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Multiplex imaging application note

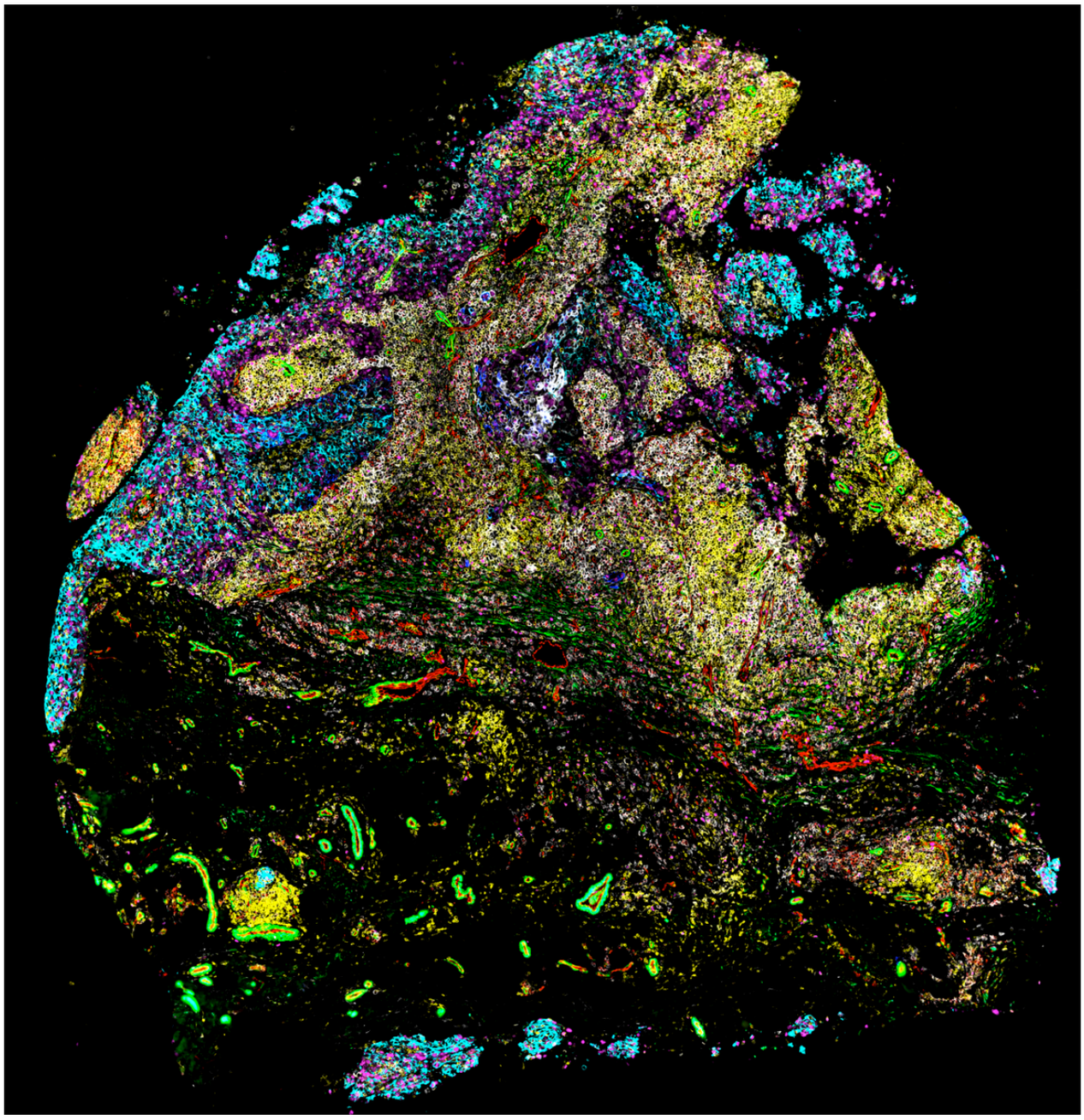

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Image credit: Dr Koei Chin, Research Associate Professor (of Biomedical Engineering), Oregon Health and Science University.

