

Fast and automated TSA-based multiplexed immunofluorescence on LabSat® Research

Introduction to Tyramide Signal Amplification (TSA)

Unlike some of the existing multiplex approaches such as next-generation sequencing, mPCR, mass spectrometry, etc. multiplex immunofluorescence -also referred to as fluorescent multiplex immunohistochemistry- provides an edge to understand the co-expression and spatial distribution of multiple targets without compromising the tissue integrity.

Tyramide Signal Amplification (TSA) is an immunostaining method designed for its high sensitivity, capable of detecting low expressed epitopes and generate precise and clear results. Tyramide is coupled to a fluorophore to generate a signal amplification. Via a reaction mediated by HRP (horseradish peroxidase) coupled with the secondary antibody (AbII), tyramide turns into its active conformation and covalently binds to tyrosine residues proximal to the target epitope¹.

To detect the next antigen, an elution step is required in order to detach the primary and secondary antibody (AbI and AbII) complex from the epitope. The already coupled tyramide remains attached to the epitope proximal region due to the covalent bonding.

Reiteration of the same process with another AbI targeting another epitope (Fig. 1) will produce a fluorescent multistaining of the different markers. Another benefit of the TSA technique in a multiplex immunostaining context is the absence of antibody cross-reactivity as protocols stain one marker at a time.

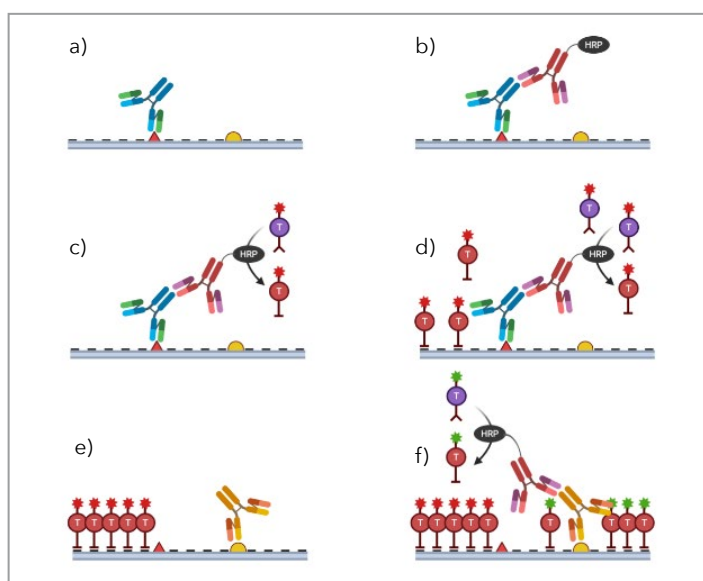


FIGURE 1 - Working principle of the TSA-based multiplexing immunofluorescence. a) Primary antibody binds to antigen of first marker; b) HRP-conjugated secondary antibody binds to primary; c) HRP catalyzes activation of fluorophore-conjugated tyramide at the site of the antigen; d) reactive tyramide fluorophore covalently binds to tyrosine residues; e) antibodies are stripped off leaving tyramide residues and a new marker detection begins; f) after following the same protocol, two different tyramide fluorophores can be detected at the sites of two markers.

¹ Lim et al., 2018; Wang et al., 1999

Take control of your research

LabSat® Research is an automated tissue immunostainer device, allowing high-quality fast multiplexing up to 6 markers + DAPI within a few hours. Lunaphore's core technology, the Fast Fluidic Exchange (FFeX), utilizes a microfluidic "Staining Chip" that delivers reagents sequentially onto a tissue sample. The staining chamber is filled almost instantaneously, preventing different areas of the tissue to be incubated unevenly hence providing a great degree of signal uniformity in an ultra-controlled environment allowing more robust and reliable results. With this purpose, multiplexing protocols benefit from a special non-static incubation, where reagents flow dynamically inside the staining chamber (Fig. 2). The system is pressurized and provides temperature control in order to finetune and optimize staining conditions. This is key for image processing purposes with digital pathology tools such as signal quantification.

