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Chapter

# Immune Cell Profiling in Cancer Using Multiplex Immunofluorescence and Digital Analysis Approaches

*Edwin Roger Parra*

## Abstract

During the last years, multiplex immunofluorescence (mIF) has emerged as a very powerful tool in multiple epitope detection to study tumor tissues. This revolutionary technology is providing an important visual technique for tumor examination in formalin-fixed paraffin-embedded specimens for a better understanding of tumor microenvironment, new treatment discoveries, cancer prevention, as well as translational studies. The aim of this chapter is to highlight the use of tyramide signal amplification methodology in mIF and image analysis to identify several proteins at the same time in one single tissue and their spatial distribution in different tumor specimens including whole sections, core needle biopsies, and tissue microarrays. This type of methodology associated with image analysis can perform high-quality throughput assay in translational research studies to be applied in cancer prevention and treatments.

**Keywords:** tyramide signal amplification, conventional IHC protocol, immunoprofiling, cancer tissues, image analysis, spatial analysis

## 1. Introduction

In the last years, novel and effective immunotherapies for patients with different tumor types are becoming clinically important, because of the remarkable clinical efficacy observed with several immune checkpoint inhibitors such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and the programed death receptor 1 (PD-1) or its ligand (PD-L1) [1–12]. Whereas anti-CTLA-4 antibodies (ipilimumab and tremelimumab), anti-PD-1 antibodies (nivolumab and pembrolizumab), and anti-PD-L1 antibodies (atezolizumab, avelumab, and durvalumab) have produced remarkable results, increasing the survival prognosis in many cancer types, it is still unknown why some tumors do not respond to or relapse after this type of treatment. In this way, increased observations suggest that tumors rich in tumor-associated immune cells (TAICs) may respond to therapies targeting immune system inhibitory or stimulatory mechanisms, and tumors with non-TAICs may require additional interventions aimed at promoting optimal inflammation and innate immune activation in the tumor microenvironment [13–15]. Indeed, characterization of different immune checkpoints as well as tumor microenvironment in patients with cancer has become

a fundamental step in providing evidence for the presence of distinct immunologic phenotypes, based on the presence or absence of various immune cells [1, 16, 17] that can predict the response to the therapy. In this way, the study of immune checkpoints and TAICs and their interaction prompt the need for multiplexed analyses of tumor tissues. To address this need, in the last years, multiplex imaging platforms have emerged as an important tool to provide critical information about cancer microenvironment, prognosis, therapy, and relapse [18–22]. Different components in the tumor microenvironment can be examined simultaneously using multiplex methodologies, providing insight into biological cross-talk present at the tumor-host interface and from subcellular levels to entered cell populations. In addition, the precision of these new techniques can be used to evaluate the special distribution of multiple biomarkers detected simultaneously, and their coexpressions or interactions between cells are becoming a essential tool to study tumor tissues [22] and to ultimately enhance disease diagnosis and better inform timely patient care [23].

Multiplex technologies are being used to identify the presence of multiple biological markers as immune checkpoint and TAICs on a single tissue sample [24]. The multiplex imagining techniques provide unique biological information that in many cases cannot be obtained by other imaging methods or by single immunohistochemistry (IHC) techniques. As mentioned, individual cells can be accessed with extraordinary fidelity equal to that achievable in the bulk population, such that even rare cell populations can be studied to understand their important role in translational research, and this knowledge can be applied in cancer prevention and treatment. In this chapter, we will discuss one of the most reliable and a very well-known methodology to identify simultaneous biomarkers in formalin-fixed, paraffin-embedded (FFPE) specimens as well as its imaging analysis platform as an important tool for potential application in future cancer immunotherapy biomarker discoveries.

## 2. Tyramide signal amplification for multiplex staining in FFPE tissues

Tyramide signal amplification (TSA) was described in the 1990s by Bobrow and colleagues [25, 26]. It is an enzyme-linked signal amplification method that is used to detect and localize low copy number of proteins present in tissues by conventional IHC protocol, using, most commonly, alkaline phosphatase or horseradish peroxidase (HRP) enzymatic reaction to catalyze the deposition of tyramide-labeled molecules at the site of probe or epitope detection. Tyramides are conjugated to biotin or fluorescent labels and revealed by streptavidin-HRP system [27, 28]. The HRP catalyzes the formation of tyramide into highly reactive tyramide radicals that covalently bind to electron-rich tyrosine moieties close to the epitope of interest on FFPE tissue. Tissue surfaces with anchored biotinylated tyramide must be further treated with fluorescent- or enzyme-tagged proteins that have a high affinity for biotin, such as streptavidin, before microscopic visualization [27, 28]. The detection of the proteins is increased more than ten times compared to standard biotin-based staining methods [29].

