DISCOVERY
LIFE SCIENCES

# USER GUIDE

## Dissociated Tumor Cells (DTCs)

### GENERAL OVERVIEW

The foundation of the future of biomedical research requires access to highly-annotated primary human biospecimens. Primary tissue is an integral part of therapeutic and diagnostic research, but the logistical barriers of acquiring fresh tissue remain an impediment to advances in medicine, requiring the coordination of not only the tissue collection but also the downstream applications in the laboratory. Dissociation and cryopreservation of solid diseased and normal tissue provides the opportunity to understand the cellular composition of these complex three-dimensional structures at the single cell level without the need to source new fresh tissue for each analytical assay. These single cell suspensions remain viable following cryopreservation and ease the demands on large-scale experimental assays. Furthermore, these cells provide the ability to screen new biomarkers and therapeutic targets as they are uncovered. The Discovery Dissociated Tissue Cell (DTC) User Guide describes the process of tissue acquisition, processing, and cryopreservation, as well as best practices for handling these specimens and preparing them for downstream analysis.

In addition to providing cryopreserved DTCs, Discovery has optimized analytical pipelines for the following services:

- Custom Tissue Processing
- Flow Cytometry
- Cell Culture
- Single-Cell Sequencing

# TISSUE ACQUISITION AND PROCESSING

Discovery Life Sciences manages a global clinic network of cancer centers and local hospital sites to acquire primary human biospecimens, including blood, bone marrow, and tissue. Unlike blood and bone marrow, which are drawn multiple times from a patient, tissue is often resected only once. While core needle biopsies and fine needle aspirates are more routinely performed, the amount of material present in these specimens is minimal and restricts the amount of downstream processing and analysis that can be performed. Resection material, on the other hand, provides enough material for in depth analysis, but the logistical demands of sourcing this tissue and getting it to a central laboratory for processing are immense. To alleviate this issue, Discovery has established two central processing facilities - one in the United States and one in Europe - that receive and process tissue specimens (**Figure 1.1**). Following resection, the tissue is immediately placed in a tissue transport solution and shipped to the central laboratory in a controlled shipping container to maintain temperature at 4°C. Tissue is received in the processing facility at 24-72 hours post-resection and immediately processed. The tissue is weighed and evaluated for necrotic and adipose tissue, which are removed before processing the sample.

After gross evaluation of the tissue, the specimen is minced and placed into a proprietary enzymatic and mechanical dissociation process that has been optimized for each indication (**Figure 1.2**). Following this process, which occurs at 37°C and takes 1 hour, the cells are strained and washed prior to counting the specimen to determine cell yield and viability. The amount of tissue received and total number of cells generated per gram of tissue varies greatly both within and across indications (**Figure 1.3**). Grossly, two groups of indications have been identified based on tumor weight and cell yield, and cells are cryopreserved at different cell concentrations between these indications (**Table 1.1**). Currently, Discovery has dissociated ~4,500 unique tumors across numerous indications (**Table 1.2**).

DTCs are cryopreserved in CryoStor CS10, slow-frozen overnight at -80°C, and stored long term in the vapor phase of liquid nitrogen. After a minimum of 8 hours in liquid nitrogen, a vial from each lot is removed for post thaw quality assessment, including cell count and viability. High viability is observed both immediately following dissociation, which is largely maintained following the cryopreservation process (**Figure 1.4**), and the cell counts from multiple vials from the same parent sample are very similar (**Figure 1.5**). In addition to cell count and viability, flow cytometric analysis of the cellular composition of the sample is also performed during the post thaw quality assessment. Flow cytometry analysis evaluates the relative percentage of tumor and immune cells in the specimen, with the immune cell compartment being further dissected into the major lymphoid (CD4+ T cells/CD8+ T cells/B cells/NK cells) and myeloid (CD11b+ myeloid cells/CD14+ monocytic cells/CD15+ granulocytic cells) populations. All of this data is generated immediately following thawing and dilution with media and does not occur after washing the specimen to ensure the most accurate representation of the original contents of the sample. Finally, all DTCs are annotated with base patient data (age, sex, race, ethicity) as well as the subtype and stage of the specimen. Additional data, including prior treatment history, are also available with some samples. All of these data are required before DTCs are passed into inventory to allow for precise sample selection for downstream studies.

