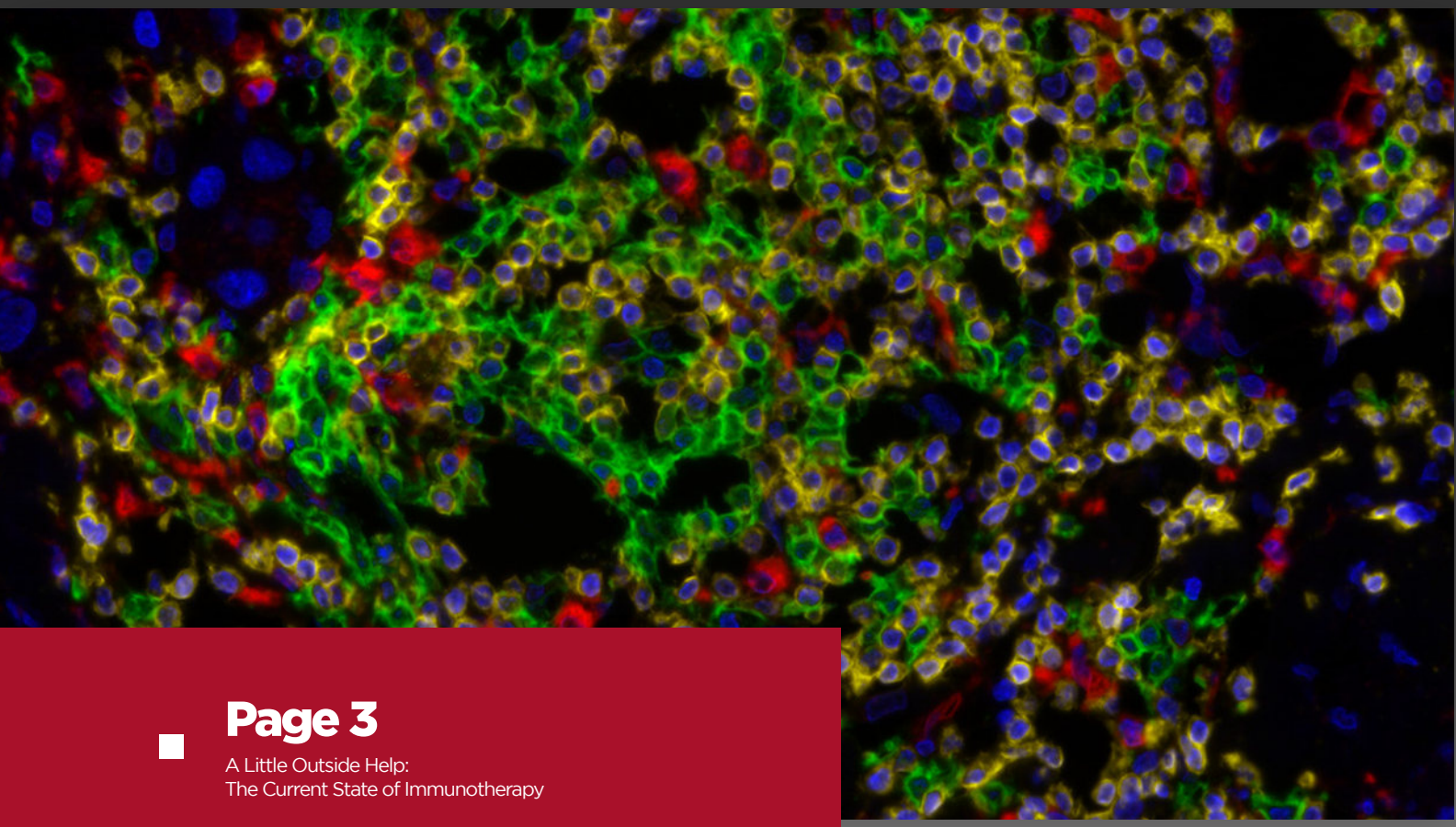
ANTIBODY DETECTION FOR CANCER RESEARCH



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Weller, MG, Analytical Chemistry Insights:*11, 21-27 (2016).*
Antibodies shown: CD3E (A700-016) & CD20 (A500-017A)

