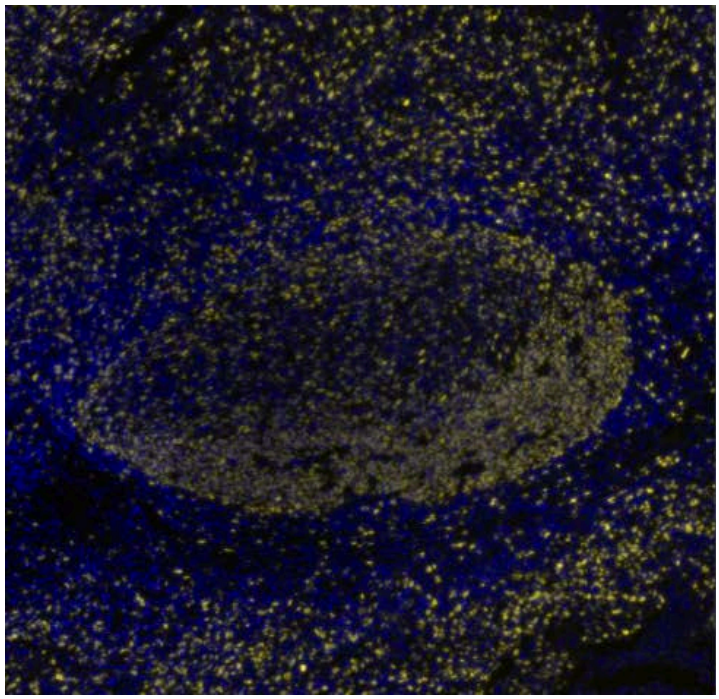
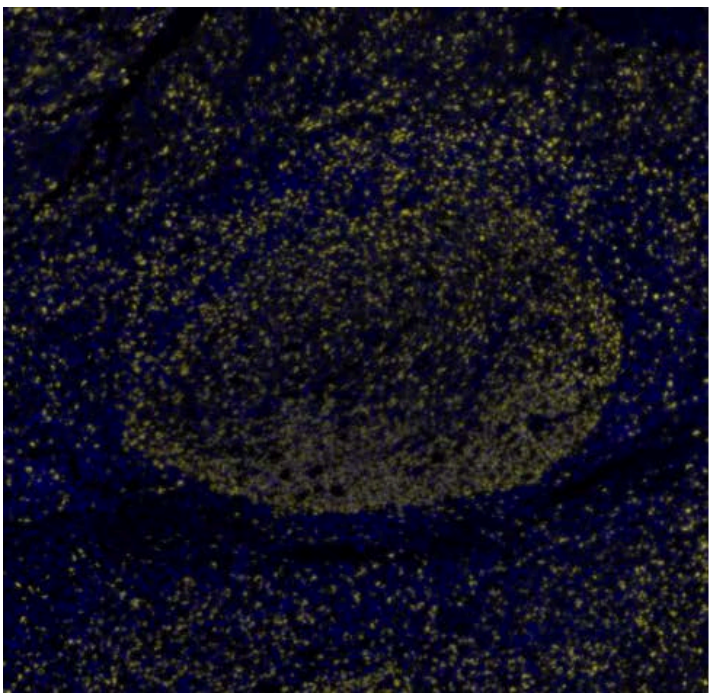


780 Channel: CNT produces weaker signal than Akoya. Both methods yield similar localization quality.

CNT



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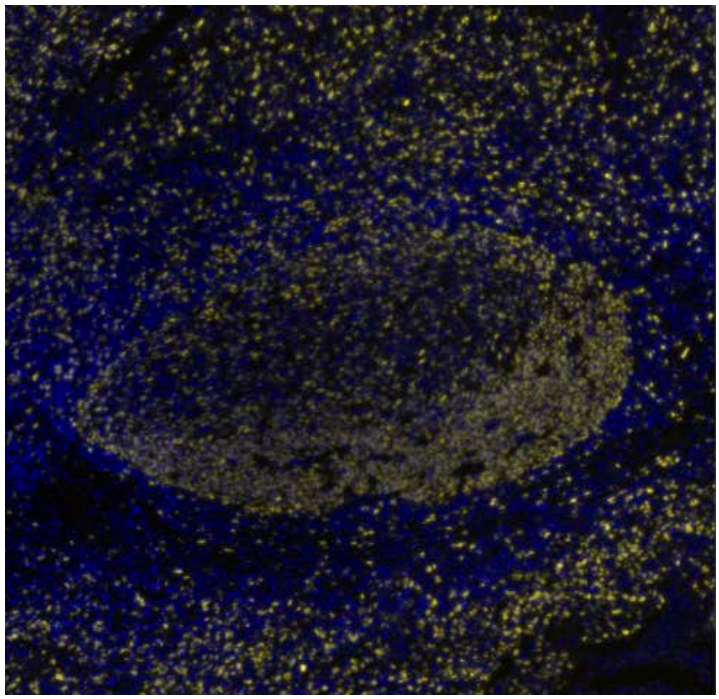
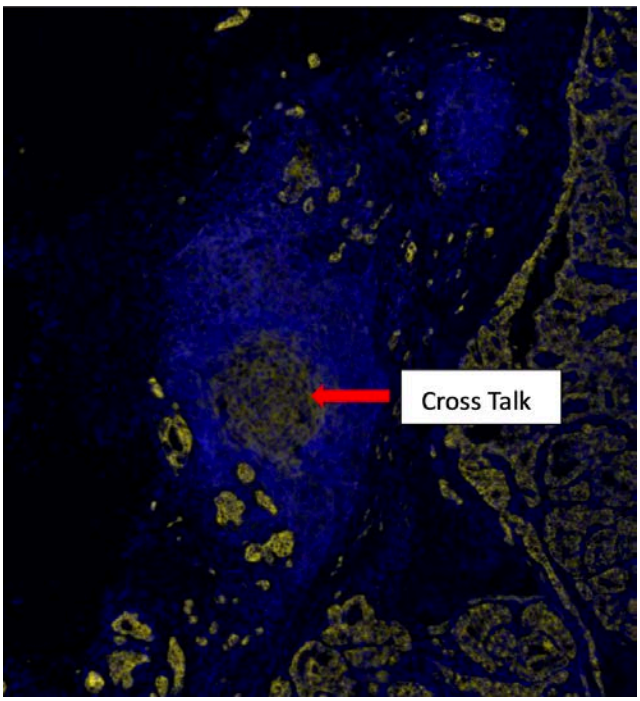