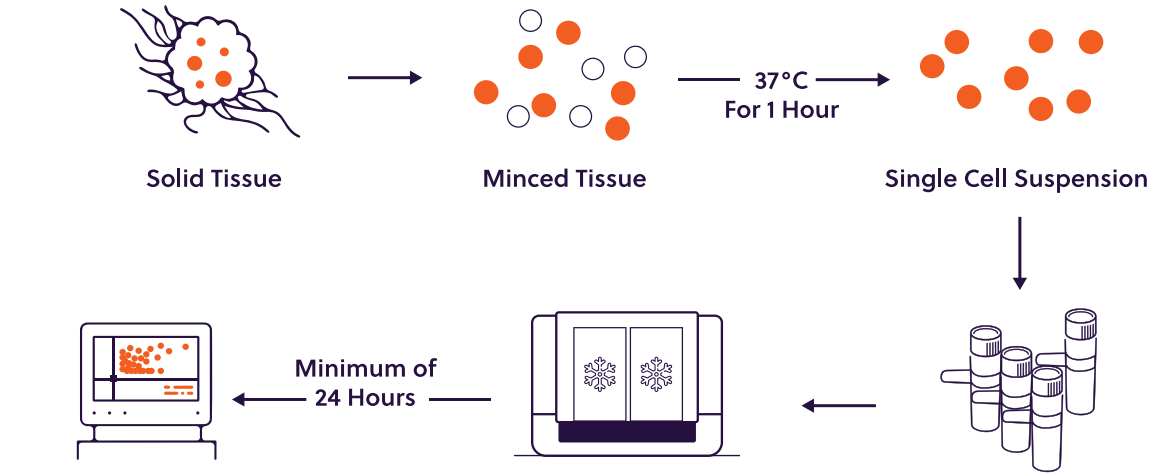
## FIGURE 1.1

Clinical Network Collection Sites

Shipped immediately in controlled 4°C container

Received within 24-72 hours and immediately processed

## FIGURE 1.2




Solid Tissue

Minced Tissue

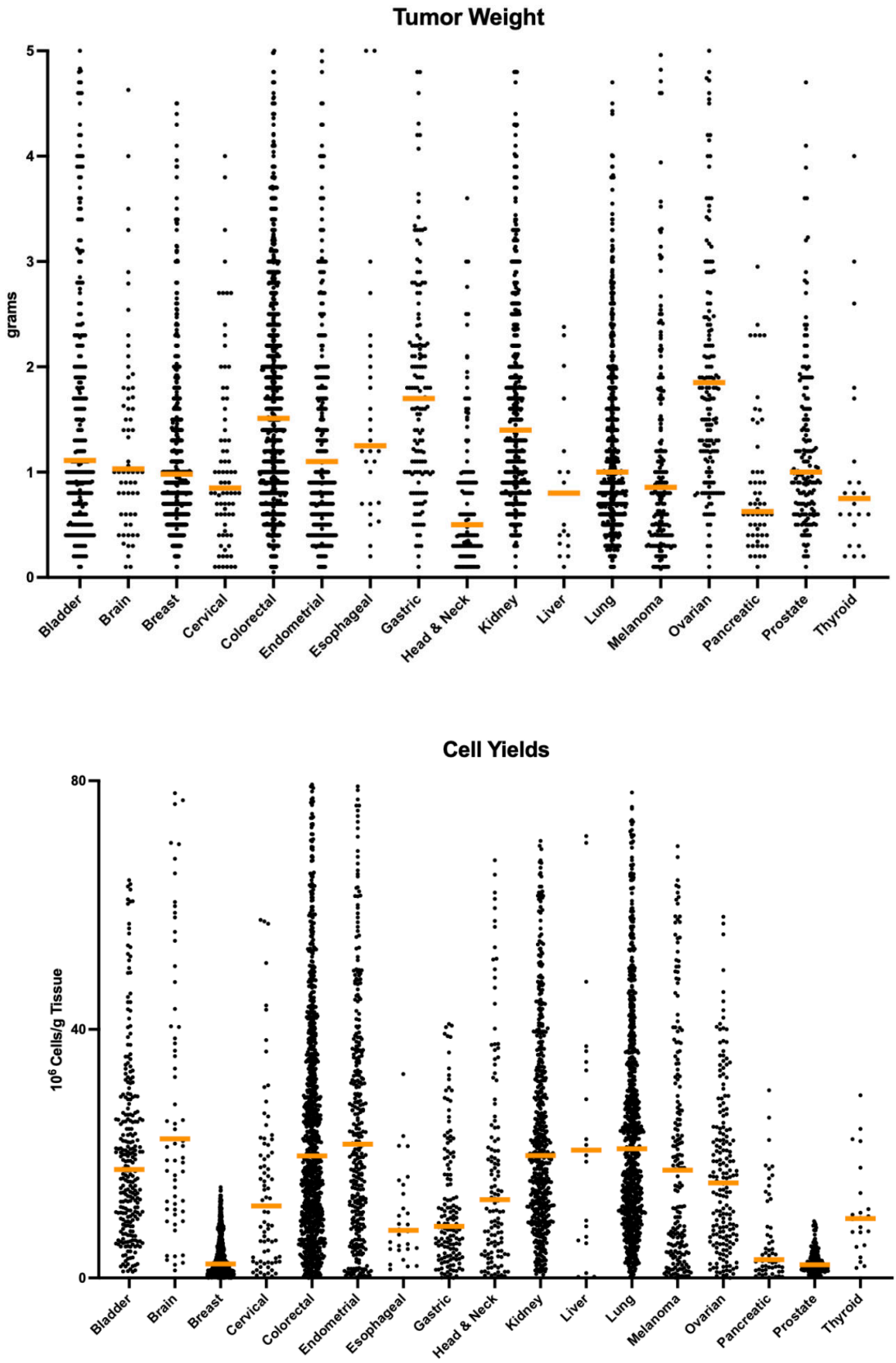
Single Cell Suspension

Aliquot per Indication in CS10

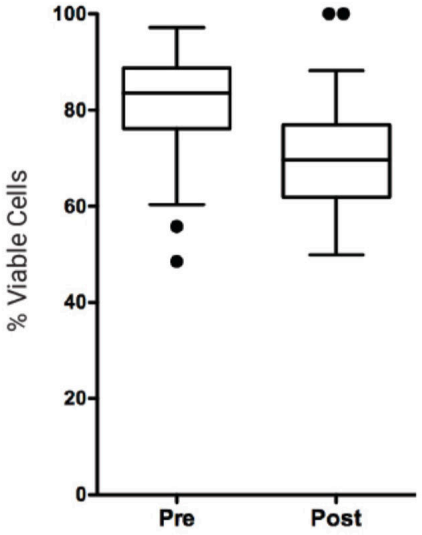
Slow Freeze at -80°C

Post Thaw QC Cell Count
Viability Flow Cytometry

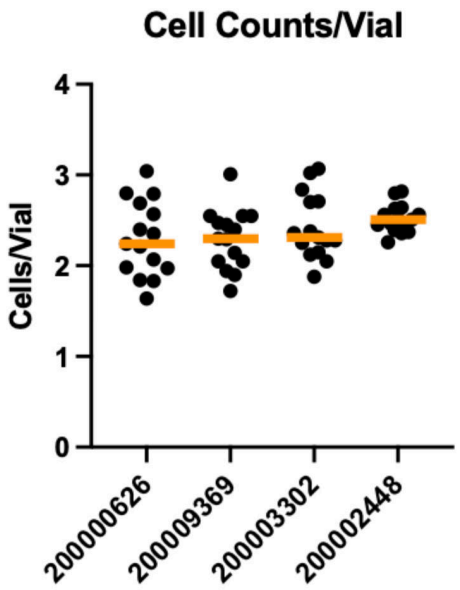
## FIGURE 1.3



## FIGURE 1.4




## FIGURE 1.5




## TABLE 1.1

| 3x10⁶ Cells/Vial | 1x10⁶ Cells/Vial |
|---|---|
| Bladder | Breast |
| Brain | Cervical |
| Colorectal | Esophageal |
| Endometrial | Head & Neck |
| Gastric | Liver |
| Kidney | Pancreatic |
| Lung | Prostate |
| Melanoma | Thyroid |
| Ovarian | |

## TABLE 1.2

| Primary Diagnosis | Count |
|---|---|
| Bladder Cancer | 260 |
| Brain Cancer | 67 |
| Breast Cancer | 573 |
| Cervical Cancer | 77 |
| Colorectal Cancer | 953 |
| Endometrial Cancer | 329 |
| Esophageal Cancer | 28 |
| Gastric Cancer | 147 |
| Head and Neck Cancer | 124 |
| Kidney Cancer | 525 |
| Liver Cancer | 20 |
| Lung Cancer | 826 |
| Melanoma | 202 |
| Ovarian Cancer | 188 |
| Pancreatic Cancer | 56 |
| Prostate Cancer | 172 |
| Thyroid Cancer | 22 |
| **Total** | **4569** |