Perkin Elmer developed the Opal™ workflow (**Figure 1**), which allows simultaneous staining of multiple biomarkers within a single paraffin tissue section. Multiplex immunofluorescence (mIF) allows researchers to use antibodies raised in the same species, and different panels combined with different targets can be created using this technology [21, 27]. The approach, in the manual protocol, involves detection with fluorescent TSA reagents, followed by microwave treatment that removes the primary and secondary antibodies between cycles and any nonspecific staining that reduces tissue autofluorescence for each antibody cycle. The correct ordering of the different antibodies in a panel is still challenging and is only solved by trial and error to obtain a perfect staining. In the automated protocol using Leica Bond RX or another

autostainer, the time of staining is reduced drastically when compared with manual staining. The possibilities for mIF are expanding our knowledge of tumor immune contexture **(Figure 2)** in different types of cancers. Mapping the tumor microenvironment and the predictive and/or prognostic value of immune checkpoint expression on malignant cells and TAICs has been carried out in patients with melanoma, lung cancer, breast cancer, gastric cancer, Hodgkin lymphoma, and others by mIF [30–34]. Similar to other multiplex techniques, in the TSA mIF method, our experience showed that the approach to different targets requires diligent optimization, first in conventional chromogenic IHC validation and then in the simplex IF, before mIF

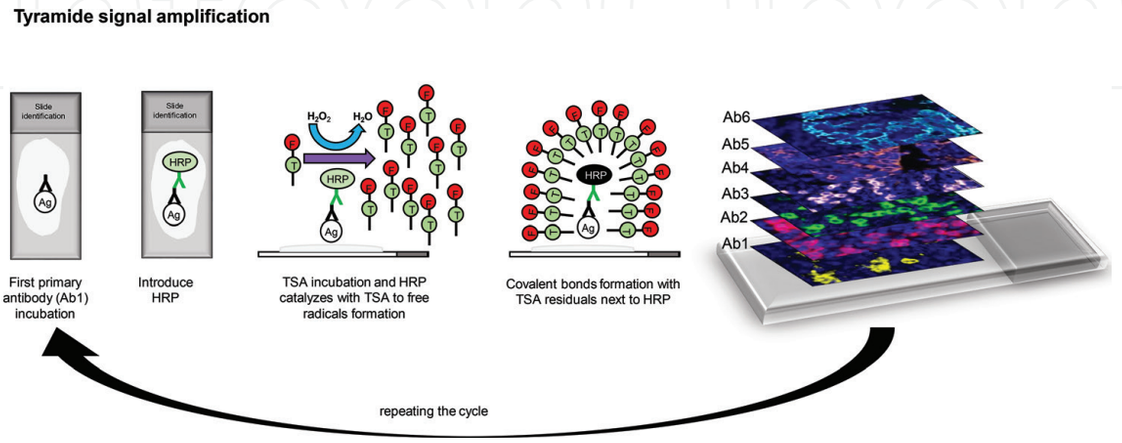



**Figure 1.**
*Tyramide signal amplification workflow. After primary antibody (Ab), the HRP-conjugated secondary antibody binds to an unconjugated primary antibody specific to the target/antigen of interest. Detection is ultimately achieved with a fluorophore-conjugated tyramide molecule that serves as the substrate for HRP. Activated tyramide forms covalent bonds with tyrosine residues on or neighboring the protein of interest and is permanently deposited upon the site of the antigen. The method allows for serial cycles of the primary/secondary antibody pairs, while preserving the antigen-associated fluorescence signal, making this process amenable to multiple rounds of staining in a sequential fashion.*