Introduction

Top summary

- Multiplex imaging of tumor tissue samples allows for improved biomarker discovery and increased accuracy in the prediction of patient therapeutic responses.
- Abcam offers highly specific, recombinant antibody reagents to support multiplex imaging of key targets within the tumor microenvironment (TME).
- Our extensive range of antibodies to established and emerging immuno-oncology targets can be effectively applied to different platforms and combined with different labeling solutions to offer a variety of flexible solutions to multiplex imaging.

Following the success of monotherapy checkpoint inhibitors for the treatment of cancer, researchers are beginning to look at combinations of immunotherapies and new immunotherapies to extend the success of these treatments to increased patient numbers. However, leveraging the host immune system to fight cancer requires a more complete understanding of how components within the tumor microenvironment interact to promote or block tumor progression.

Tumor progression and pathogenesis are directly influenced by the spatial architecture of the tumor microenvironment. Spatial analysis of tumors has also been shown to be effective for predicting treatment responses to the PD-1/PD-L1 checkpoint blockade[1-5].

Immunohistochemistry (IHC), a popular method used to analyze the tumor microenvironment, can only support a small number of biomarkers studied per sample. To overcome this limitation, many have transitioned to multiplex imaging, combining the study of multiple biomarkers in a tissue section with the capability to profile the spatial organization of each biomarker alone or in combination to determine the tissue motifs of each tumor.

Multiplex immunofluorescence spectral unmixing techniques have expanded the number of fluorophores that can be analyzed to seven within a single sample. To further improve the spectral resolution of immunostaining, cyclic immunofluorescence (CycIF) methods use cycles of fluorescent labeling, imaging, and inactivation of fluorescence (or quenching fluorescence), which work to increase multiplexing capabilities, with the ability to re-image a single sample through multiple cycles of staining [6].

Discovery and validation of high-quality biomarkers in this field are important to support further developments, offering the potential to increase diagnostic accuracy, improve patient stratification, and offer new targets for next-generation immunotherapies[7,8]. Highly specific, fit-for-purpose, recombinant antibodies to various immune checkpoint receptors, tumor, and immune cell markers complexed in a single assay can provide a holistic view of a tumor to guide personalized medicine. Ensuring antibodies are target specific, show robust performance in the assay, and provide reproducible data across samples and data are essential to yield informative results in multiplex analysis [9,10].

Here we highlight work from Abcam's in-house research laboratories and Drs Koei Chin and Joe Gray, Department of Biomedical Engineering at the Oregon Health and Science University (OHSU) in the immuno-oncology field. It demonstrates the successful implementation of Abcam antibodies to key immuno-oncology targets in different multiplex imaging solutions, including commonly used commercial labeling kits and across different analytical platforms.

6-plex IHC staining of immuno-oncology targets in tonsil tissue and tumor microarrays

Methods

To determine the presence, localization, and abundance of six different immune checkpoints and immune cell phenotype markers in parallel, we used a selected panel of Abcam antibodies in conjunction with Opal™ 7 color automation IHC kit from Akoya Biosciences (Table 1).

Table 1. Antibody labeling and experimental conditions with PD-L1 / PD1 multiplex IHC-IF antibody panel (PD-L1, PD1, CD68, CD3, Ki67, panCK) **(ab269812)** used in multiplex staining. Antibodies within the panel are also available for individual purchase.

Order of staining	Antibody target	Fluorescent label	Antibody concentration	abID
1	Recombinant anti-PD1 antibody [CAL20]	Orange	1/500	ab237728
2	Recombinant anti-PD-L1 antibody [CAL10]	Green	1/500	ab237726
3	Recombinant anti-CD68 antibody [SP251]	Yellow	1/300	ab192847
4	Anti-CD3 antibody [SP7]	Red	1/300	ab16669
5	Recombinant anti-Ki67 antibody [SP6]	Light blue	1/200	ab16667
6	Anti-pan cytokeratin antibody [C11]	Gray	1/200	ab7753