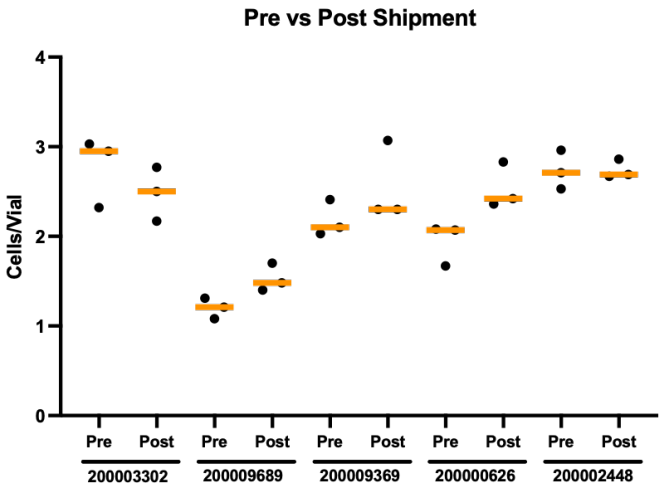
# DTC RECEIPT AND HANDLING

Dissociated tissue cells (DTCs) are cryopreserved for long term storage in the vapor phase of liquid nitrogen. DTCs are shipped on dry ice and should be used immediately upon receipt or stored in liquid nitrogen vapor phase. It is not recommended to store DTCs for long term at -80°C. Shipment of DTCs on dry ice does not negatively impact the cell counts of the samples (**Figure 2.1**). To thaw DTCs, the vial is gently swirled in a 37°C water bath until only a small ice crystal remains. The vial is sprayed thoroughly with 70% ethanol or isopropanol, dried and transferred into a biosafety cabinet. Depending on the total number of cells present in the vial, the contents are diluted in 1-4mL of media to ensure that the cell counts can be accurately determined. Cell count and viability are determined immediately after thawing and before washing using a dual nucleation dye system, specifically acridine orange, which stains all nucleated cells, and propidium iodide, which only stains dead cells. We do not recommend the use of trypan blue exclusion systems for evaluating cell counts and viability of DTCs, as non-cellular material is generated during the dissociation process. For more information, see Best Practices for Counting Dissociated Tissue. After cell counts are determined, the samples should be washed to remove any remaining cryopreservative from the sample prior to performing. Washing DTCs results in an ~30% reduction in the corresponding cell counts (**Figure 2.2**) and should be factored when calculating the total number of DTCs required for a downstream assay.

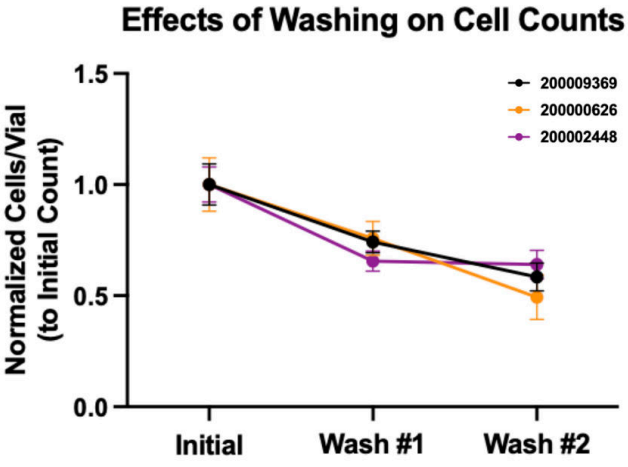
## BEST PRACTICES FOR DTC RECEIPT AND HANDLING

- Store in the Vapor Phase of Liquid Nitrogen
- Thaw Quickly in 37C Water Bath
- Dilute in 1-4mL Media to Maintain Cell Counts in Linear Range
- Use Dual Nucleation Dye Cell Counting Methods
- Avoid Trypan Blue Exclusion Counting Methods

## FIGURE 2.1




## FIGURE 2.2



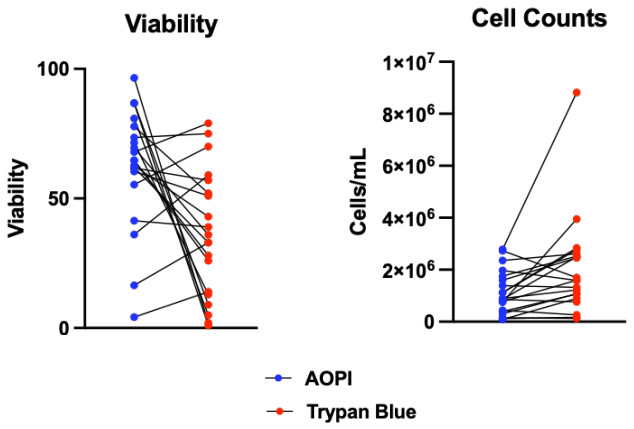
The process of generating single cell suspensions from these tissues yields not only viable and non-viable cells, but also non-cellular material, cellular debris and tissue fragments, making overall cell counts and viabilities difficult to determine. Multiple different cell counting methodologies, including trypan blue exclusion, dual nucleation dye staining, and flow cytometry, have been evaluated to determine their utility in assessing cell counts and viability of dissociated human tissue, and based on these studies, it is recommended that dual nucleation cell countings be used to ensure only nucleated live and dead cells are identified. For flow cytometry analysis of dissociated tissue, at a minimum the use of a live/dead discriminator, such as DAPI or PI, is highly recommended. Additionally, if possible within the study being performed, the addition of a nucleation dye, such as DRAQ5, can aid in separating nucleated cells from non-nucleated material and improve study results.

To evaluate the cell count and viability of dissociated human tissue, two popular cell counting techniques - trypan blue exclusion and acridine orange/propidium iodide (AO/PI) staining - were evaluated. Twenty unique dissociated human tumor samples were counted using automated cell counters utilizing both methods, and the resulting cell counts and viabilities were compared. The live cell counts, presented as cells per milliliter, were comparable using either cell counting method (**Figure 2.3**). However, the overall viability of the samples varied greatly when analyzed using the trypan blue and AO/PI counting methods. In most samples, the viabilities reported using AO/PI were higher than those reported using trypan blue. When the brightfield and fluorescent images from the trypan blue and AO/PI counting methods were evaluated, some significant differences were observed (**Figure 2.4**).

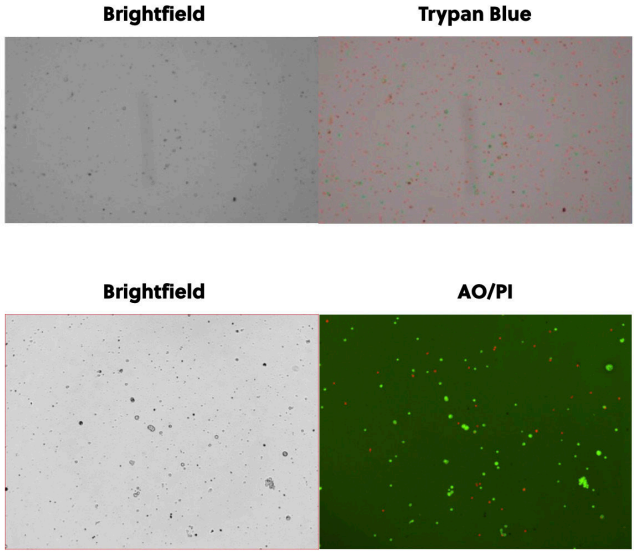
The brightfield images on both automated cell counters showed a significant amount of material present. Using trypan blue, a majority of this material was identified as dead cells, with a small proportion being identified as live cells. However, using AO/PI staining, the majority of this material was acellular, as it did not stain with either acridine orange (green, representing all nucleated cells) or propidium iodide (red, representing dead cells). By only factoring in actual nucleated cell events, the overall cellular viability was greatly improved, and it is recommended that dye nucleation dye systems be utilized when analyzing cell counts and viabilities of dissociated tissue.