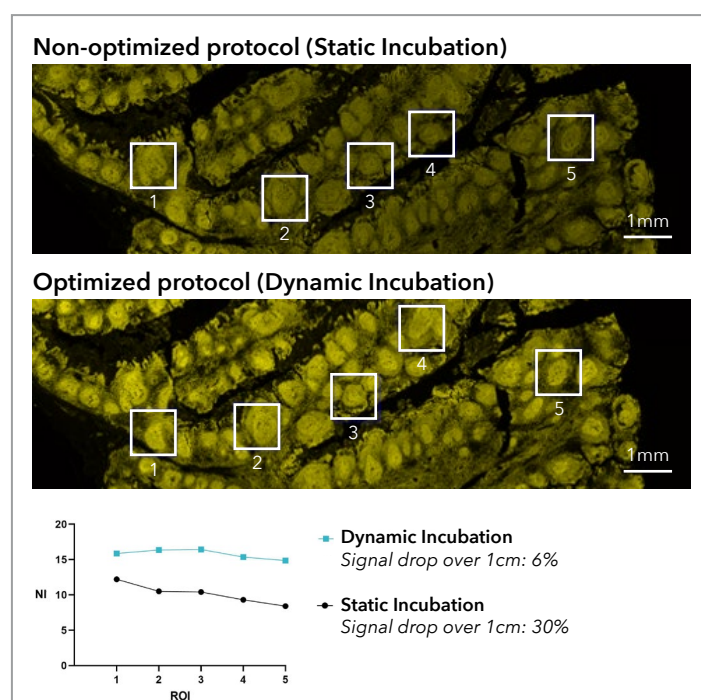


FIGURE 2 - Normalized signal intensity with respect to regions of interest. Comparison between non-optimized and optimized protocols on LabSat® Research. Uniformity is improved thanks to the Dynamic Incubation. FFPE IF, CD20 (Opal® 540), tonsil.

LabSat® Research is an open and flexible system, where different reagents can be loaded and staining parameters modified accordingly. Here two use cases are presented for LabSat® Research with TSA kits:

1. Thermo Fisher TSA-SuperBoost™ Kit
2. Akoya Opal® 7-color IHC Kit

It is important to note that the system being adaptable can be used with other detection kits.

USE CASE: 3-plex with Thermo Fisher TSA-SuperBoost™

For multiplexing users analyzing a low number of markers, it is not required to use a spectral microscope capable of deconvolution. In this first scenario, a slide scanner (3DHISTECH Panoramic Midi II) with four available channels was used. As described in (Fig. 3), FFPE sample preparation and pre-processing (baking, dewaxing) was not performed on LabSat® Research. The Thermo Fisher TSA-SuperBoost™ kit was used and the fully automated 3-plex protocol was run on LabSat® Research in 2 hours and 20 minutes on a tonsil tissue sample (Fig. 4). For comparison, a manual single plex and counterstaining protocol would take around 4 hours and 40 minutes, and would not allow the study of biomarker spatial relationships.

High-quality visualization of proteins enabling the identification of different cell populations in combination with the spatial information showed macrophage presence (CD68) into the germinal center.

USE CASE: 6-plex with Akoya Opal® 7-color IHC Kit

For the second use case, we used the Akoya Opal® 7-color IHC kit on an FFPE tonsil sample. Images were acquired with Mantra™, a spectral microscope with deconvolution capability of overlapping emission/excitation spectra, thus allowing to analyze up to 6 markers and provide data stratification to support research.

A 6-plex and DAPI staining was performed on LabSat® Research (Fig. 5) in a total staining time of 4 hours and 14 minutes, including one “reagent reservoir swap” step consisting of a two-minute hands-on procedure (Fig. 6). The results obtained with LabSat® Research displayed several qualitative and quantitative features. Direct visualization of immunomodulator cell types like T-reg (FoxP3), PD-L1+ and PD1+ cells as well as immune cells like macrophages (CD68) and cytotoxic T-cells (CD8) holds potential for a deeper understanding of cell interactions and complex phenotypes.

Obtain high quality stainings

Following a single-plex optimization, a bright and specific signal, as well as a high signal-to-background ratio, can be achieved with LabSat® Research for each marker (Fig. 7).

Workflow: 3-plex with Thermo Fisher TSA-SuperBoost™



FIGURE 3 - Workflow: 3-plex with Thermo Fisher TSA-SuperBoost™ Kit. Indicated in the blue box the steps automated by LabSat® Research.