6 Targets 7 Colors (Breast Cancer, 480-CD8, 520-CD20, 570-CD4, 620-CD3, 780-CK)

780 Channel: Akoya and CNT both have cross-talk issue.

CNT



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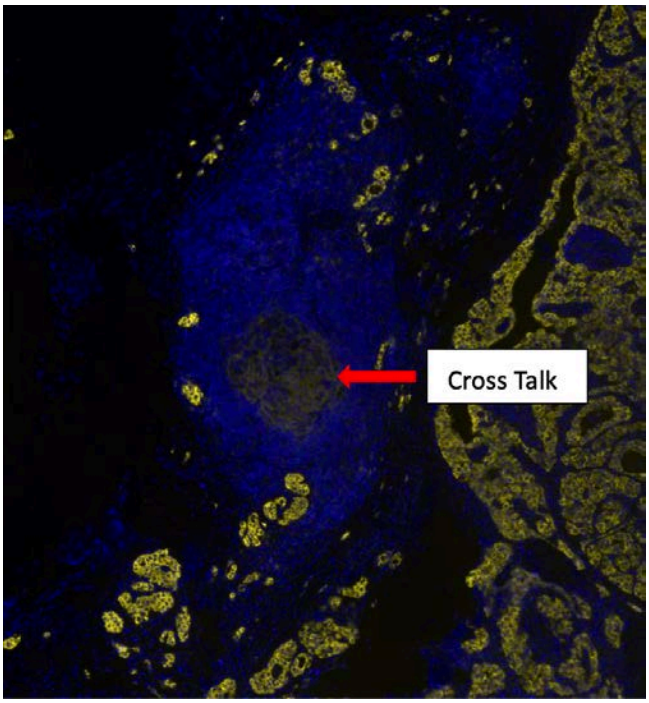
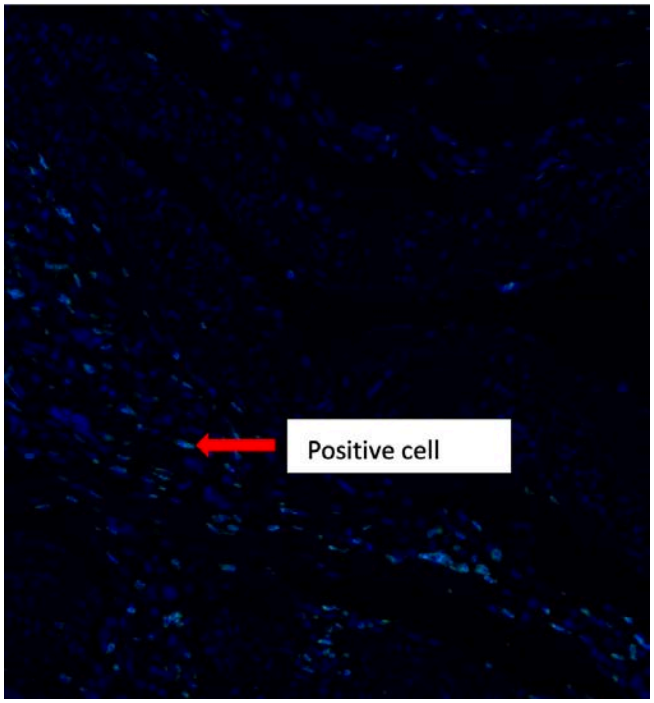


6 Targets 7 Color (Stomach cancer, 480-CD8, 520-CD163, 570-FOXP3, 620-CD3, 780-CK)

480 Channel: Akoya produces non-specific staining in 480 channel and loses some positive signal whereas CNT produces accurate results.