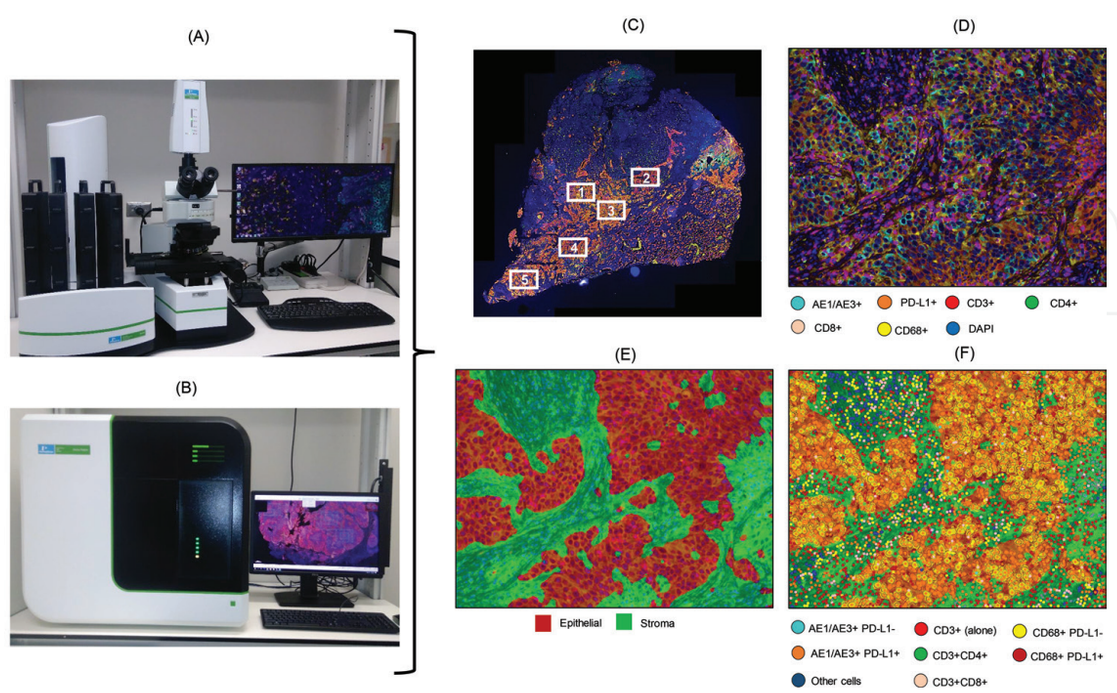



**Figure 2.**
*(A) Vectra® and (B) Polaris™ scanner systems, (C) low magnification image, showing the selection of five intratumoral areas of interest to be analyzed, (D) composite image of lung cancer tissue showing seven color markers to identify different cell populations, (E) tissue segmentation (epithelial and stromal compartments), and (F) cell populations' immune phenotyping.*

staining in control tissues. The use of specific, very well-standardized, and validated antibodies, as well as the careful use of other components, as right antibody titration, incubation time, and antigen retrieval during staining, is important to obtain good and reproducible results using different panels [27]. The use of very well-known control tissues during each staining and for each created panel that allows all the markers is important and essential to detect possible staining errors during the process in each mIF panel. Properties of the FFPE material, such as sample age, method of preservation, storage conditions, and tissue type, are very important factors to be considered to obtain high-quality mIF staining and good data. Pathologists play a key role in making sure that tissue samples collected are appropriate for diagnostic and research purposes. The tissues need to be processed adequately, that is, fixed in 10% formalin and stored in good conditions to avoid antigenic deterioration that can influence the process when targeting several proteins using this methodology. Type of tissue, is another important factor to be considered, sometimes as a limitation factor for a quality staining with this technique. We observed that some tissues that have abundant fat as breast tissue or cartilage in some type of cancers or bone component that were submitted a decalcification procedures, are more challenges during the mIF staining, showing frequently artifacts of staining like background, folds, caused by tissue detached and unspecific or not clear staining on the cells, causes by the decalcification procedures. Antibodies with very good performance in decalcified tissues are limited, and those need an exhaustive validation in IHC before creating a new panel to stain these samples. No less important, the size of the sample is another factor to be considered during mIF staining; small biopsies as core needle biopsies (CNBs) less than 1.0 × 0.2 cm are more challenging and have high probability to be lost during standard mIF staining than bigger tissues as whole sections (~1.0 × 1.0 cm). The minimum number of malignant tumor cells required for mIF marker analysis has not been well established and is another factor to be considered during staining and analysis.