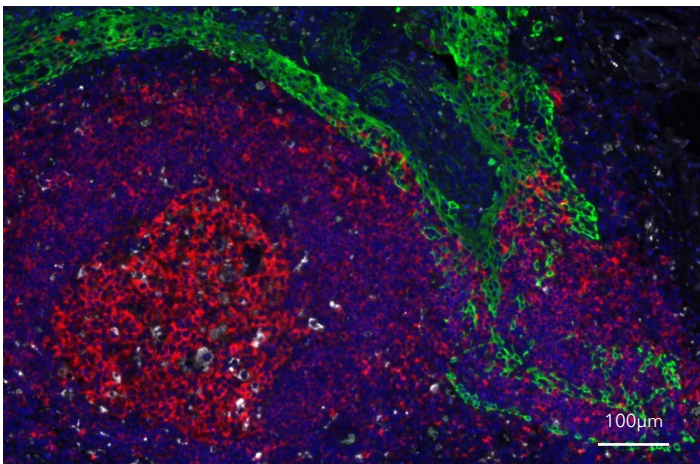


FIGURE 4 - FFPE, tonsil: CD20 - red (AF555), panCK - green (AF647), CD68 (AF488) - white and DAPI - blue. No optimization was required.

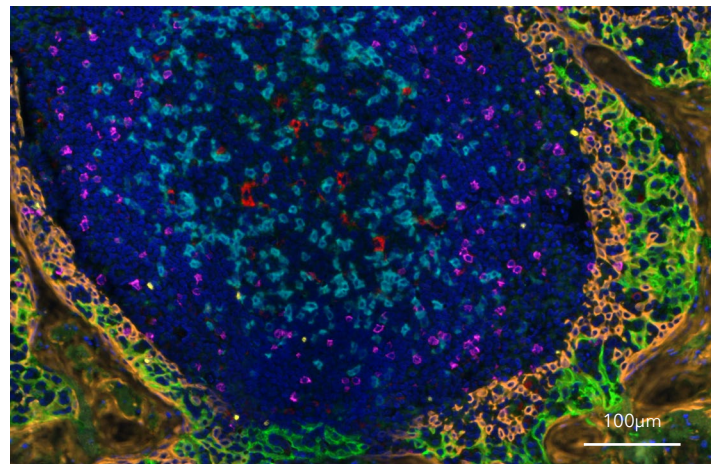
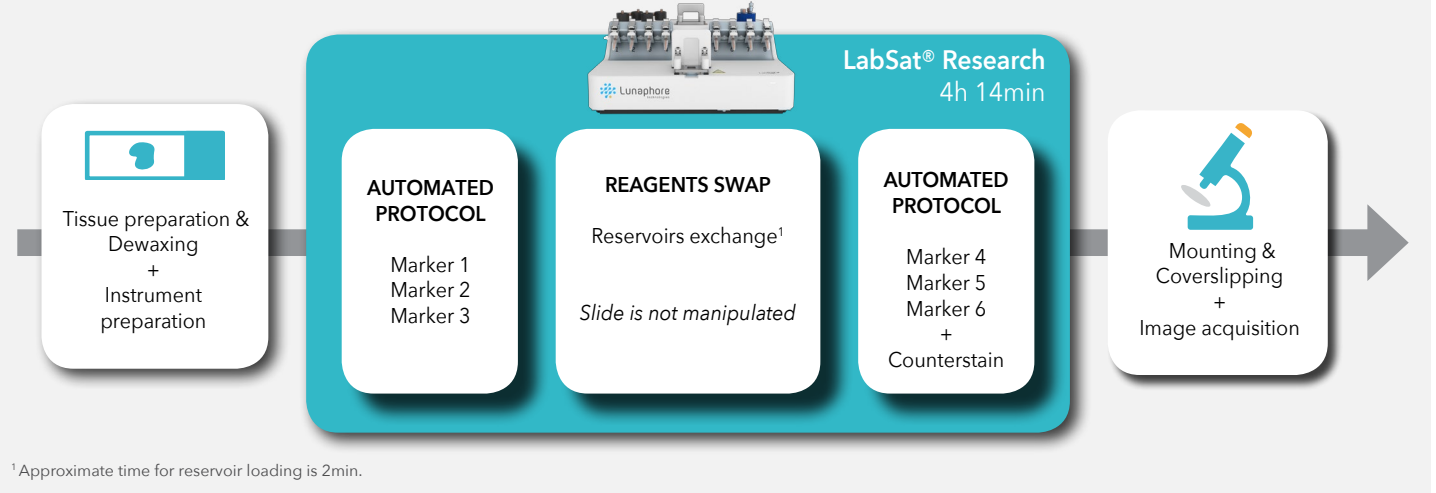


FIGURE 5 - FFPE, tonsil: FoxP3 - yellow (Opal® TSA 570), PD-L1 - green (Opal® TSA 520), PD1 - cyan (Opal® TSA 690), CD68 - red (Opal® TSA 620), CD8 - purple (Opal® TSA 540), CK - orange (Opal® TSA 650), DAPI - blue.