Another method for evaluating cellular viability flow cytometry. To evaluate the viability of dissociated human tissue, DRAQ5 was used to identify nucleated cells, while DAPI was used to distinguish live versus dead cells. Representative flow diagrams for a low debris sample and high debris sample are presented in **Figure 2.5**. The presence of debris in dissociated tissue is problematic when trying to evaluate not only the viability of the sample, but also accurately determining the cellular composition of the tissue. By using DRAQ5, the nucleated cells (DRAQ5+) and the non-nucleated debris (DRAQ5-) fraction in the specimen can be identified. Also, by gating on the DRAQ5+ cells and evaluating DAPI staining, DAPI- live cells and DAPI+ dead cells can also be identified. This staining profile provides the opportunity to understand how these three populations - live cells (DRAQ5+ DAPI-), dead cells (DRAQ5+ DAPI+), and non-cellular material (DRAQ5-) - are evaluated on forward and side scatter profiles. The presence of non-cellular material made identification of live and dead cells by forward and side scatter problematic and very difficult to interpret, especially if the debris proportion is high. To better evaluate the live cell fraction within dissociated tissue, DAPI was plotted against forward scatter. By using these parameters, the live cell fraction (DRAQ5+ DAPI-, blue) could be better distinguished from the dead cell (DRAQ5+ DAPI+, orange) and non- cellular fraction (DRAQ5-, red), and therefore more easily gated on for downstream analysis. It is recommended that, at a minimum, a live/dead discriminator be used when analyzing DTCs by flow cytometry.

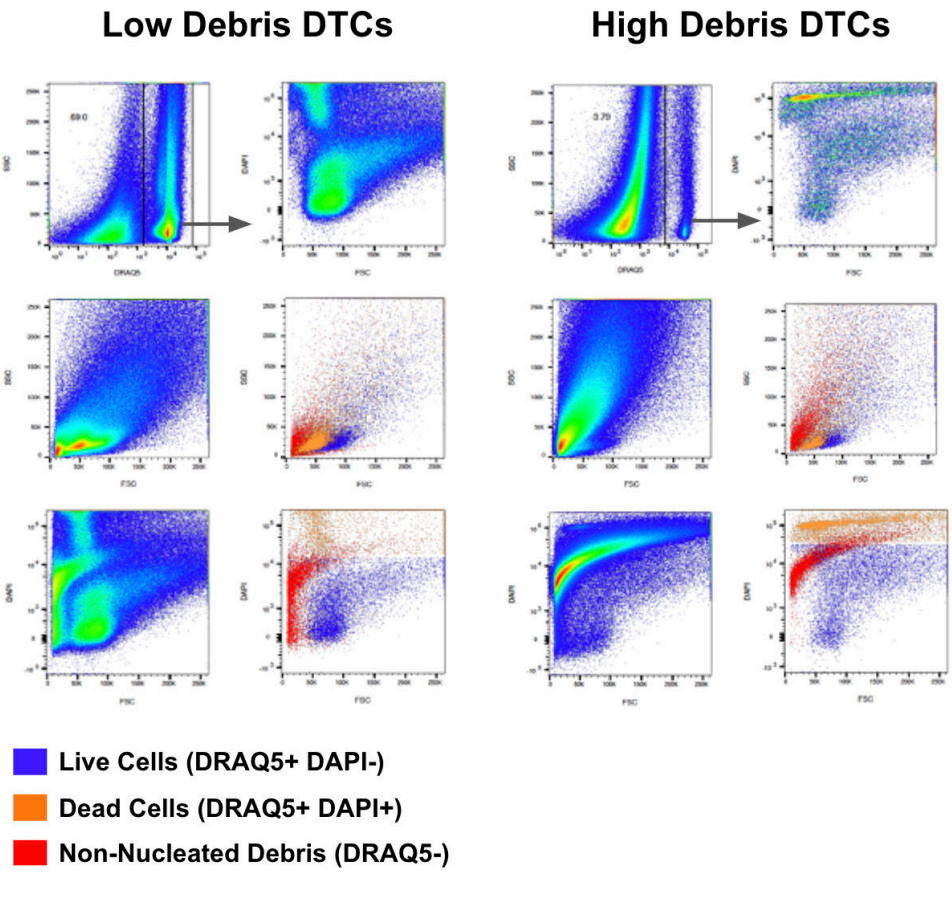
## FIGURE 2.3




## FIGURE 2.4




## FIGURE 2.5



# FLOW CYTOMETRIC ANALYSIS OF DISSOCIATED TISSUE

Dissociated tissue samples represent the entire cellular composition of the parent solid tissue. As viable, single cell suspensions, DTCs are amenable to analysis by flow cytometry to understand the complex cellular components of the tissue, as well as the surface expression of key therapeutically relevant receptors. In total, over 4,000 unique tumors have been profiled by flow cytometry, and best practices for analyzing DTCs by flow cytometry have been established to streamline processing and analysis. Through profiling such a large volume of unique dissociated tumors, indication-specific trends are observed, specifically in terms of tumor and immune content, as well as the specific immune cell subsets present. Finally, as a proof of principle, the expression of PD1/PDL1, which are currently being therapeutically targeted in a number of solid tumor indications, is examined on both normal and diseased dissociated tissue to demonstrate the utility of dissociated tissue in evaluating novel druggable targets.

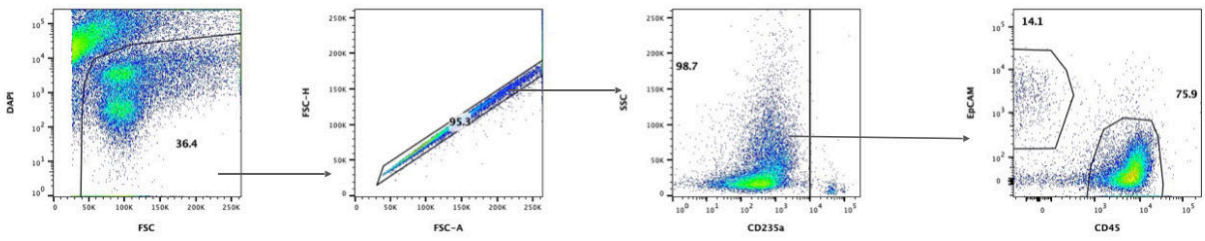
## Considerations for Flow Cytometry Analysis of Dissociated Tissue

The process of dissociating primary human tissue generates viable, single cell suspensions that are amenable to flow cytometry analysis; however, careful handling of these specimens is important to ensure the highest quality data are obtained. Due to the presence of aggregates, it is recommended that DTCs be filtered through a 70-100 µm cell strainer prior to running on the flow cytometer to prevent clogging issues. Myeloid cells within the tumor microenvironment non-specifically bind antibodies, so the use of Fc blocking reagent, such as Human TruStain FcX from BioLegend, is highly recommended. Red blood cells are not lysed during the preparation of dissociated tumor cells; If possible, the addition of a marker against red blood cells, such as CD235a, can aid in data interpretation. Finally, given the dead cells and non-cellular debris generated during dissociation, either a DNA-based or amine-reactive live/dead discriminator should be used to remove any dead cells or debris from the analysis. Using these practices, a gating strategy has been established and utilized for all dissociated tissue being evaluated at Discovery Life Sciences (**Figure 3.1**). In this strategy, dead cells and debris are first removed by gating on DAPI-negative cells when DAPI is plotted against FSC. Once live cells are identified, doublets are removed using the height and area parameters off of FSC. Finally, any red blood cells that remain are excluded using a CD235a by SSC plot. Once these initial gates are performed, the DTCs are further analyzed based on the specific study design.