A Little Outside Help: The Current State of Immunotherapy

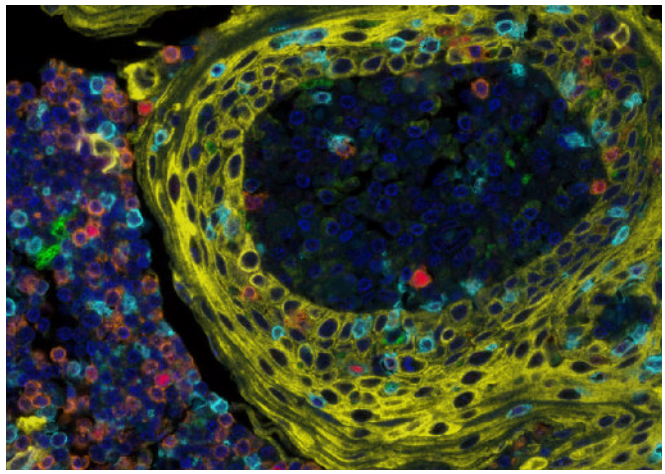
Under normal physiological circumstances, the immune system is the greatest defense against cancer. Immune cells detect and eliminate cancer cells at the onset of oncogenic mutations. In response, cancer cells adapt to circumvent immune detection, leading to the failure of immunosurveillance and subsequent tumor formation. This phenomenon, "immune evasion," is a major problem for treatment strategies that aim to enhance or restore natural immune anti-cancer responses.

Cancer cells predominantly evade the immune system in two ways: they prevent immune cells from detecting them and they deactivate immune responses prematurely. To accomplish the former, cancer cells downregulate the surface expression of key antigens identified by immune cells. For the latter, they create immunosuppressive local environments by secreting anti-inflammatory cytokines and chemokines, promote the activity of regulatory immune cells over effector subtypes, and directly activate immune checkpoints, the "shut down" switches on immune cells[1].

Immunotherapeutic Approaches Fight Back

Rather than directly augmenting immune responses, modern immunotherapy approaches seek to inhibit or circumvent cancer cell immune evasion mechanisms. For example, checkpoint inhibition therapy—one of the most popular immunotherapies used in clinics today—uses antibodies to antagonize checkpoint receptors such as CTLA-4 and PD-1, thus preventing immune deactivation[2]. Checkpoint inhibitors are currently approved for clinical use against a wide range of solid tumor cancers, ranging from breast, lung, and gastrointestinal cancers to melanomas, and scientists and clinicians are exploring how they complement other therapeutic approaches to maximize treatment efficacy[2].

Cytokine-based therapeutic approaches are another way to prevent immune evasion. Several cytokines, including IFN-α and IL-2, can directly inhibit cancer cell proliferation or induce apoptosis[3]. However, cytokines are chiefly valued for their ability to potentiate other immunotherapeutic approaches, especially through stimulating pro-inflammatory and pro-cytotoxic immune responses. Scientists pursuing new cytokine-based approaches are looking for synergistic combinations with existing checkpoint inhibitors, improved persistence in vivo (e.g., longer circulatory half-life and tumor concentration), and local administration capability[3].



Since the 1960s, scientists have been investigating adoptive cell transfer (ACT), where immune cells are injected into patients as an anti-cancer therapeutic strategy[4], but low cell survival and poor targeting affinity limited effectiveness. The advent of chimeric antigen receptors (CARs) changed that. Attaching custom engineered CARs to T cells gave researchers a multitude of potential immunotherapeutic avenues. CAR T cells activate endogenous T cells without using mechanisms that cancer cells target, or they directly target cancer cells through cell surface antigens that cancer cells have not learned to downregulate. Continued advancements in both CAR engineering and CAR T cell generation practices have greatly improved clinical treatment efficacy and safety since CARs were first created in the 1980s[4].

The Future of Immunotherapy

Scientists and clinicians have made great strides in identifying how cancer cells evade immune responses and deploying new countermeasures to prevent their escape. Nonetheless, the battle against cancer continues, and numerous challenges, such as tumor heterogeneity and cellular plasticity, still remain. With each passing day, research continues to unveil new information on how tumors and immune cells interact with one another, drawing out roadmaps for new therapeutic approaches.

For references, please see page 7

Understanding Change: Cellular Plasticity in Cancer

With technological advances enabling more in-depth and cell-level specific investigations, researchers are starting to recognize how diverse and dynamic multicellular tumors are. Heterogeneity within tumor cell and immune cell populations exists at the genetic, epigenetic, and phenotypic levels, and is a leading limiting factor for the efficacy of targeted therapeutic approaches[1]. Moreover, tumor and immune cells are highly sensitive to extracellular signals, whether from other cells or from elements of their local environment. This complexity creates difficulties when characterizing cellular populations and individual cells.