Workflow: 6-plex with Akoya Opal® 7-color IHC Kit



¹Approximate time for reservoir loading is 2min.

FIGURE 6 - Workflow: 6-plex with Akoya Opal® 7-color IHC Kit. Indicated in the blue box the steps automated by LabSat® Research.

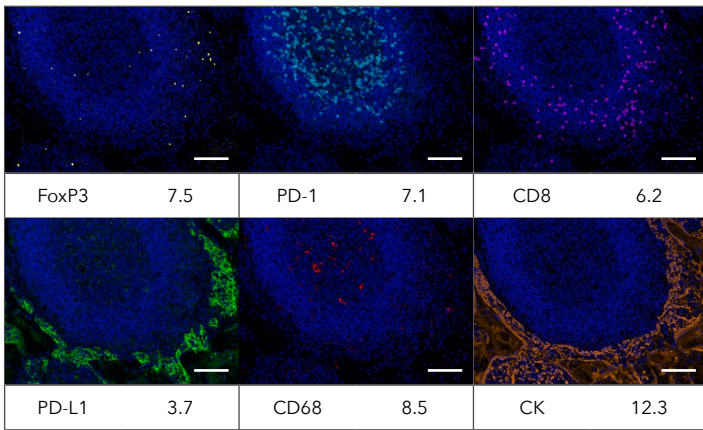


FIGURE 7 - Quantification of the six different markers. Mean Normalized Intensity computed with Inform® on three ROI per slide. FFPE, tonsil. Scale bar: 100µm.

A robust technology

LabSat® Research verification tests were performed through several rounds of experiments to ensure rigorous assay reproducibility and repeatability. The robust system along with automated protocols offers a high degree of consistency in results (less than 12% of signal variability) (Fig. 8).

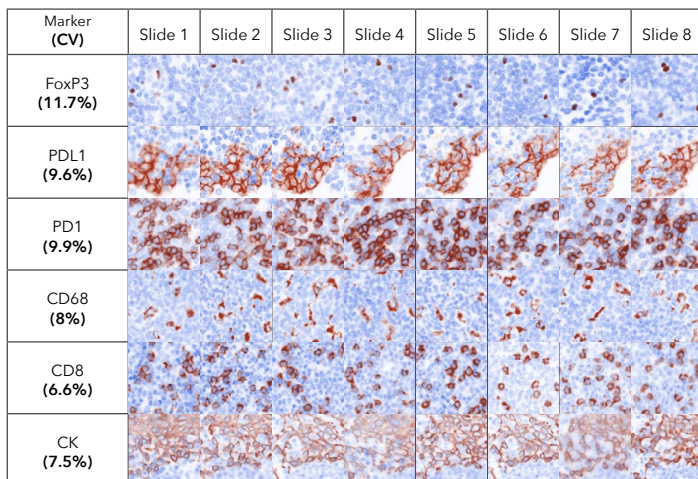


FIGURE 8 - Reproducibility study: coefficient of variation of signal among 8 sequential tonsil slides. Software-reconstructed brightfield view (Pathology view, Inform®, AKOYA). FFPE, tonsil.

Preserve your tissue with an efficient antibody strip-off system

The elution step on LabSat® Research consists of an active heating-cooling cycle after each marker detection, which successfully strips off antibodies. The elution efficiency was over 99% for all six markers of the panel (Fig. 9).

	FOXP3	PD-L1	PD-1	CD68	CD8	CK
Before elution						
After elution						
Efficiency	100%	100%	99%	99%	100%	100%

FIGURE 9 - Elution efficiency: over 99% for all 6 markers of the panel. Software-reconstructed brightfield view (Pathology view, Inform®, AKOYA). FFPE, tonsil.