The immunostaining of formalin-fixed paraffin-embedded (FFPE) tissue sections of normal tonsil and a tissue microarray was performed on a Leica Biosystems BOND® RX instrument incubated in six rounds of staining (Table 1), each using a separate fluorescent tyramide signal amplification system. Sodium citrate antigen retrieval (Leica ER1, pH6.0, 30 minutes) was used in between rounds of tyramide signal amplification to remove the antibody from the previous round and avoid any cross-reactivity. DAPI (dark blue) was used as a nuclear counterstain. Microscopy with spectral unmixing of the seven channels and pseudo-coloring of individual Opal™ dyes was performed using a Vectra Polaris.

Results

Flexible labeling and compatibility of immuno-oncology antibodies for multiplex IHC

Multiplex staining of FFPE healthy tonsil tissue showed distinct staining patterns for each of the antibodies used, demonstrating that the antibodies are compatible with the commonly used Opal™ multiplex IHC fluorescent kit and that they function correctly within multiplex IHC to show the expected specific staining pattern in the tissue samples (Figure 1).

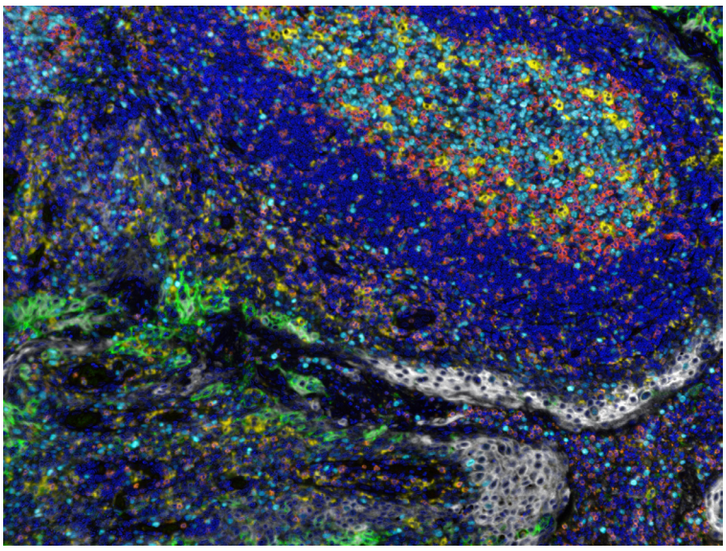

Figure 1. Multiplex IHC staining of healthy tonsil tissue. (a) 7 color IHC-IF multiplex image on FFPE tonsil using 6-plex multiplex IHC-IF panel (ab269812) (PD1 [CAL20] – Orange (ab237728), PD-L1 [CAL10] - Green (ab237726), CD68 [SP251] - Yellow (ab192847), CD3 [SP7] - Red (ab16669), Ki67 [SP6] – Light blue (ab16667), PanCK [C-11] - Gray (ab7753) and DAPI nuclear counterstain - Dark blue). Image acquired with a Vectra® Polaris™ Automated Quantitative Pathology Imaging System.

When performing multiplex staining, antibodies must be validated both independently and co-dependently within the same assay to ensure their correct function under the assay conditions. The resolution enabled by the multiplexing method in this assay demonstrates that the antibodies show the desired robust performance under the multiplex assay conditions.

Specific and robust antibody performance to assess tumor heterogeneity

It is widely recognized that tumors are not homogenous tissues. Even tumors arising from the same genetic mutations display variation between patients and metastatic sites within one patient [10-12]. The use of multiplexed imaging in tumors allows an increased understanding of cancer heterogeneity, including the many active signaling pathways and dynamic interactions that occur within the TME. This tumor heterogeneity may explain the variable response of different lesions to immunotherapies [15, 16]. The contextual relationship between cancerous, stromal, and immune cells in the tumor milieu may provide certain advantages and liabilities to tumor cells. Understanding the specific characteristic of tumors in this manner allows for the personalized treatment of cancer patients according to their specific phenotype [10].

The use of the tumor tissue microarray demonstrated the heterogeneity between lymphoma sections from different patients, where the same antibody panel was used to label each of the tissue sections, with highly variable staining patterns observed between the different patient sections (Figure 2).