### BEST PRACTICES FOR FLOW CYTOMETRY ANALYSIS OF DTCS

- Filter through 70-100um Cell Strainers
- Fc Block Prior to Antibody Staining
- Add CD235a to Gate Out Any Remaining Red Blood Cells
- Use a DNA-Based or Amine-Reactive Live/Dead Discriminator

### FIGURE 3.1



## Indication-Specific Trends in Tumor and Immune Cells in Dissociated Tumor Cells

Discovery Life Sciences has performed flow cytometric analysis on over 4500 unique patient samples across 17 different indications. For these evaluations, tumor cells and total immune cells are profiled, and the immune cell compartment is further evaluated for the presence of CD4+ T cells, CD8+ T cells, B cells, NK cells, monocytic cells, and granulocytic cells. Through this large-scale immunophenotyping endeavor, trends have been observed across indications in terms of the presence or absence of different cellular subsets.

Epithelial cell adhesion molecule, or EpCAM, is a well-established marker of tumor cells and is overexpressed in numerous oncology indications, making it a suitable marker for identifying tumor cells in epithelial cell-derived indications. However, while EpCAM is a particularly good marker of tumor cells in many indications, melanoma cells do not express EpCAM. However, three other cell surface markers – CD63, CD146, and CD166 – have been reported to be expressed on melanoma cells. When the non-immune cell (defined as CD45-) and non-red blood cell (defined as CD235a-) fraction of melanoma dissociated tumor cells was examined, all three of these markers were expressed on melanoma tumor cells (**Figure 3.2A**).

While CD166 expression was low, there was high expression of both CD146 and CD63, and there was a high correlation in the percentage of tumor cells identified by CD146 and CD63. When CD63 and CD146 expression was examined on CD45+ immune cells, however, a subset of CD45+ immune cells was identified that express CD63 (**Figure 3.2B**). In contrast, very few CD45+ immune cells express CD146, and this marker was utilized for further studies on melanoma samples. In general, higher tumor cell percentages were observed in DTCs generated from bladder, breast, colorectal, endometrial, melanoma, and ovarian cancer samples, while lower tumor cell percentages were present in head & neck, kidney, liver, and lung cancer samples (**Figure 3.3**). Melanoma samples consisted of both primary melanoma as well as metastatic melanoma to peripheral lymph nodes. There was a higher proportion of melanoma tumor cells present in the primary tumors, although tumor cells were also present in the metastatic cases as well (**Figure 3.4**). In addition to tumor cells, the tumor microenvironment consists of additional cellular populations (often grouped together as tumor stromal cells) including cancer-associated fibroblasts and endothelial cells. Both fibroblasts and CD31+ endothelial cells were present in dissociated tumor cells (**Figure 3.5**). Immune cells represent a major component of the tumor microenvironment. Immune cell percentages (as defined as CD45+ cells) were the inverse of those observed for the tumor cell compartment, with the highest immune percentages being observed in cervical, head & neck, kidney, liver, and lung specimens, while bladder, brain, and melanoma tumors had the lower immune cell percentages (**Figure 3.6**).