| Specimen type | Size (cm) | Viable tumor cells (N) | Necrosis (%) | Fat/cartilage/bone (%) | Adequacy for mIF staining* |
|---|---|---|---|---|---|
| Whole section | >1.0 × 1.0 | >100 | 0 or <10 | 0 or <10 of any component | 100% in our series |
| Whole section | >1.0 × 1.0 | >100 | 0 or >10 | >50 of any component | 80% in our series |
| Small biopsies | >1.0 × 0.2 | >100 | 0 or <10 | 0 or <5 of any component | 100% in our series |
| Small biopsies | <1.0 × 0.2 | >100 | <10 | <50 of any component | 70% in our series |
| Small biopsies | <1.0× 0.2 | <100 | >10 | >50 of any component | 50% in our series |
| TMA (by core) | >0.1 | >100 | 0 or <5 | 0 or <5 of any component | 100% in our series |

*A preliminary quality control to establish the samples by a pathologist is strongly recommended to optimize the preparation of tissue for multiplex immunofluorescence staining and ultimately to guaranty a quality data. Each case needs to be considered separately and can be influenced by several characteristics. In general, the quality of the samples including the fixation process of the FFPE tissues, storage and cutting procedures, will influence the quality of multiplex staining (*). There are, however, according to our experience, different tissue characteristics that need to be considered as challenges for staining and analysis, and these are considered sometimes as limitations of the staining. By understanding much better these tissue limitations, we can avoid wasted effort, resources, and funds of the laboratory as well as preserve the high-quality data obtained by this technique.*

**Table 1.**
*Quality criteria's samples for multiplex immunofluorescence.*

According our experience, samples with at least more than 100 malignant cells are preferred, to avoid errors in the interpretations of different markers, especially when the targets of study are malignant cells. Necrotic areas in more than 50% of the entire sample can compromise the quality of the staining and when compromise the quality of the sample and the staining need to be considered judiciously by the pathology as excluded criteria to preserve the quality of the analysis and data (**Table 1**). The quality control of pathology, as a first step, is essential to avoid wasted effort, resources, and funds of the laboratory and to preserve the high-quality data obtained by this methodology.

## 3. Image approaches and data analysis

Although the methodology of TSA is available for FFPE material and can enable multiparametric readouts from a single tissue section, they sometimes have limited scalability and throughput, related to limited number of markers allowed per panel compared with other multiplex methodologies like imaging mass cytometry and multiplexed ion beam imaging [35, 36]. The scanner system (**Figure 2**) Vectra® [27] from PerkinElmer provides high quality of scanning with high-resolution and multiband filter cubes that provide greater flexibility associated with the multispectral camera, to match with the sample. The new generation of scanner Polaris™ (PerkinElmer) scan system supports multiple filters using tunable LED excitation, similar to confocal microscope, and the captured signals are assembled in a composite image [37]. After acquiring the panoramic low-magnification images at ×4 or ×10, the specimens can be sampled using different ROI sizes by the phenochart (PerkinElmer) software viewer to scan high-resolution images at ×20 or ×40. Although, the scanner system Vectra®-Polaris™ can capture different regions of interest (ROIs) using the filters and the multispectral camera at high quality resolution [36], it is still impossible to accelerate the process of scanning or scan the whole tissue section as a unique image for the analysis. The time for scanning the sample is variable and depends on the number of markers used in the panel, number of ROIs captured per sample, and size of the ROI and can take from minutes to several hours according these parameters [38] (**Table 2**). According our experience, the TSA

| Magnification | Scanner | | ROI (Vectra®)/(Polaris™) (seven markers) |
|---|---|---|---|
| | Vectra® (Time/minutes) | Polaris™ (Time/minutes) | |
| 4×* | ~9 | – | Panoramic view |
| 10×* | ~18 | ~13 | Panoramic view |
| 20× | ~6 | ~5 | 1×1 (669×500 μm)/(931×698 μm) |
| 20× | ~12 | ~14 | 2×2 (1338×1000 μm)/(1862×1396 μm) |
| 20× | ~22 | ~35 | 3×3 (2007×1500 μm)/(2793×2094 μm) |
| 40× | ~4 | ~8 | 1×1 (334×250 μm)/(465×249 μm) |
| 40× | ~10 | ~20 | 2×2 (669×500 μm)/(931×698 μm) |
| 40× | ~19 | ~36 | 3×3 (1004×750 μm)/(1396×1047 μm) |