CNT



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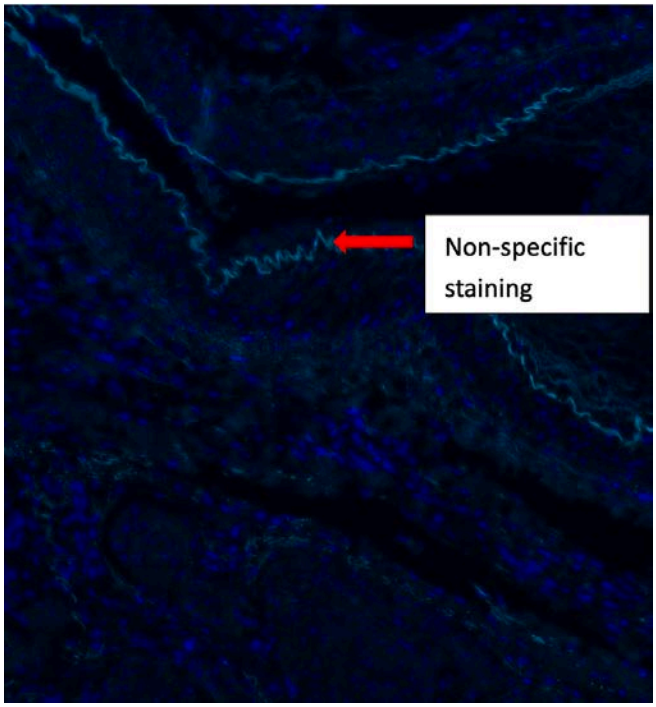
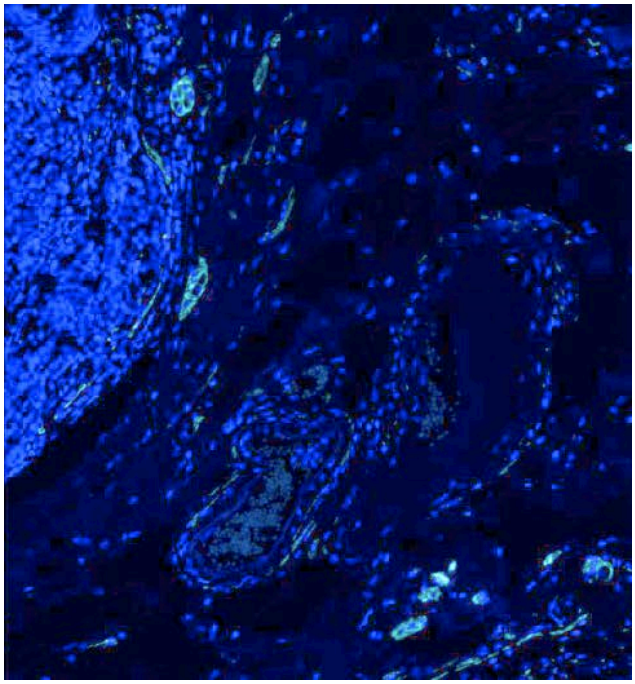


Comparison of Single-color Labeling

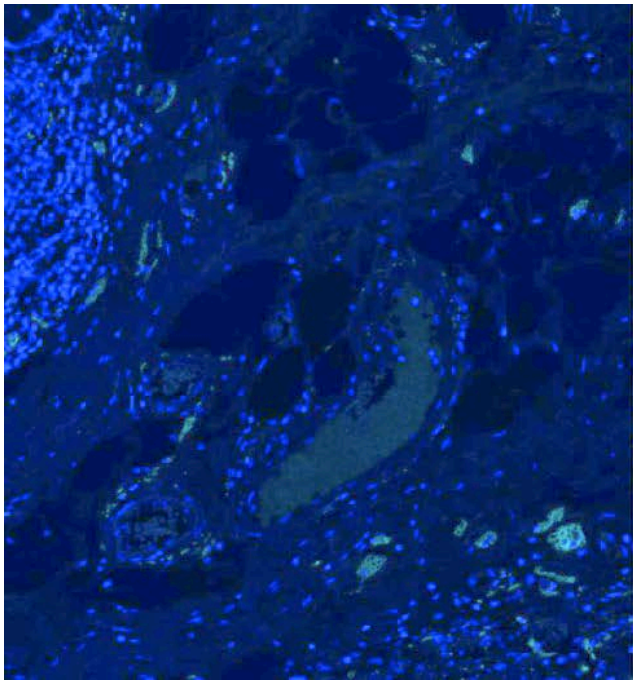
Vendor	Reagent	Catalog	Comment
AKOYA	Opal Polaris - 7 Color Manual IHC Kit	NEL861001KT	Use Individual Channel for Each Target
CNT	TSA Dye – CM480	FT480	/
	TSA Dye – CM520	FT520	/
	TSA Dye – CM570	FT570	/
	TSA Dye – CM620	FT620	/
	TSA Dye – CM690	FT690	/
	TSA Dye – CM780	FT780	/
	DAPI Solution	FTDAPI	/

480 Channel: Appendix Tissue, This Tissue Contains Many Red Blood Cells

CNT



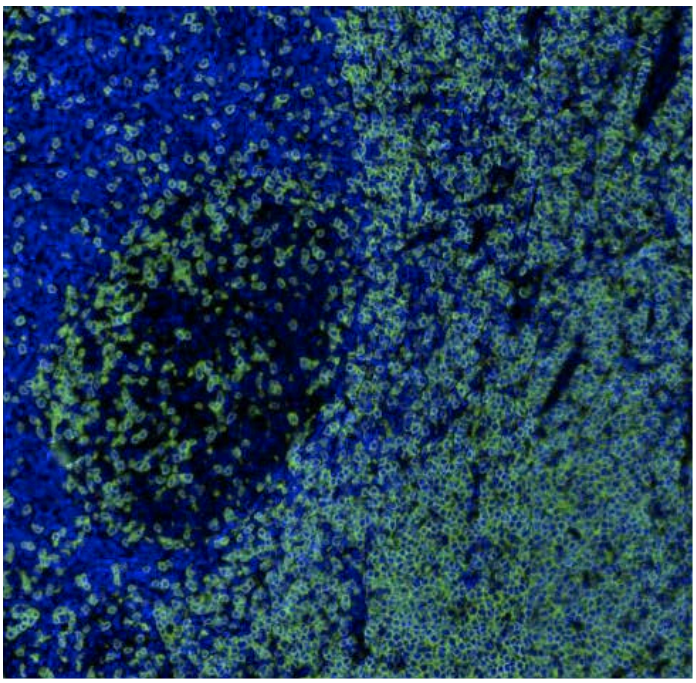
Akoya



Conclusion: Using the same primary antibody and tissue, each with its respective staining kit and identical image acquisition parameters, it can be observed that CNT's 480 channel staining is stronger than AKOYA's. Both show weak nonspecific staining of red blood cells.