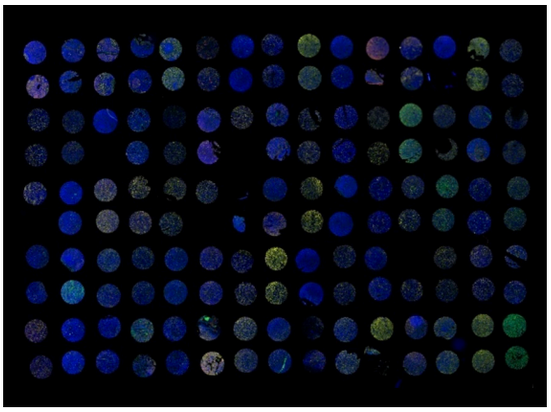

Figure 2. Microarray of 150 patient lymphoma FFPE sections using 6-plex multiplex IHC-IF panel (ab269812) (PD1 [CAL20] – Orange (ab237728), PD-L1 [CAL10] - Green (ab237726), CD68 [SP251] - Yellow (ab192847), CD3 [SP7] - Red (ab16669), Ki67 [SP6] – Light blue (ab16667), PanCK [C-11] - Gray (ab7753) and DAPI nuclear counterstain - Dark blue). Image acquired with a Vectra® Polaris™ Automated Quantitative Pathology Imaging System.

Higher magnification analysis of three different samples from the tumor microarray indicates the discrete staining patterns observed among the different tissue samples (Figure 3), with different patient samples showing a predominance of different markers. While all three samples demonstrate expression of the monocyte/macrophage marker CD68, indicating immune cell infiltration of the tumor, two of the samples show high levels of expression of the tumor proliferation and growth marker Ki67 (Fig. 3a, c), two show expression of the immune checkpoint ligand, PD-L1 (Fig 3 b,c) and high levels of the T cell co-receptor, CD3, are present in only one of the samples (Fig 3b).

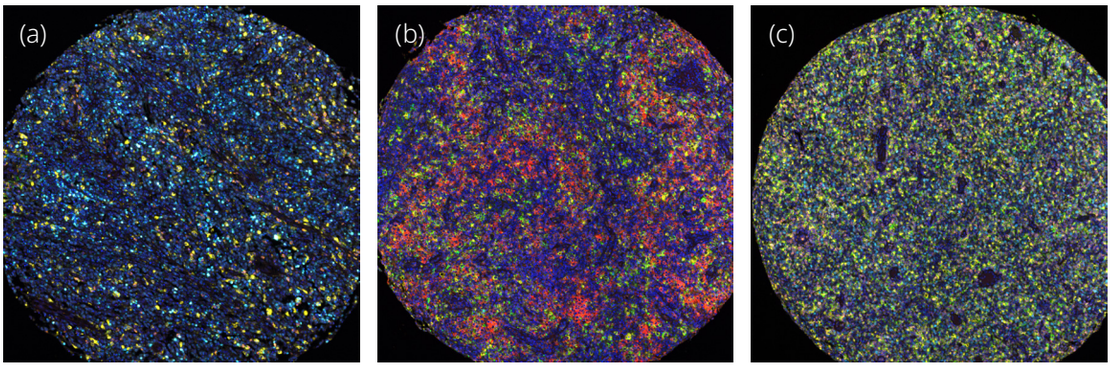

Figure 3. Multiplex IHC staining of three different patient FFPE lymphoma samples using 6-plex multiplex IHC-IF panel (ab269812) (PD1 [CAL20] – Orange (ab237728), PD-L1 [CAL10] - Green (ab237726), CD68 [SP251] - Yellow (ab192847), CD3 [SP7] - Red (ab16669), Ki67 [SP6] – Light blue (ab16667), PanCK [C-11] - Gray (ab7753) and DAPI nuclear counterstain - Dark blue). Image acquired with a Vectra® Polaris™ Automated Quantitative Pathology Imaging System.