**FIGURE 3.2A**

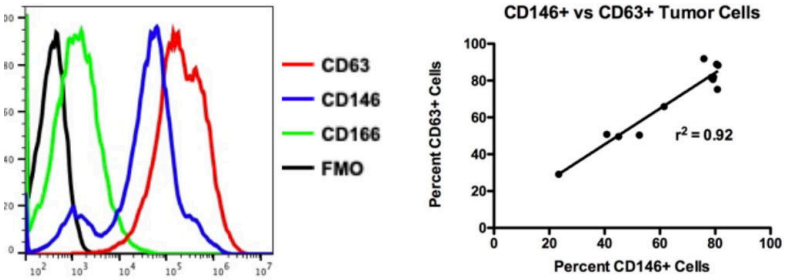



**FIGURE 3.2B**

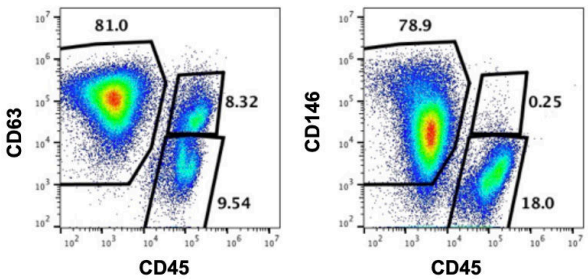


**FIGURE 3.3**

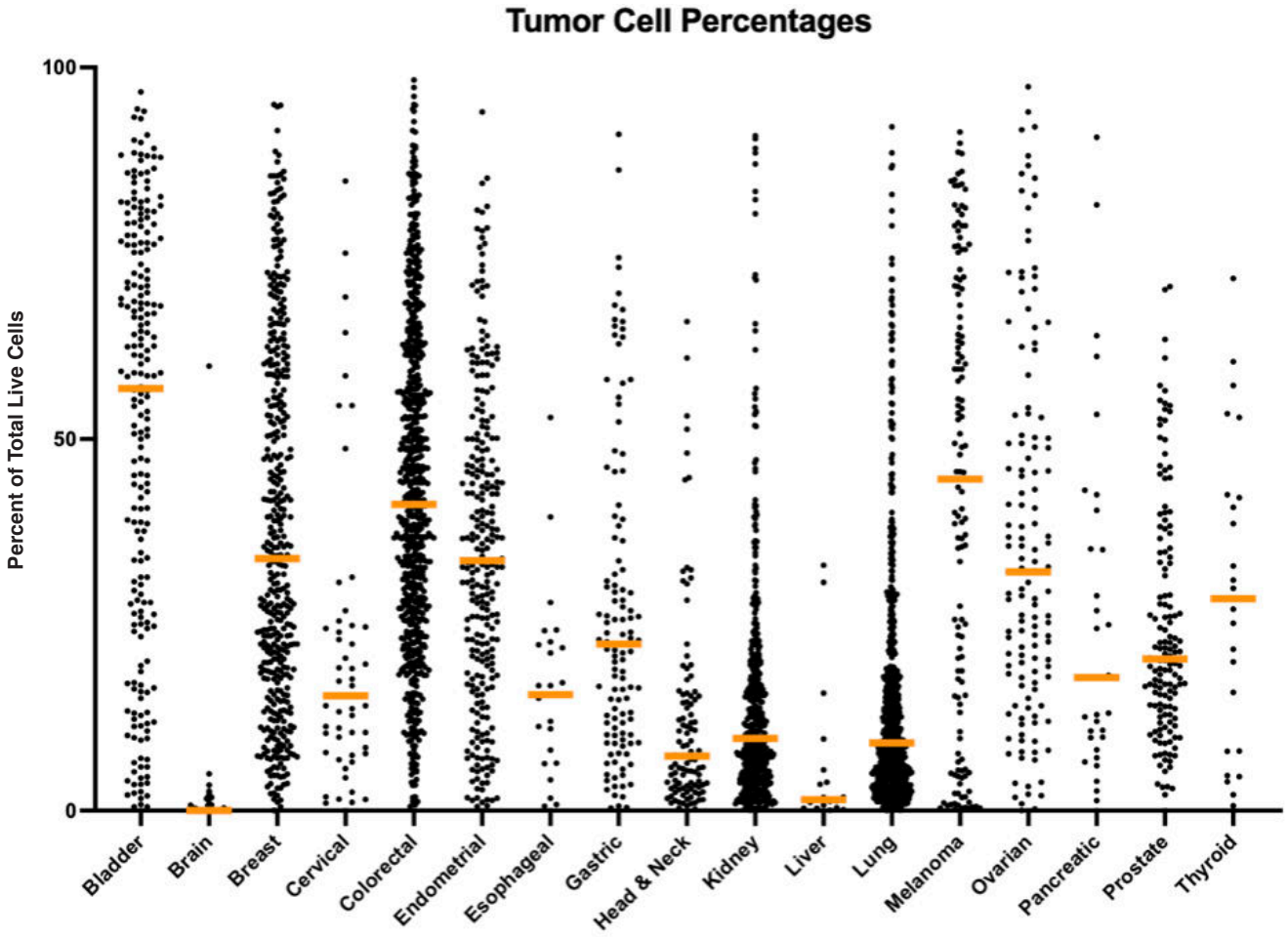



**FIGURE 3.4**

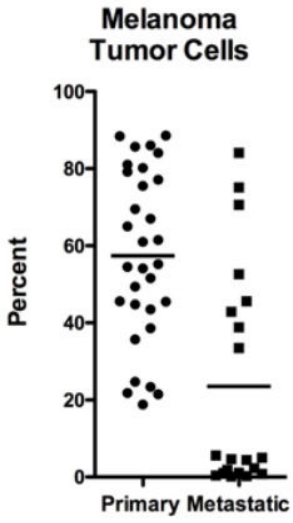
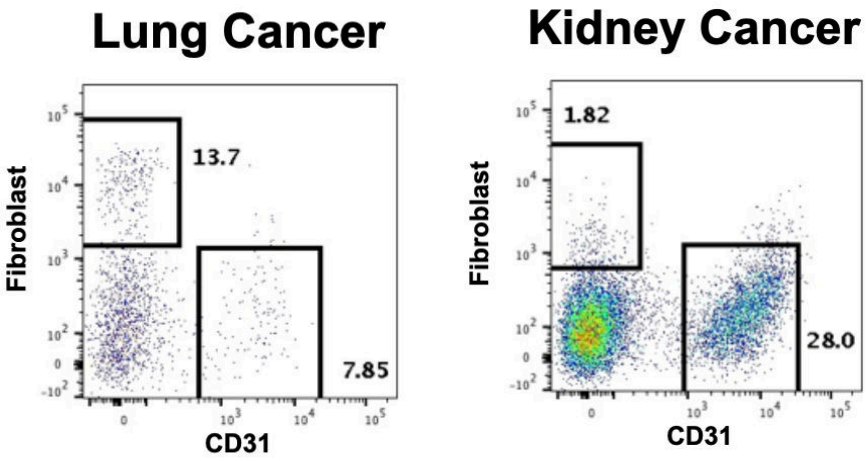
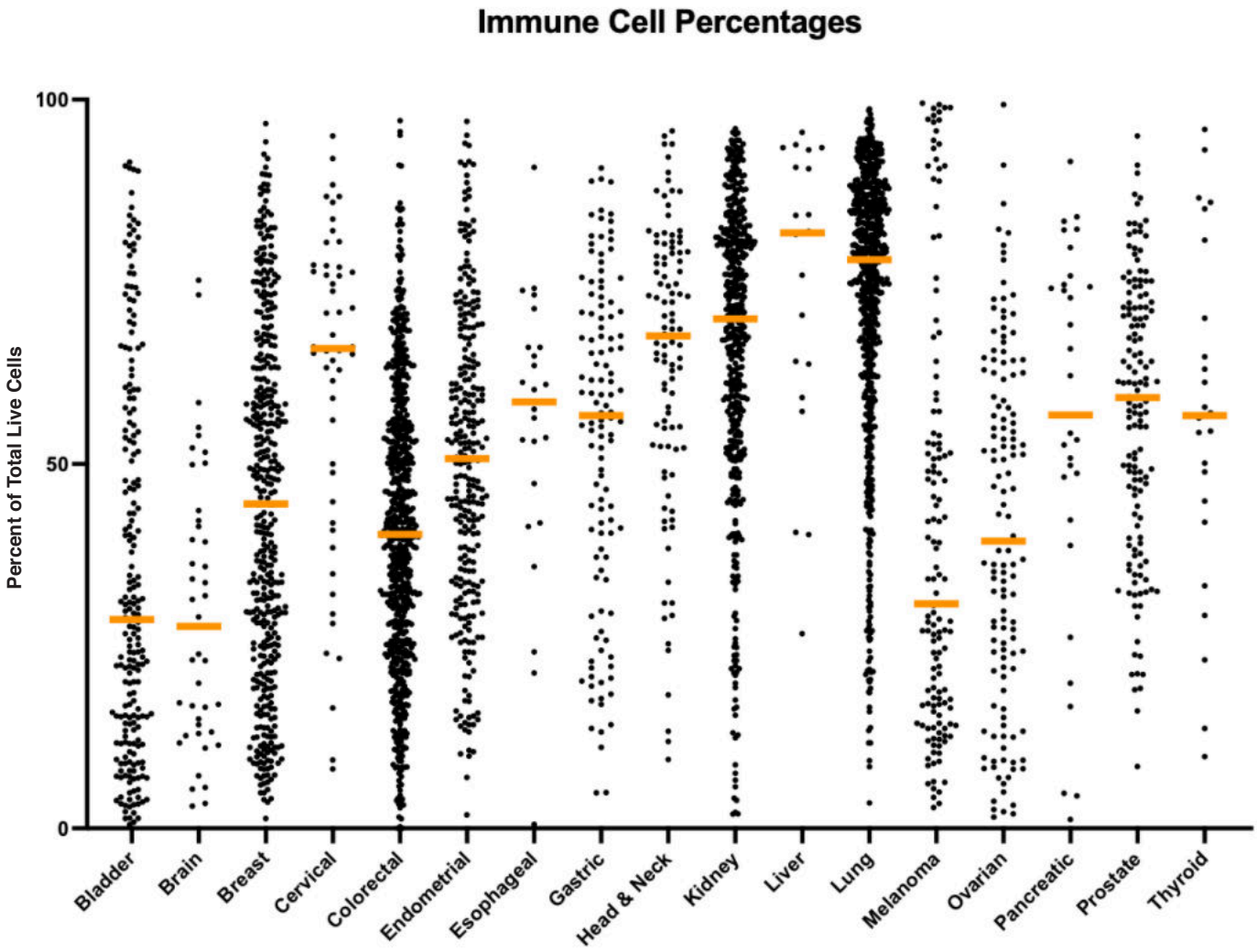