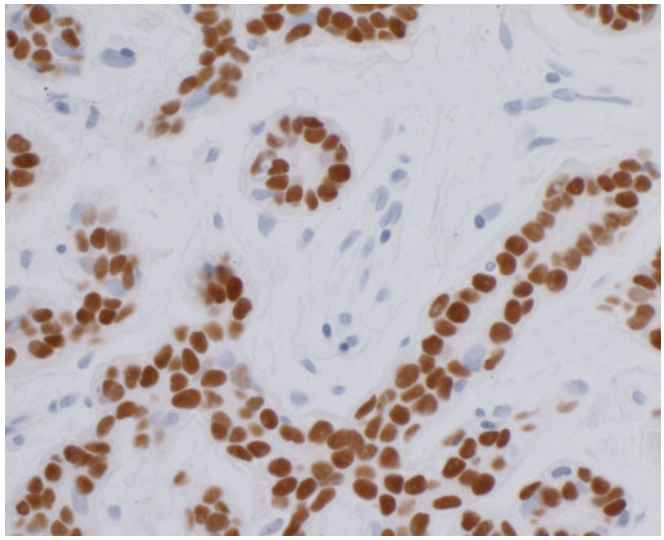
A Moving Target

Initially, scientists believed that intercellular differences between tumor cells were either the product of accumulated mutations or caused by cancer stem cells, a tumoral subset with self-renewal capabilities and pluripotency. However, recent research makes it clear that cancer cells can shift between differentiated and multi/pluripotent states based on intrinsic and extrinsic signaling cues[2]. This dynamism makes it difficult for scientists to characterize cancer cell behavior and identify potential therapeutic targets.

Genetic and epigenetic alterations are a key source of these cues. For example, loss of p53 or PTEN function induces cellular plasticity, while epithelial and filament gene modulation can promote epithelial-mesenchymal transition (EMT) and subsequent metastasis[2]. Mutational extent combined with compensatory mechanisms creates numerous intermediate phenotypes along the EMT gradient[2].

Role Reversal

Cancer cell signaling also hijacks the innate adaptability of immune cells, creating many tumor-specific immune cell variants. T cells are the most prominent anti-tumor immune cells. Cancer cells evade cytotoxic T cell killing through immune evasion, but also by recruiting and activating regulatory T cells (T_{regs}), which then deactivate effector T cells and suppress the immune response[3]. Likewise, activated macrophages normally adopt either a pro-inflammatory phagocytic phenotype or an anti-inflammatory growth-promoting phenotype. Tumor-based signaling drives them heavily towards the latter, creating tumor-associated macrophages (TAMs) that promote tumor growth and deactivate other effector immune cells. Similarly, tumor-associated neutrophils (TANs) are pushed away from their normal pro-inflammatory activities towards phenotypes that suppress T cell and facilitate tumor growth[3].



Finally, the combination of cancer cells and pro-tumorigenic immune and stromal cell phenotypes create a tumor microenvironment (TME) that serves as a positive feedback loop for cancer survival and proliferation[2]. Cancer-associated fibroblasts and other TME stromal components secrete growth factors (e.g., TGFβ, EGF, FGF) and angiogenic factors (e.g., VEGF), while TAMs and mesenchymal stem cells induce stem-like phenotypes in cancer cells through prostaglandin, TNF, and interleukin signaling. Finally, hypoxia, resulting from poor tumor vascularization, also induces stemness in cancer cells and promotes self-renewal[2].

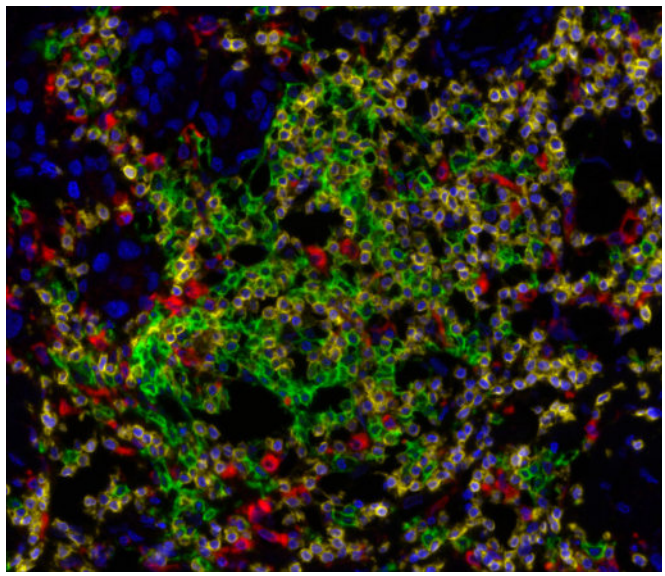
The Need for More Information

Researchers rely heavily on cell surface markers to define cell types. The sheer number of different marker permutations on any given cancer or immune cell makes it difficult to clearly characterize both cell compositions and functions within a tumor. Increasing understanding of cell heterogeneity and plasticity highlights the requirement for greater detection bandwidth for generating comprehensive marker profiles.