The application of multiplex IHC in this manner and analysis of the presence, absence, and abundance of specific markers across each sample demonstrates the specificity and robust performance of Abcam antibodies to work in multiplex imaging, and the flexibility of these antibody reagents for labeling with different universal labeling kits, such as the Opal™ labeling kits.

Cyclic immunofluorescent staining of breast cancer tissue

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OHSU

Results

CycIF to uncover the tumor microenvironment architecture with 11-plex labeling

Triple-negative breast cancer (TNBC) is a uniquely aggressive and highly heterogeneous cancer with high rates of relapse due to resistance to chemotherapy. This cancer type shows triple-negative staining in IHC for HER2, estrogen, and progesterone receptors [17, 18]. Higher levels of PD-L1 are known to be expressed in TNBC compared to other breast cancer subtypes; this is being targeted with anti-PD-L1/PD-1 monoclonal antibodies (mAbs) and further efforts to identify actionable biomarkers that may allow for combination therapies with immune-checkpoint blockade [19].

CycIF of TNBC tissue was performed by Dr Koei Chin, Center for Spatial Systems Biomedicine and Knight Cancer Institute at Oregon Health and Science University, Oregon, USA, for multiplex image analysis of 11 different tumor, stromal, and immune cell markers in three cycles (Figure 4). These results demonstrate the heterogeneic nature of tumor tissue. Hematoxylin and eosin staining revealed areas of tumor nest and microenvironment with varying cellular density, with the lower portion of the tissue section being largely absent of cells, while high cell density was seen in the top half of the tissue section. This is reflected in the expression patterns observed in the composite image of the various cell and cancer hallmark markers analyzed, which shows corresponding spatial organization of cells expressing Ki67, CK19, and CD45, suggesting TNBC with high levels of tumor-infiltrating leucocytes within the upper portion of the tumor section.

Methods

Immunofluorescence: direct labeled primary antibodies were applied to a triple-negative breast cancer FFPE tissue, which was deparaffinized, unmasked antigens in a heat-mediated method, and blocked in PBS with normal goat serum and BSA. Following overnight incubation, the tissue was washed and mounted with coverglass in DAPI solution for DNA counterstaining. The whole tissue was scanned with 20x objective (0.8NA) on the AxioScan.Z1 (Zeiss, Germany). After successful multi-channel image acquisition, the fluorophore signal on the tissue was inactivated in PBS with 20 mM sodium hydroxide (NaOH) and 3% hydrogen peroxide (H_2O_2), and the subsequent cycle of immunofluorescence staining was initiated[11,12]. Visualization: Each acquired image was registered based on DAPI features in each cycle of staining[13], and was overlaid using Zen Lite software (Free version from Zeiss). Spatial, single-cell analysis: In-house software was used to generate nuclear, cell, and membrane segmentation masks by classifying pixels based on a combination of marker expression to identify cells and membranes respectively[12]. Extracted single-cell features included centroids and mean intensity of each marker from its biologically-relevant segmentation mask, E-cadherin and CD45 in membrane and Ki67 in nuclei. The last round of DAPI images was used to filter out cells lost during previous rounds of CycIF staining. The computational biology tools for downstream analysis, especially for large cohort studies, were developed and publicly available to download[14].