The elution time is optimized to ensure ultra-rapid incubation times, while limiting the exposure of tissue to harsh conditions. The results show no apparent degradation of epitope stability or tissue morphology (including nuclear structures) (Fig. 10).

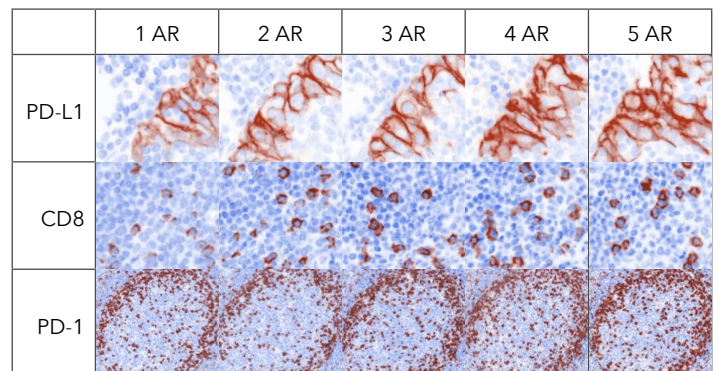


FIGURE 10 - Five elution cycles show no apparent degradation of tissue morphology. Software-reconstructed brightfield view (Pathology view, Inform®, AKOYA).

Speed up your research with ultra-rapid turnaround times

Thanks to the unique microfluidic technology, FFEx, an active

flow of reagents produces a fast exchange at the tissue surface, reducing the required incubation times dramatically. As shown in Fig. 11, the experiment time requirements for the LabSat® Research protocol and the manual procedure were significantly different. Furthermore, by increasing the number of markers, the time divergence between the two processes is also increased due to a higher time-increase-coefficient of the manual procedure over LabSat® Research (Fig. 12). Typically, this time gain will greatly impact optimization phases, where it will be possible to test one condition on one marker protocol every 30 min.

The LabSat® Research staining chamber is closed and pressurized, thus preventing evaporation and allowing better temperature control even above 100°C.

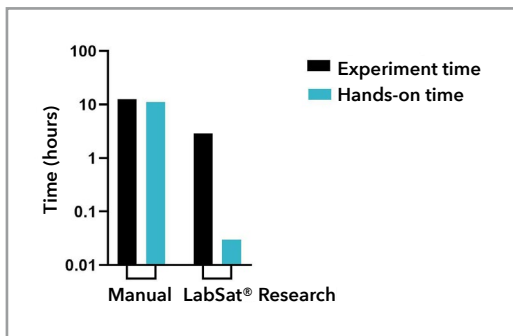


FIGURE 11 - Comparison of time requirements between manual stainings and automated stainings performed on LabSat® Research. FFPE, 4-plex + DAPI.

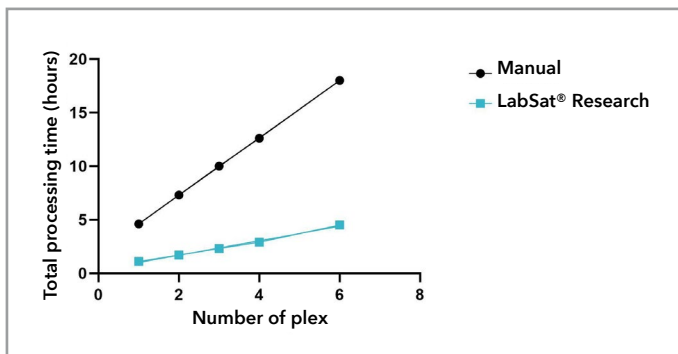


FIGURE 12 - Difference in time between manual protocols and automated protocols performed with LabSat® Research.

Simple protocol transfer onto a new tissue type

LabSat® Research offers the possibility to optimize and transfer protocols to different tissue types, following only a few steps. The procedure starts by applying the optimized protocol onto a sample of the new tissue type. The performance is evaluated in order to define which markers need re-optimization. Finally, the protocol parameters such as antigen retrieval temperature, incubation times, reagent titration, etc. are modified to reach the desired performance (Fig. 13).