520 Channel: Tonsil Tissue, CD3

CNT



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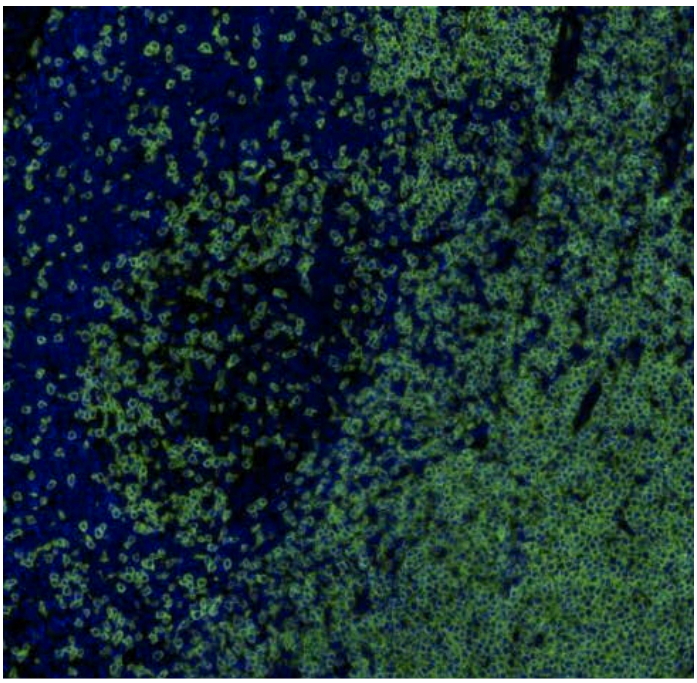
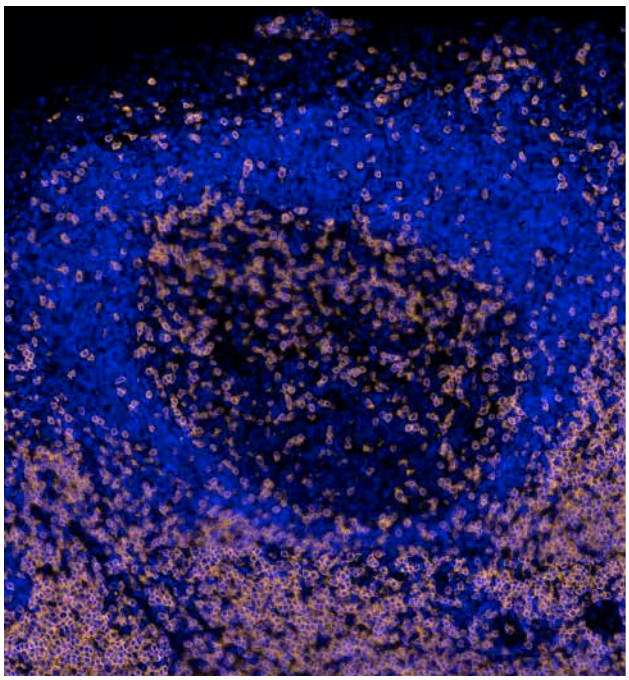


Conclusion: Using the same primary antibody and tissue, each with its respective staining kit and identical image acquisition parameters, it can be observed that CNT's 520 channel staining is relatively stronger, brighter, and better defined cellular localization.

570 Channel: Tonsil Tissue, CD3

CNT



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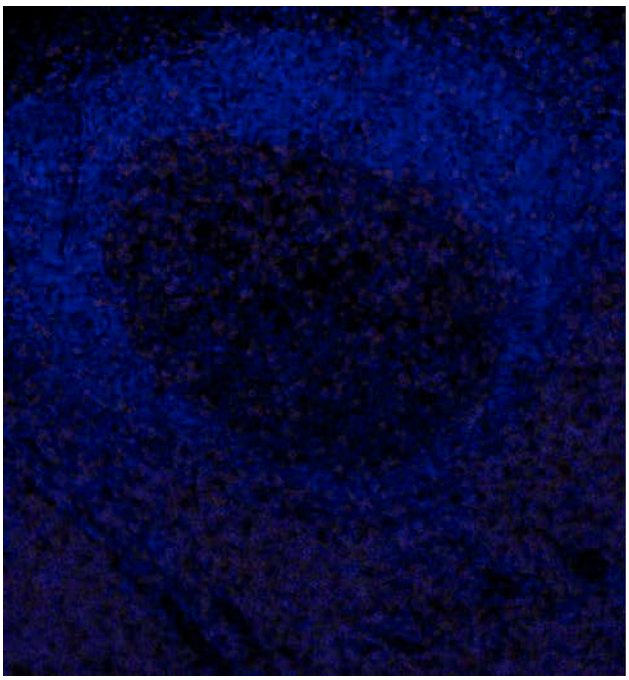
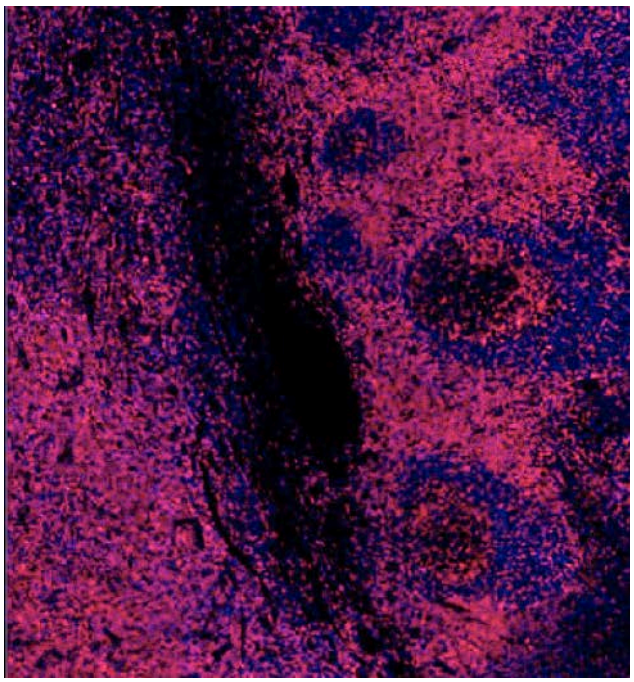