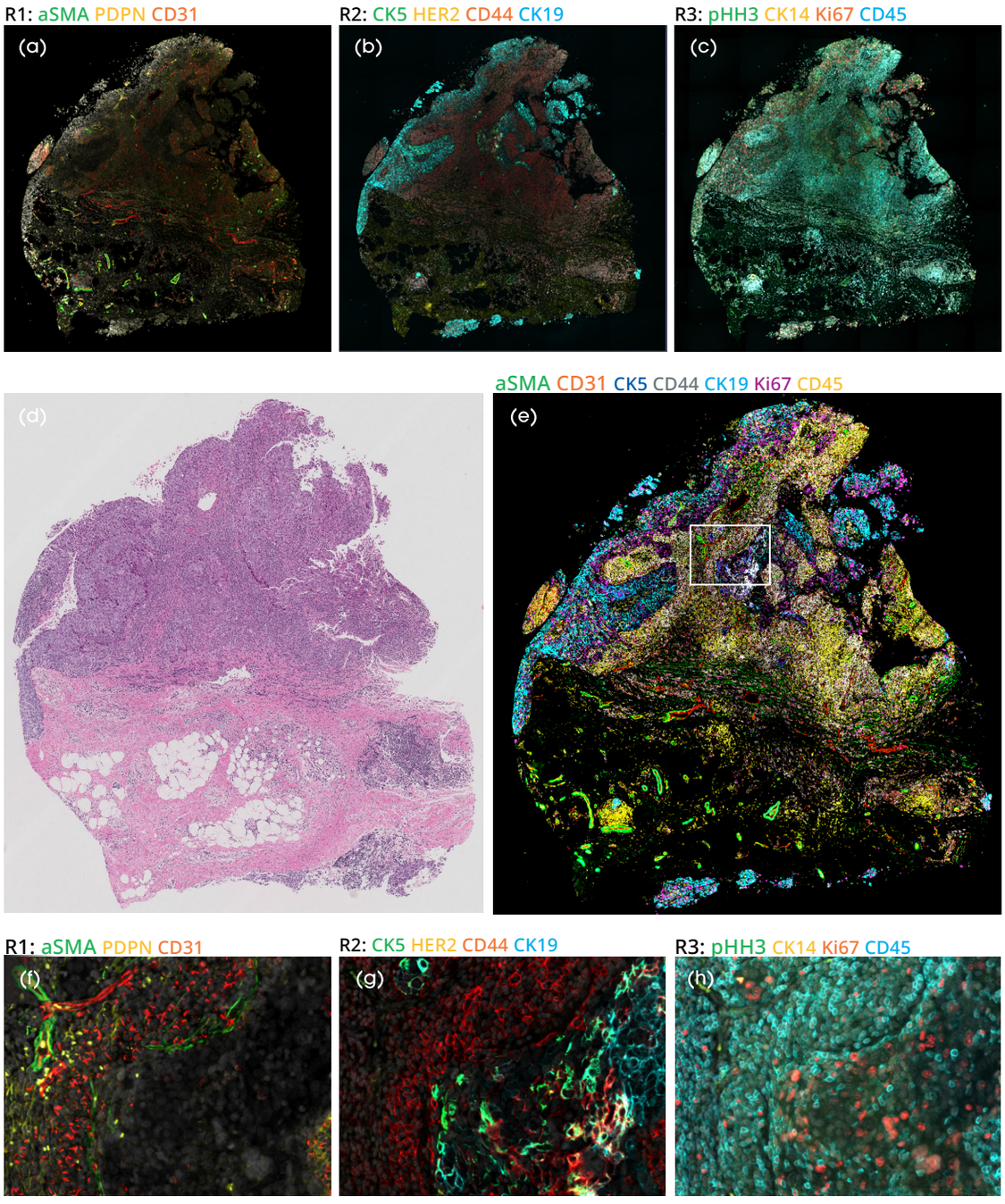

Figure 4. CycIF labeling of FFPE TNBC tissue sections with immuno-oncology and immune cell marker antibodies. (a) First cycle labeling of a-smooth muscle actin (green), Podoplanin (yellow), and CD31 (red). (b) Second cycle labeling of CK5 (green), HER2 (yellow), CD44 (red), and CK19 (cyan). (c) Third cycle labeling of phospho-histone H3 (green), CK14 (yellow), Ki67 (red), and CD45 (cyan). (d) Hematoxylin and eosin staining of tissue section. (e) compound image of a-smooth muscle actin (green), CD31 (red), CK5 (blue), CD44 (Gray), CK19 (cyan), Ki67 (magenta), CD45 (yellow). ROI delineated by white box. (f) Magnification of ROI with first cycle labeling, aSMA (green), Podoplanin (yellow) CD31 (red). (g) Magnification of ROI with second cycle labeling, CK5 (green), HER2 (yellow - negative), CD44 (red), CK19(cyan). (h) Magnification of third cycle labeling, phospho-histone H3 (green), CK14 (yellow), Ki67 (red), CD45 (cyan).