*The time for scanning the sample is variable and depends on the number of markers used in the panel, number of regions of interest (ROI) captured per sample, and size of the ROI using Vectra® or Polaris™ system, as well as whether the system stores the image locally or in a server. *Available only in Vectra®.*

**Table 2.**
*Approximate time for image scanning using Vectra® or the Polaris™ scanner system.*

| Vendor | Program name | Method | Availability |
|---|---|---|---|
| PerkinElmer | InForm | Color-based colocalization, tissue, cell segmentation | Licensed |
| Definiens | Tissue Studio | Imaging segmentation, marker intensity measurement, and statistical analysis | Licensed |
| Indica Labs | HALO | Membrane, colocalization, immune cell proximity, spatial analysis | Licensed |
| Visiopharm | Visimoph Tissuemorph | Signal intensity, area, counting objects, statistical analysis | Licensed |
| Spot Imagine | Spot advanced | Color-based colocalization | Licensed |
| FARSIGHT | Nucleus Editor | Multichannel-based object identification/ toolkit | Free |
| NIH | Image J | Color-based, user interactive segmentation | Free |
| HistoRx | AQUAnalysis | Signal intensity per unit area and per layer | Licensed |
| CompuCyte | iCyte | Nucleus segmentation or phantom contouring, measuring associated signals | Licensed |

**Table 3.**
*Image analysis software systems available for multiplex immunofluorescence.*

staining system for mIF when combined with multispectral image analysis software, such as InForm (PerkinElmer), can provide a powerful tool for analysis of multiple markers in one single slide [21, 39]. However, there are many available software in the market that can be used for the analysis of mIF images generated by the InForm software from the Vectra®-Polaris™ scanner systems, and it is important to know that the InForm software is essential to generate the individual unmixed tyramide fluorochrome with a positive signal without noise or aberrant background staining and with high resolution performance across the different ROIs from the scanning systems [40]. For the analysis, image analysis software need to be accessible (**Table 3**), with easy automated capabilities of detection, including tissue segmentation, compartmentalization of the staining (e.g., nuclear, membranous, or cytoplasmic) (**Figure 2**), and spatial colocalization of cell distribution, critically important to study different markers included in different panels (**Figure 3**). In the same way, comprehensive evaluation using this different image analysis software is needed not only for clear antigen demarcation and good staining procedures but also for good interpretation of the results. Pathologists are very important and need to standardize the possible interobserved variation [41, 42] when using different image analysis platforms during the colocalization of proteins.

## 4. Multiplex immunofluorescence staining from translational research

Despite the evolution in the last years, in different levels of cancer research, concerning prevention, diagnosis, therapeutic options, and follow-up methods, cancer diseases are still the major public health problem worldwide [43]. Profiling immune cells is currently a powerful metric for tumor subclassification and predicting clinical outcomes. A great variety of cancer research screening tools is applied to diagnose tumors and has been established for different tumors. Simultaneous quantification of more than one biomarker at the same time has become more and more interesting in cancer research using different multiplex technologies. Multiplex TSA can allow different biomarkers in one single slide, targeting different