Conclusion: Using the same primary antibody and tissue, each with its respective staining kit and identical image acquisition parameters, it can be observed that CNT's 570 channel staining is very strong and the localization is clear and crispy while AKOYA's staining is much weaker.

620 Channel: Tonsil Tissue, CD3

CNT



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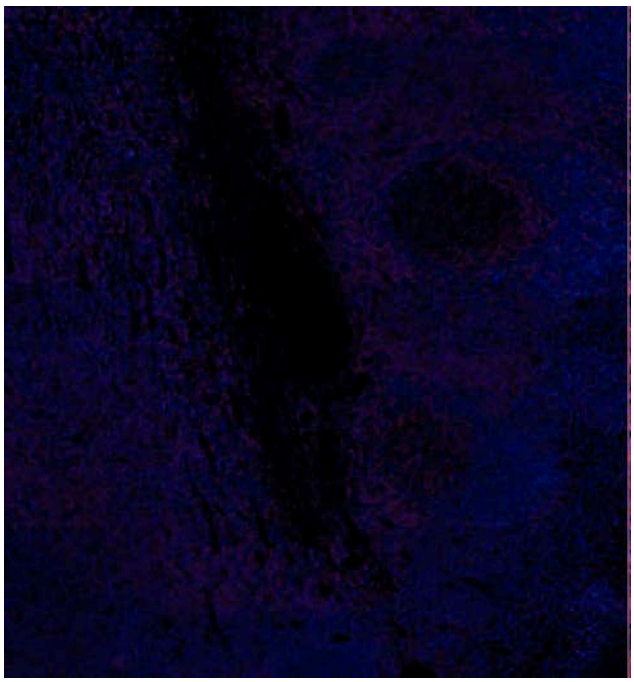
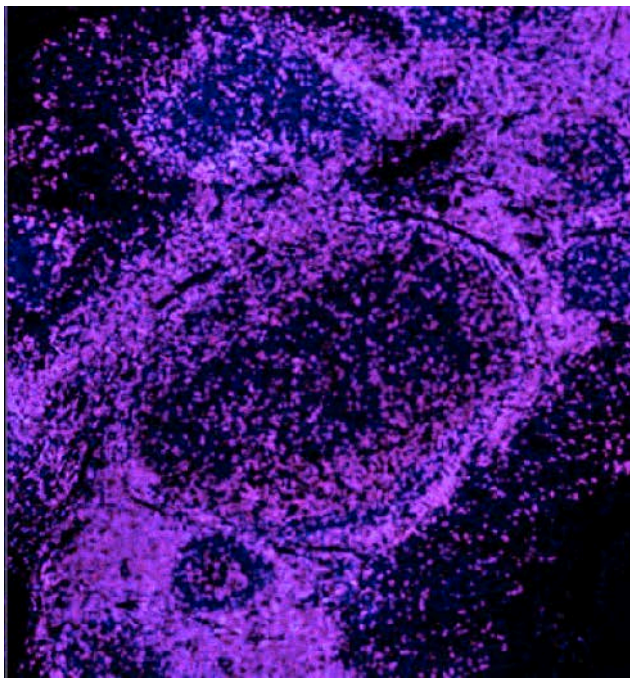


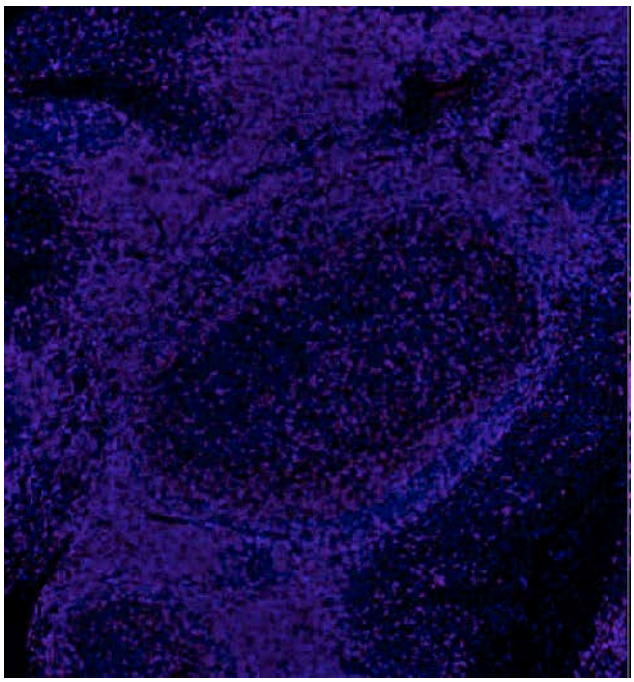
Conclusion: Using the same primary antibody and tissue, each with its respective staining kit and identical image acquisition parameters, it can be observed that CNT's 620 channel staining is very strong and well localized while AKOYA's staining is much weaker.