This analysis showed that each of the antibodies was able to specifically label their target antigen in the diseased tissue, with distinct localization observed for each antibody used. No loss in performance due to the harsh conditions of CycIF labeling and fluorophore removal was observed.

Table 2. Antibodies, fluorochromes, and experimental conditions validated for cyclic IF of breast

Stain cycle	Biomarkers	Fluorophore	Antibody concentration	abID	Clone
Cycle-1	aSMA	Alexa Fluor® 488	1/200	ab184675	1A4
	Popoplanin	Alexa Fluor® 555	1/50		D2-40
	CD31	Alexa Fluor® 647	1/100	ab218582	EPR3094
Cycle-2	Cytokeratin 5	Alexa Fluor® 488	1/200	ab193894	EP1601Y
	HER2	Alexa Fluor® 555	1/50		3B5
	CD44	Alexa Fluor® 647	1/200	ab194988*	EPR1013Y
	Cytokeratin 19	Alexa Fluor® 750	1/200		A53-B/A2
Cycle-3	Phospho- Histone H3	Alexa Fluor® 488	1/100		D2C8
	Cytokeratin 14	Alexa Fluor® 555	1/400	ab7800	LL002
	Ki67	Alexa Fluor® 647	1/400		D3B5
	CD45	Alexa Fluor® 750	1/100	ab214437	EP322Y

*ab194988 is unavailable, we recommend using ab232556

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Conclusion

Contextual analysis of immuno-oncology biomarker expression with the tumor microenvironment has shown to be important for a full understanding of tumor biology and can offer increased accuracy in targeting immunotherapies for specific cancers [1-5]. Multiplex IHC is important to enable profiling of the TME architecture, providing a comprehensive overview of the interplay between tumor cells and the microenvironment, as well as the many immune checkpoints and cell markers that compose specific cancer signatures and biomarkers [20].

The increased interest in multiplex imaging across the field of immuno-oncology to identify new biomarkers and improve the accuracy of patient stratification has revealed a requirement for specific and reliable tools to perform these analyses. For optimal performance in multiplex imaging, antibodies must be target-specific, sufficiently sensitive to provide an accurate signal within the dynamic range of the assay, robust to ensure accurate performance within the harsh assay conditions, and offer reproducible results [9, 10]. The results presented here demonstrate the suitability of Abcam antibodies for multiplex imaging with highly specific, sensitive, and robust antibodies to both established and emerging targets in immuno-oncology. The antibodies performed accurately and effectively across both multiplex IHC and CycIF assays and showed compatibility with common labeling tools for flexible ease of use across multiple platforms.

Further research into the abundance and distribution of these markers in parallel across various healthy and cancerous tissues via multiplex imaging may aid in the understanding of which therapies offer the greatest efficacies for each specific tumor phenotype.

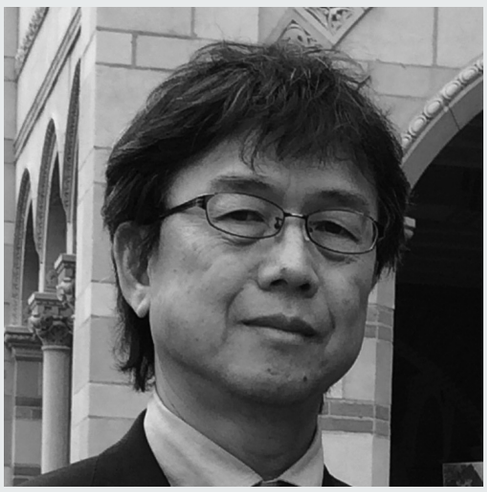
Antibodies validated for multiplex imaging

Table 3. Abcam antibodies validated by Dr Koei Chin, Center for Spatial Systems Biomedicine and Knight Cancer Institute for multiplex imaging