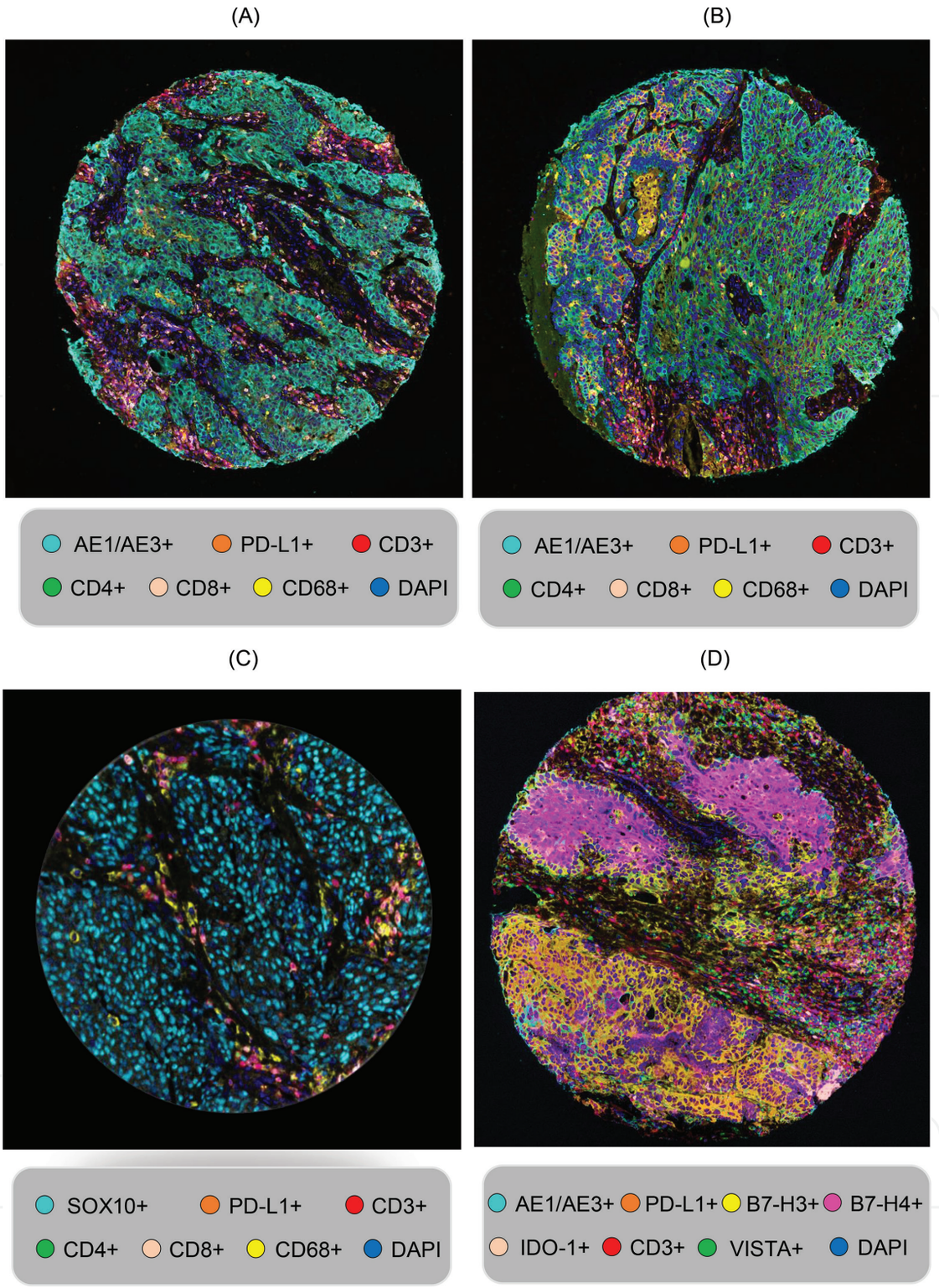



**Figure 3.**
*Microphotographs of representative examples of multiplex immunofluorescence in tumor tissues using different markers, (A) lung adenocarcinoma, (B) lung squamous cell carcinoma, (C) malignant melanoma, and (D) lung squamous cell carcinoma. ×20 magnification.*

systemic processes, such as inflammation, immunocheckpoints, angiogenesis, or cell death using tumor markers (**Figure 3**), to improve cancer prevention, diagnostic accuracy, and treatment. We demonstrated that this method can offer important advantages, such as high-throughput performance, low material requirement, wide range of applications, and cost- and time-effective multiplex for several parameters in different panels [23, 44, 45]. Several biomarkers can be cancer-specific since malignant cells of different histologic types can produce different patterns of