690 Channel: Tonsil Tissue, CD3

CNT



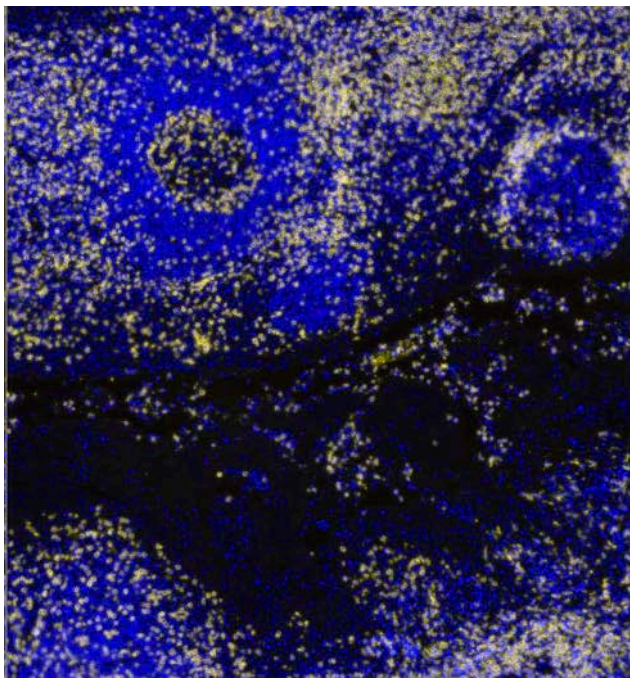
Akoya



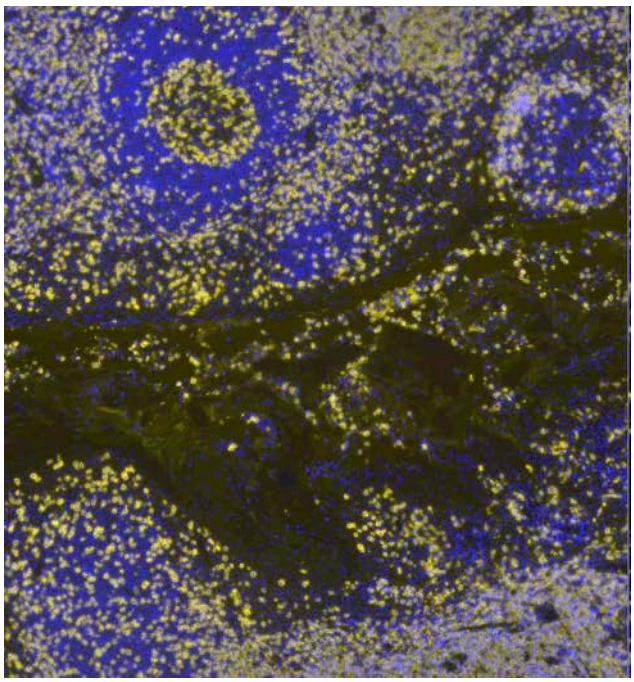
Conclusion: Using the same primary antibody and tissue, each with its respective staining kit and identical image acquisition parameters, it can be observed that CNT's 690 channel staining is very strong and well localized, while AKOYA's staining is much weaker.

780 Channel: Tonsil Tissue, CD3

CNT



Akoya



Conclusion: Using the same primary antibody and tissue, each with its respective staining kit and identical image acquisition parameters, it can be observed that CNT's 780 channel staining is slightly weaker than AKOYA's. However, CNT shows clearer localization without nonspecific staining whereas AKOYA exhibits some degree of nonspecific staining.

Tips for Optimal Results

1. CM dye working solution prepared according to the manufacturer's instructions is good for 3 days at room temperature.

2. When using CM480 for labeling, a microwave antibody stripping step is necessary after the labeling step regardless of whether it is a single color or multiplexed experiment, which ensures fully removal of non-specific interactions. DAPI stain and mounting steps can be carried out as usual after the antibody stripping step.

3. When doing 6 targets/7 colors labeling, CM780 should be the last target for optimal results.

4. Currently, it is not recommended to perform multiplexed labeling experiments on frozen tissue or cell culture-based samples because these samples have a higher tendency of detaching from the cover glass.

5. Before starting experiments, review configurations of your imaging system to make sure that it is compatible with the excitation and emission spectrums of the dyes involved.

6. To pair dye with antibody, it is generally a good practice to use brighter dye (e.g. CM480 and CM520) for sparse targets and dimmer dyes (e.g. CM620 and CM780) for those with higher levels of expression.

7. Try to label weak or sparse targets first as they will behave more similarly to single color experiments.

8. To achieve better antigen unmasking, alkaline based antigen retrieval method is preferred. Use acidic conditions for antigen retrieval only when all the primary antibodies require acidic antigen retrieval.