Antibody target	Fluorophore	Antibody concentration	abID
Anti-CD20	Alexa Fluor® 488	1/50	ab198941
Anti-CD31	Alexa Fluor® 647	1/50	ab218582
Anti-CD4	Alexa Fluor® 647	1/100	ab196147
Anti-CD45	Alexa Fluor® 647	1/100	ab200317
Anti-CD8	Alexa Fluor® 555	1/50	ab213017
Anti-Collagen I	Alexa Fluor® 750	1/100	ab215969
Anti-CSF-1-R	Alexa Fluor® 750	1/100	ab240265
Anti-Cytokeratin 17	Alexa Fluor® 488	1/100	ab185032
Anti-Cytokeratin 7	Alexa Fluor® 488/555	1/400	ab203434
Anti-Cytokeratin 8	Alexa Fluor® 488	1/200	ab192467
Anti-E Cadherin	Alexa Fluor® 750	1/100	ab201499
Anti-Estrogen Receptor alpha	Alexa Fluor® 647	1/100	ab205851
Anti-Glucose Transporter GLUT1	Alexa Fluor® 488	1/50	ab195359
Anti-Granzyme B	Alexa Fluor® 750	1/100	ab219803
Anti-PD1	Alexa Fluor® 647	1/50	ab201825
Anti-MUC1	Alexa Fluor® 488	1/100	ab196443
Anti-CAV1	Alexa Fluor® 488	1/100	ab185043
Anti-Cyclin D1	Alexa Fluor® 555	1/50	ab203448
Anti-Rb (phospho S807)	Alexa Fluor® 647	1/100	ab215947
Anti-Rad51		1/50	ab221796
Anti-gamma H2A.X (phospho S139)	Alexa Fluor® 647	1/50	ab195189
Anti-GATA3	Alexa Fluor® 555	1/50	ab210672
Anti-53BP1		1/50	ab222232
Anti-beta III Tubulin	Alexa Fluor® 488	1/50	ab195879
Anti-Chromogranin A		1/100	ab215276
Anti-CD90	Alexa Fluor® 555	1/50	ab202511
Anti-Lamin B1	Alexa Fluor® 488	1/50	ab194106
Anti-Lamin B2	Alexa Fluor® 647	1/50	ab200427
Anti-Progesterone Receptor	Alexa Fluor® 647	1/100	ab267524
Anti-MSH6	Alexa Fluor® 647	1/100	ab198334
Anti-MSH2		1/50	ab228334
Anti-HIF-1 alpha	Alexa Fluor® 647	1/50	ab190569
Anti-BMP2		1/100	ab225898
Anti-Synaptophysin	Alexa Fluor® 555	1/100	ab206870
Anti-Cytokeratin 5	Alexa Fluor® 488	1/200	ab193894
Anti-CD44	Alexa Fluor® 647	1/200	ab194988
Anti-Cytokeratin 14		1/200	ab7800

For a full list of Abcam antibodies that have been validated for multiplex imaging, contact a member of our team at immuno.oncology@abcam.com. For more resources on multiplex imaging, please visit abcam.com/multipleximaging.

Dr. Koei Chin
Research Associate Professor (of Biomedical Engineering), Oregon Health and Science University.



Dr Chin's research is focused on understanding the mechanisms of spatial heterogeneity in the tumor ecosystem by which cancer cells escape from therapeutic stress, acquire drug resistance, and metastasize.

He has led the development of highly-multiplexed imaging workflows for single-cell phenotyping (which contributes to identifying biomarkers for therapeutic targets and predicting drug response in cancer).

He received MD and PhD degrees at Nippon Medical School in Tokyo, Japan.

Additional resources

Find directly conjugated primary antibodies manufactured and validated to help you design reproducible imaging experiments. Go to **abcam.com/primary-antibodies/antibody-conjugation-simply-do-it-your-way** for more information

Achieve the highly reproducible results your research requires with recombinant monoclonal antibodies. Available in a carrier-free format for easy antibody-labeling and sandwich immunoassay development. Visit **https://www.abcam.com/primary-antibodies/pbs-only-formulations-for-recombinant-rabmab-antibodies** for more information.

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Notes

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