For references, please see page 7

More Details, More Discoveries: Multiplexed Antibody Detection

Researchers use antibody-based labeling techniques to find and track proteins in cell and tissue samples. Historically, technological limitations restricted scientists to probing only a single protein per experimental run, which was adequate as long as cells could be distinguished using one or two markers. However, as researchers discover more tumor-specific cellular subtypes, the number of markers needed to characterize and distinguish them increases. Antibody multiplexing techniques now allow scientists to simultaneously detect and examine multiple protein markers. The increased single-assay detection capacity offered by antibody multiplexing is paramount for the effective and comprehensive characterization of heterogeneous cell populations such as tumors.



Adapting Staining for Modern Science

Both immunohistochemistry (IHC) and immunofluorochemistry (IF) work by visualizing antibodies tagged with either a chromogen or fluorochrome bound to the intended target. Conventional IHC and IF multiplexing techniques use multiple antibodies to probe different targets within a sample at the same time; the results can be analyzed by light and fluorescent microscopy. For these experiments, researchers need to pay careful attention to potential cross-reactivity. It is imperative that all the antibodies involved in the assay react only with their respective protein targets. Moreover, it is also critical that the antibody-generated colors are easily distinguishable[1]. Since multiplex IHC/IF techniques generate exponentially greater amounts of data (and merge combinations) than conventional techniques, software-guided analysis is an excellent tool for processing and quantifying signal[2].

The Next Level: Tyramide Signal Amplification

Finding appropriate antibody combinations for multiplexed IHC/IF becomes more complicated as the number of targets increases. Tyramide signal amplification (TSA) offers one solution to this challenge. For TSA IHC/IF protocols, samples are treated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies, and then these secondary antibodies are treated with inactive tyramide. HRP activates tyramide, enabling it to covalently bind tyrosine residues on or near the target protein. Instead of simultaneously detecting multiple antibody-chromogen/fluorophore complexes, TSA-based techniques sequentially apply layers of signal using tyramide conjugates tagged with different fluorophores[2]. Since antibody complexes can be removed without affecting tyramide signal, cross-reactivity is no longer a concern. Bound antibody is simply removed before applying the next detection antibody.

TSA-based protocols provide great amplification potential, since each HRP-conjugated antibody can activate multiple tyramide molecules, without raising serious non-specific binding issues. This is advantageous for overcoming endogenous non-specific signal in IHC/IF, and when combined with high sensitivity techniques like flow cytometry, it enables scientists to detect even very weakly expressed proteins. TSA-based flow cytometry protocols require shorter incubation times, consume smaller antibody/probe volumes, and provide detection limits that are orders of magnitude more sensitive than conventional flow cytometry.

Bridging the Gap between Bench and Bedside with Multiplexing

Multiplexing allows scientists to probe deeper into tumor structures in order to better understand the cells and mechanisms underlying tumorigenesis and anti-tumor responses. In tandem with automation, it provides a speed and depth of information that allows clinicians to characterize patient cancer cells for diagnostic purposes. For example, immune checkpoint marker expression levels obtained using multiplexed IF are used to predict disease severity and outcome[3]. Given the heterogeneity of cancer, immunotherapy is shifting towards personalized treatment approaches. Multiplexing will be instrumental towards both characterizing an individual's cancer cells and discovering what therapeutic approaches work best against that profile[1,4].

For references, please see page 7

TYRAMIDE SIGNAL AMPLIFICATION-BASED MULTIPLEXING

Tyramide signal amplification (TSA) offers greater sensitivity and flexibility, allowing researchers to avoid cross-reactivity and signal spectral overlap complications.

Primary Antibody

Conventional and tyramide signal amplification (TSA)-based multiplexing protocols both require a primary antibody that selectively targets the protein of interest.

Secondary Antibody

Secondary antibodies for TSA protocols carry a horseradish peroxidase (HRP) tag.

HRP

CONVENTIONAL MULTIPLEXING

TSA MULTIPLEXING

Secondary Antibody

Conventional secondary antibodies carry a fluorophore tag to enable visualization. Caution: avoid antibody cross-reactivity and spectral overlap.