9. Cross-talk could be related to many factors:

   a. Choose filters with narrower range to filter out crossed emission.
   b. Cross-talk signal could be from previous rounds of labeling, this could happen when previous antibodies are not completely removed. This phenomenon is more likely to happen when the antibody has high affinity for its target, such as antibodies against cytokeratin. It could be helpful to extend antibody stripping time or increase antibody stripping temperature.
   c. When neighboring channels have a significant imbalance of signal strength, signal from the brighter channel could sometimes spill over to the weaker channel.
   d. Misuse of antibody or missed antibody stripping steps.

10. Factors that lead to non-specific labeling:

   a. Polycolonal antibody tends to give non-specific signal. It is recommended to use monoclonal antibody.
   b. Over amplifying could eventually produce non-specific signal. Try to shorten the incubation time or reduce dye concentration for the signal amplification step.
   c. Primary antibody concentration is too high. Try to lower primary antibody concentration.

Multiplex Assay Panel Table

See example below for your multiplex assay design

Project Name:				Date:			
Tissue(s):				Researcher:			
Order	Antibody	Supplier	Clone/Lot	Catalog#	Dilution Factor	CNT Pairing	AR
1	CD4	CNT	C9E15	/	Ready-to-Use	CM570	ER2 Alkaline
2	CKpan	CNT	AE1/AE3	/	Ready-to-Use	CM690	ER1 Acidic
3	CD163	CNT	C2F5	/	Ready-to-Use	CM620	ER1 Acidic
4	CD3	CNT	C3E7	/	Ready-to-Use	CM520	ER1 Acidic
5	CD8	CNT	C2B11	/	Ready-to-Use	CM480	ER1 Acidic
6	Ki-67	CNT	C3G4	/	Ready-to-Use	CM780	ER1 Acidic
7							
8							

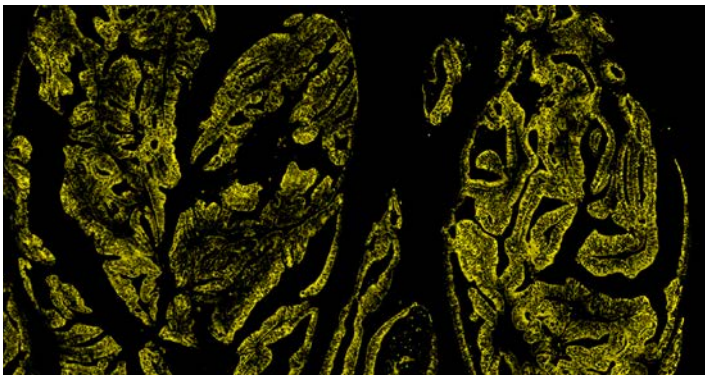
Project Name:				Date:			
Tissue(s):				Researcher:			
Order	Antibody	Supplier	Clone/Lot	Catalog#	Dilution Factor	CNT Pairing	AR
1							
2							
3							
4							
5							
6							
7							
8							