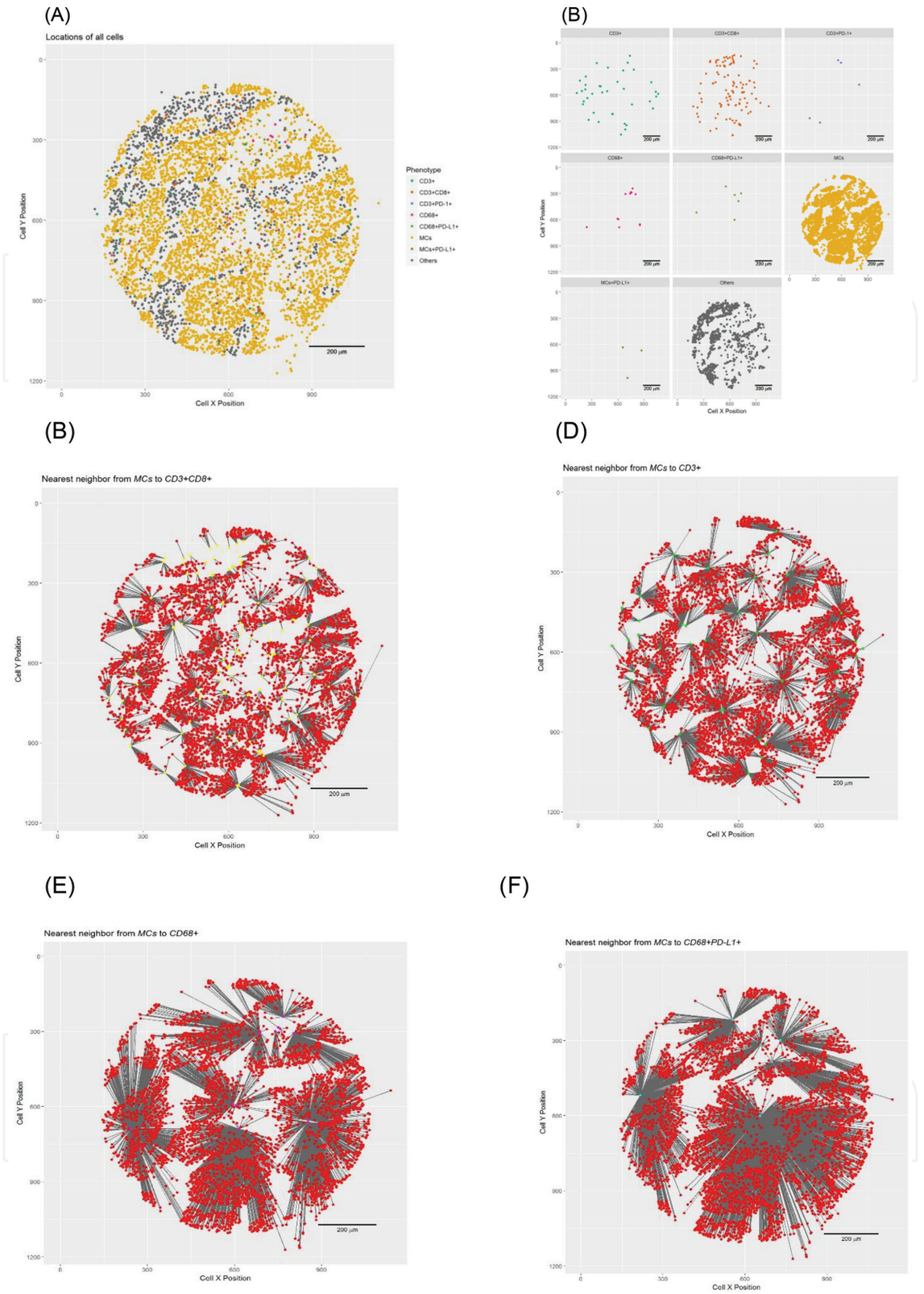



**Figure 4.**
*Microphotographs of representative examples of spatial-distribution visualization of different phenotypes analyzed. (A) distribution of individual cells using X and Y positions, (B) spatial localization of selected cells, and (C to F) distance measurements between malignant cells (MCs) and different cell populations.*

proangiogenic factors, growth factors, and immune cells that are tumor related. The study of biomarker panels (**Figure 3**) and its spatial distribution (**Figure 4**) can be used for early diagnosis and assessment of therapy response [46]. This methodology can represent an ideal method to realize personalized therapies using efficient mIF panels and help to understand much better the cancer microenvironment,

highlighting the benefit for exploring immune evasion mechanisms and finding potential biomarkers that allow researchers to assess the mechanism of action and predict and track response [47].

## 5. Conclusion

The detection of multiple markers in the same tissue section can provide important and efficient means to apply this technology in disease diagnosis, prevention, and translational research. Multiplex immunoflourescence platforms have emerged more and more from translational research labs toward the clinic, increasing the opportunity to study and understand much better the tumor-immune interactions. This methodology and different image analysis strategies can give important information about immune cells' coexpression and their spatial-pattern distribution in the tumor microenvironment. Development of multiplex immunoflourescence based-TSA system requires a very well-trained multidisciplinary team including pathologists, oncologists, and immunologists. In addition, this methodology requires automation to provide efficient and fast information as well as easy analysis methodologies for research pathologists that currently use this method.

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## Conflict of interest

The author does not have any type of competing interest.

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