FITC

HRP

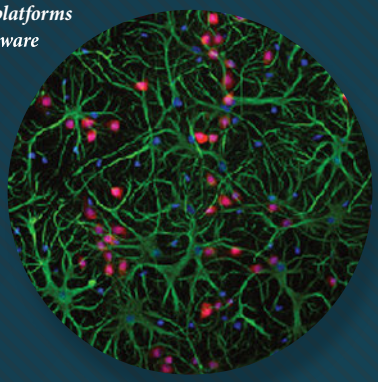
Tyramide Addition

HRP activates inactive fluorophore-conjugated tyramide in the presence of peroxide. Activated tyramide binds to tyrosine residues on or around the protein of interest.

Visualization and Analysis

Samples are visualized using microscopy after all desired probes have been applied.

Note: automated platforms and computer software can greatly assist in providing a more comprehensive and systemic image capture and analysis.



Repeat as Necessary

Samples are microwaved to remove antibody complexes without impacting tyramide binding integrity. The protocol is then repeated for each protein of interest to be probed.

Note: because the antibodies are introduced one at a time, cross-reactivity is not a concern.

Carrier-Free Antibodies

Antibodies are normally stored in solutions containing carrier proteins such as bovine serum albumin and sodium azide. These stabilizers and preservatives help maintain antibody integrity during storage, but can interfere with binding and inhibit HRP activity. Carrier-free antibodies are free from such limitations.

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Article 1: A Little Outside Help: The Current State of Immunotherapy

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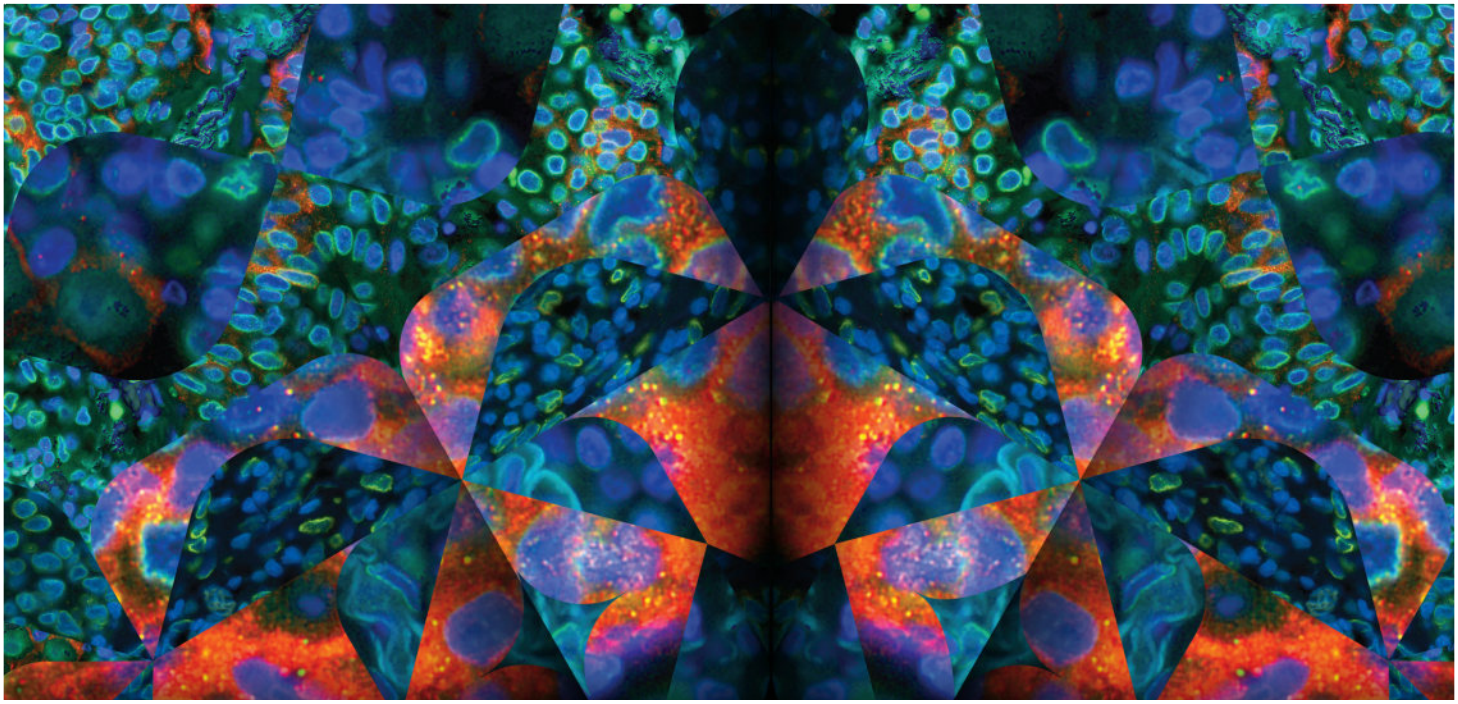

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