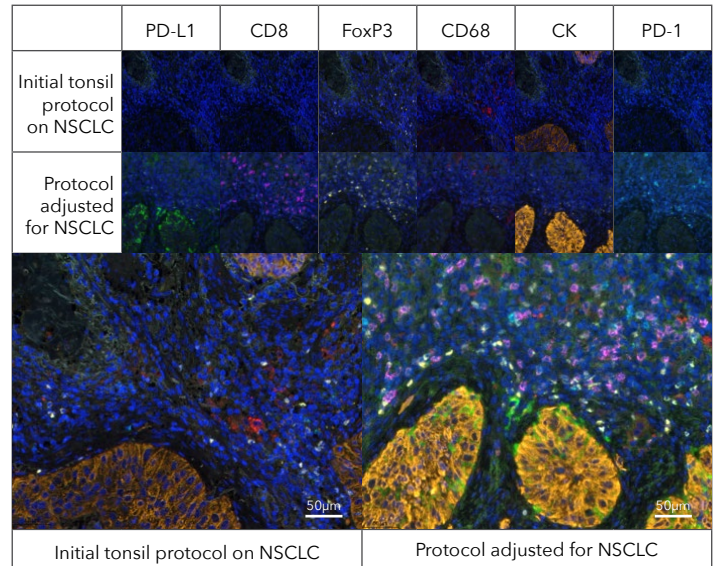


FIGURE 13 - 6-plex with Akoya Opal® 7-color IHC Kit protocol transfer to NSCLC resulted in appropriate detection of all markers. Protocol adjustments: Antigen retrieval temperature increase, Opal® 540 (CD8) concentration increase. FFPE, NSCLC: FoxP3 - yellow (Opal® TSA 570), PD-L1 - green (Opal® TSA 520), PD1 - cyan (Opal® TSA 690), CD68 - red (Opal® TSA 620), CD8 - purple (Opal® TSA 540), CK - orange (Opal® TSA 650), DAPI - blue.

Conclusion

TSA-based multiplexing is a precise tool in immunostaining to unravel the complexity of the tumor microenvironment.

The open system of LabSat® Research allows TSA multiplexing with various kits. By taking control of their experiments, scientists can simultaneously study several cell types with high quality and in an ultra-fast manner.

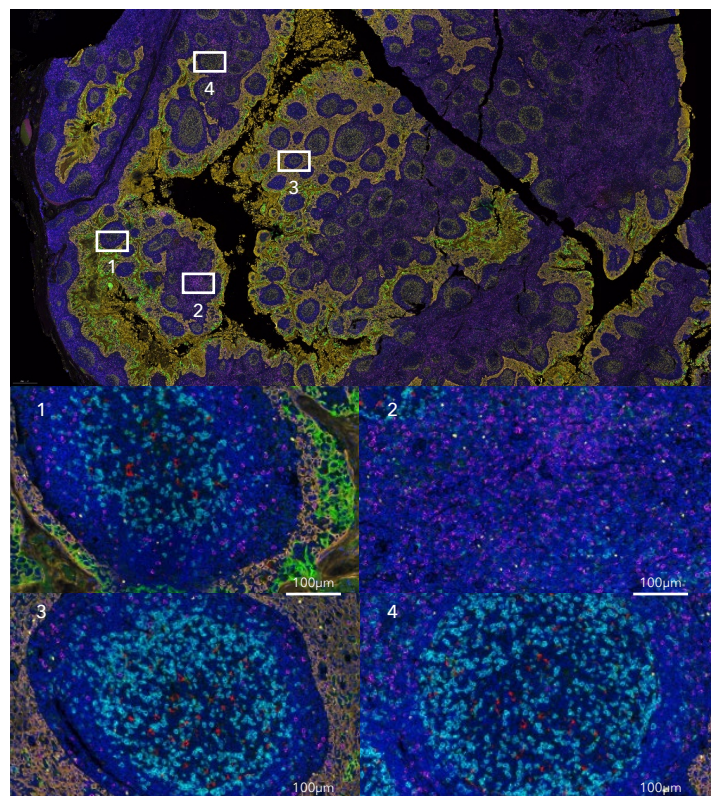


FIGURE 14 - 6-plex with Akoya Opal®. FFPE, tonsil: FoxP3 - yellow (Opal® TSA 570), PD-L1 - green (Opal® TSA 520), PD1 - cyan (Opal® TSA 690), CD68 - red (Opal® TSA 620), CD8 - purple (Opal® TSA 540), CK - orange (Opal® TSA 650), DAPI - blue.



Interested in LabSat® Research?

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