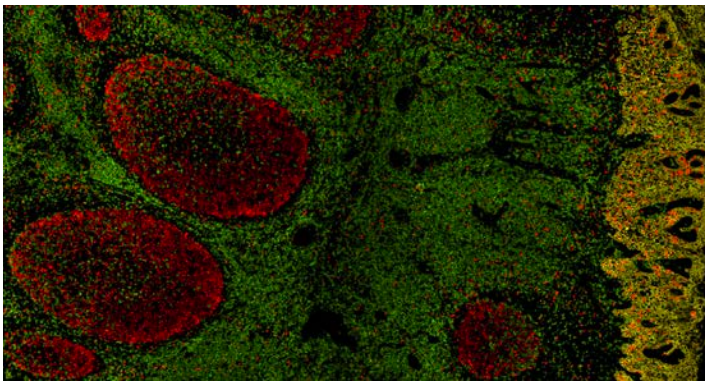
Conclusion

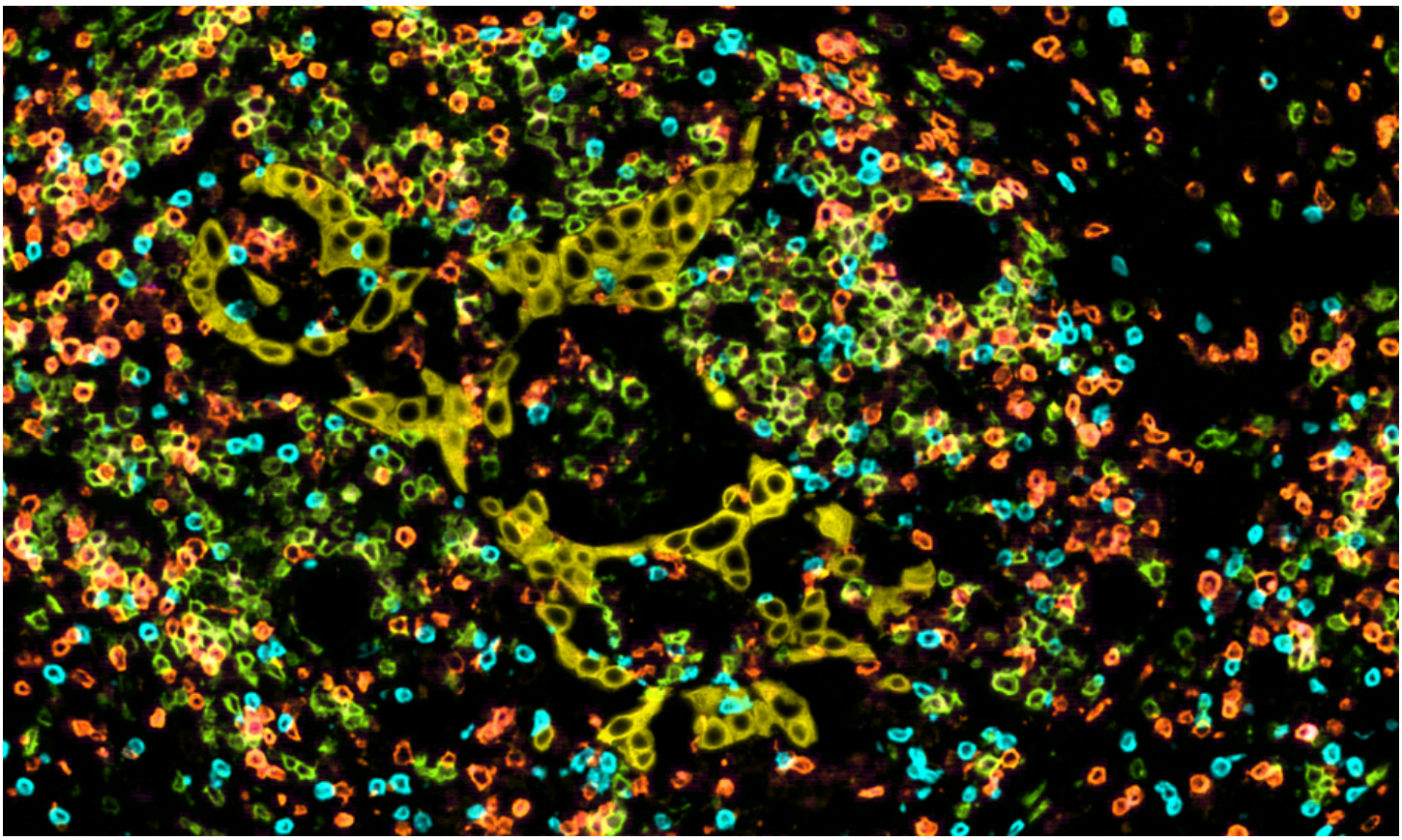
The CNT multiplex immunofluorescence (mIHC) kit offers an adaptable and high-performance solution for researchers looking to achieve clear, multi-channel staining results. Through careful protocol design, we have ensured minimal differences between our process and established systems like Akoya Opal, making it easy for users to adopt our product with minimal workflow disruption.

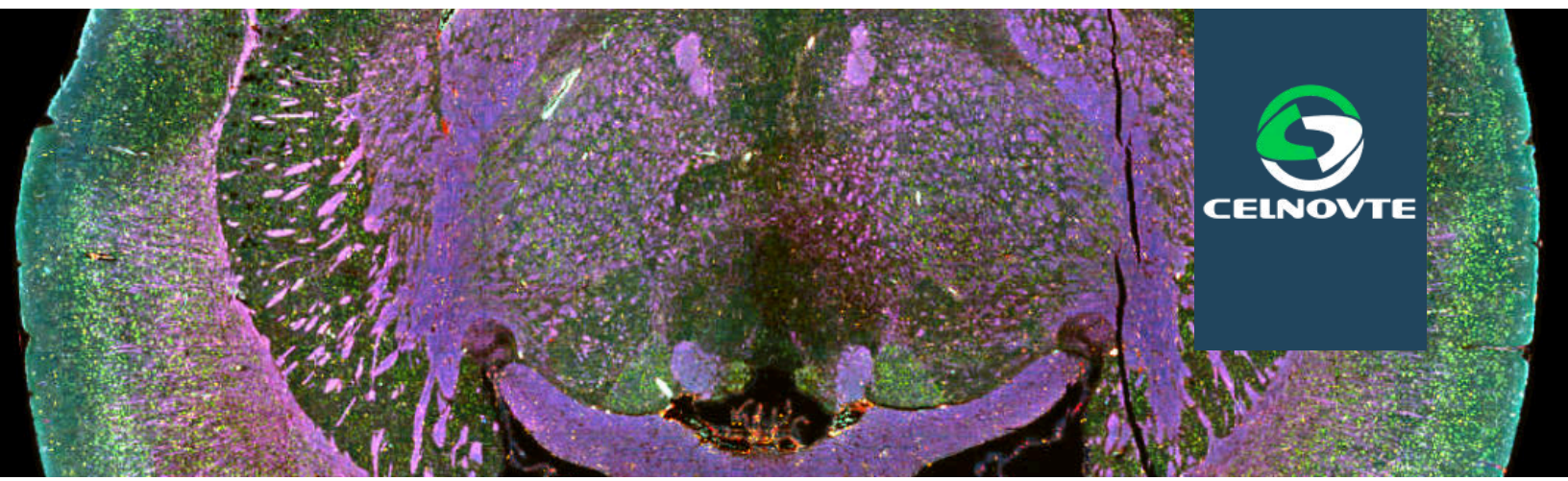
The comparative analysis shows that CNT's mIHC kit consistently delivers stronger signal intensities, better signal-to-noise ratios, and clear biomarker localization, particularly in key channels such as 480 and 690. This performance, combined with the reliability of our reagents, ensures reproducible results and long-term fluorescence stability.

By following this guide, users can seamlessly integrate CNT's mIHC solutions into their existing immunostaining workflows, whether using automated platforms like the Leica Bond RX or manual staining techniques. This tech note highlights the minimal protocol adjustments required and demonstrates how CNT's superior performance enhances multiplexed imaging results. Researchers can now confidently expand their staining capabilities, gaining deeper insights into complex biological systems with greater precision.





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