**FIGURE 3.5**



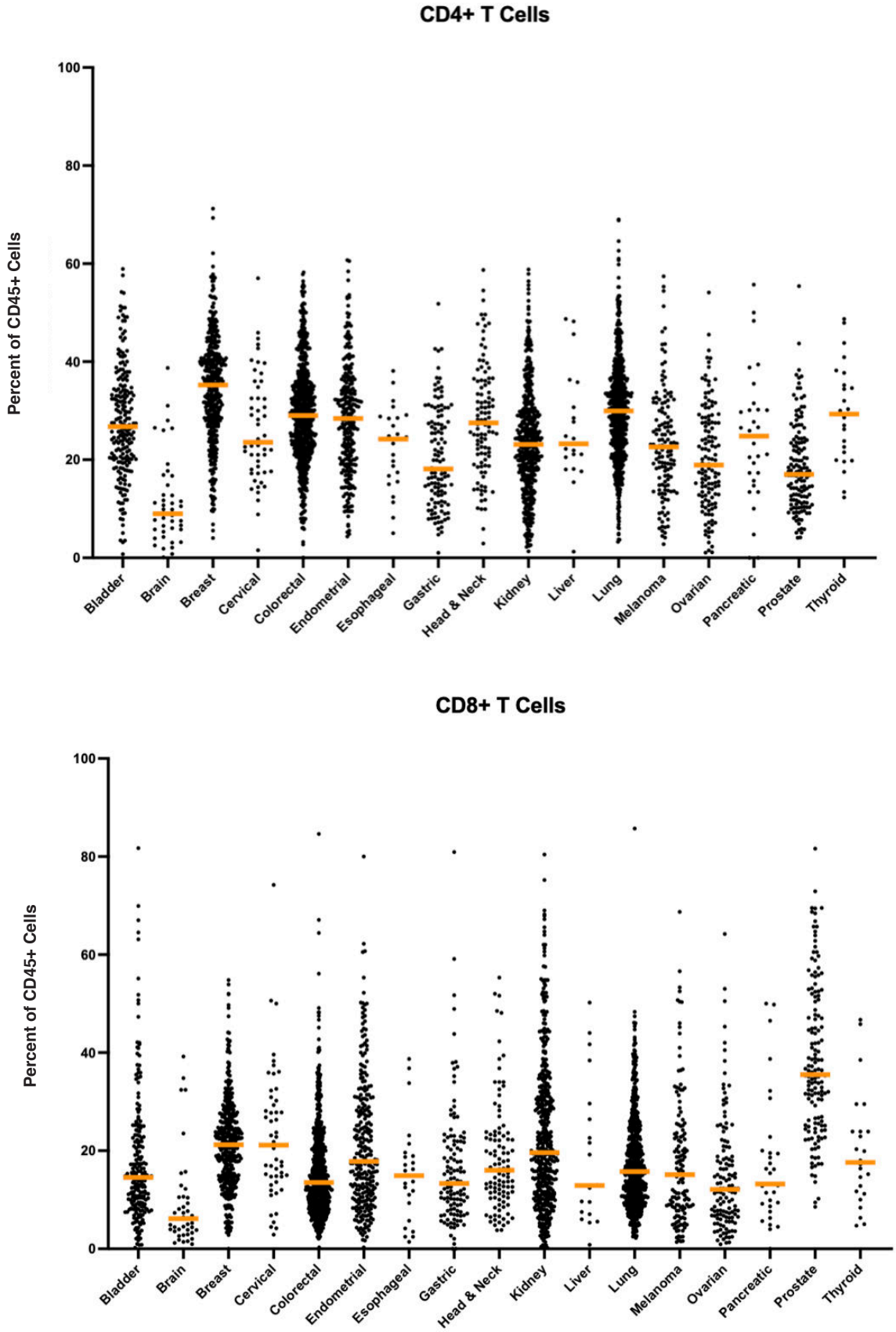
## FIGURE 3.6



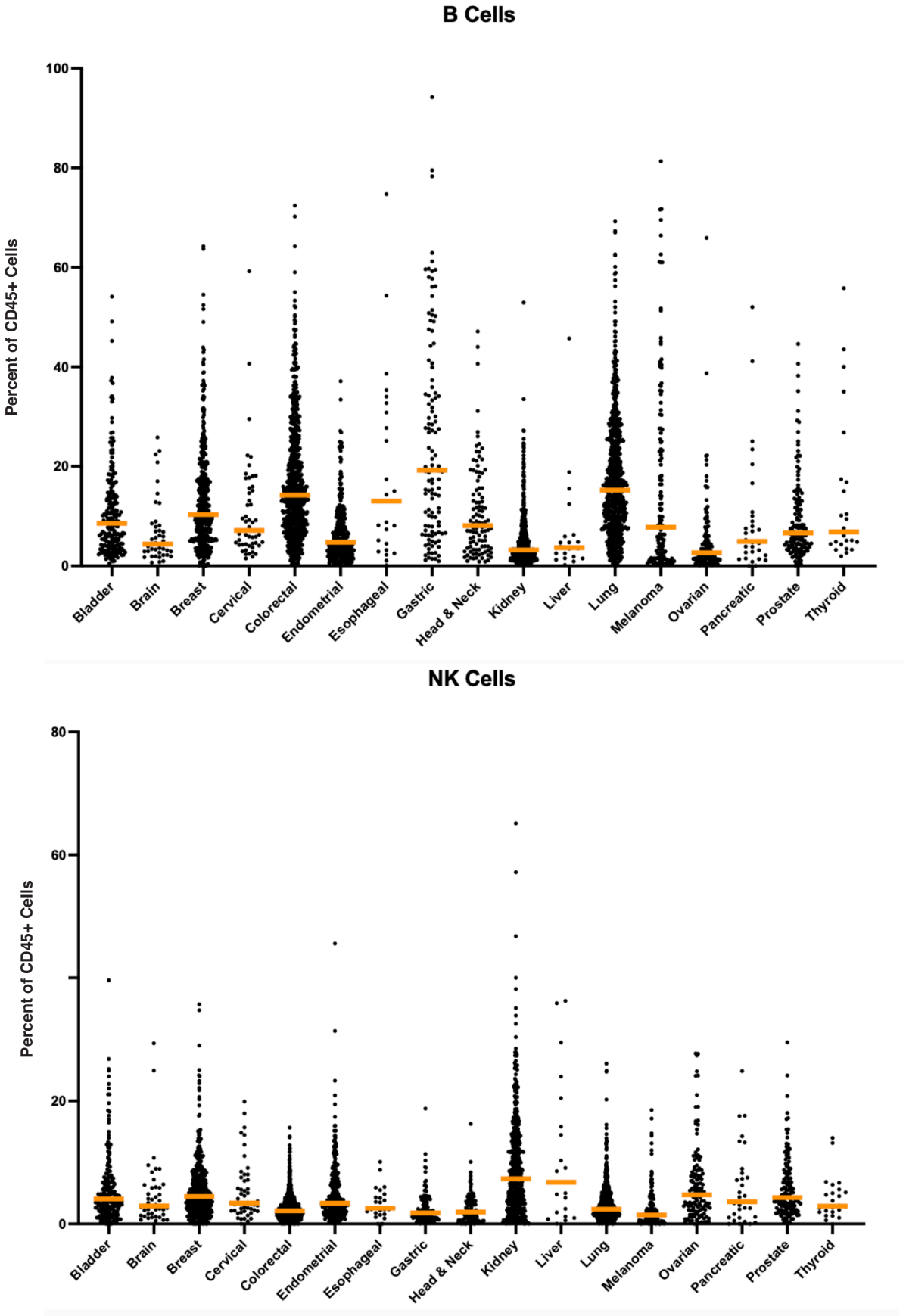
The immune cell compartment in the tumor microenvironment is composed of numerous innate and adaptive immune cell subsets. T cells represented the largest component of the CD45+ immune cell population in dissociated tumor cells, with both CD4+ T cells and CD8+ T cells observed (**Figure 3.7**). The lowest percentage of CD4+ T cells was present in the brain tumor samples, with the remaining indications ranging from 20-40% of the CD45+ immune cell compartment. Similarly, the lowest percentages of CD8+ T cells were observed in brain DTC samples, with prostate cancer samples having the highest percentage of CD8+ T cells. In addition to T cells, two additional lymphocyte populations, B cells and NK cells, are present in the tumor microenvironment. Both B cells (defined as CD19+) and NK cells (defined as CD3- CD56+) are present in dissociated tumor cells and, in general, were present at lower percentages than T cells. Across the indications, B cells were present at lower percentages in brain, endometrial, kidney, liver and ovarian DTCs, while the highest percentages were observed in colorectal, gastric, and lung dissociated tumor cells (**Figure 3.8**). NK cells, on the other hand, were very rare across all the indications analyzed, although relatively higher percentages were observed in kidney and liver cancer samples. In total, these results highlight that all major lymphocyte subsets are present in cryopreserved dissociated tumor cells and provide insight into indication-specific trends that are crucial for the next-generation of immunotherapies.

Myeloid cells are also represented within the immune cell fraction in dissociated tissue samples. Myeloid cells, defined as CD11b+, were present in all the indications tested, with the highest percentages observed in brain and ovarian tumor samples (**Figure 3.9**). Myeloid cells can be further subdivided in CD14+ monocytic and CD15+ granulocytic cells. CD14+ monocytic cells were identified in all indications, with brain, kidney, and ovarian tumor samples displaying the highest percentages of monocytic cells. Granulocytes were present in viable cryopreserved dissociated tumor cells, although at lower percentages than observed for monocytic cells. There were higher proportions of CD15+ granulocytic cells present in esophageal, head & neck, liver, and pancreatic dissociated tumor cells. In contrast, lower percentages of granulocytic cells were observed in bladder, breast, melanoma, and thyroid cancer samples. Taken together, these results demonstrate that, like their lymphocyte counterparts, myeloid cells represent a significant portion of the tumor microenvironment, and given their role in mediating the immunosuppressive environment within the tumor, highlight the need to understand the cellular composition of the tumor when deciding on the appropriate immunotherapy.

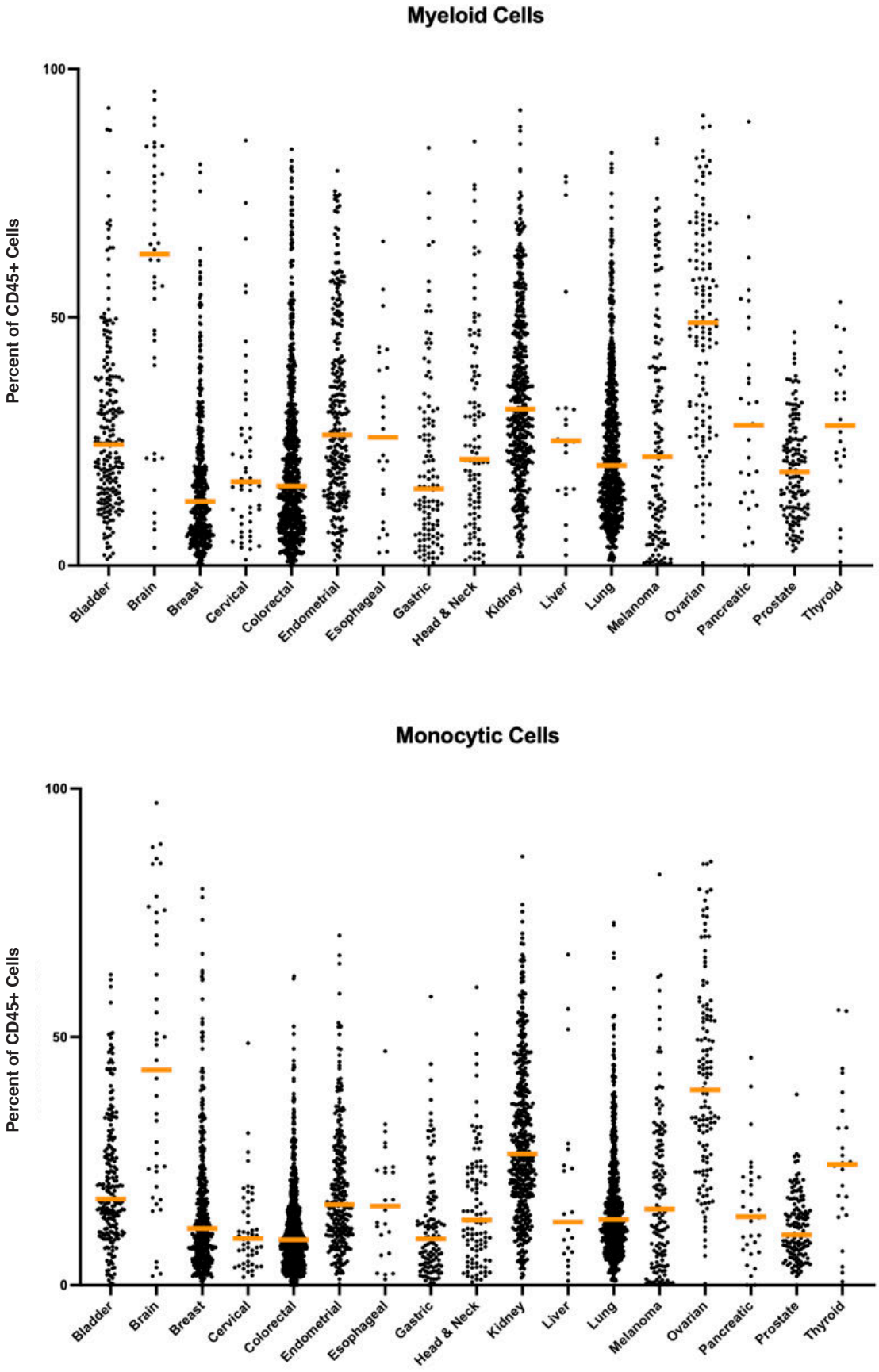
## FIGURE 3.7



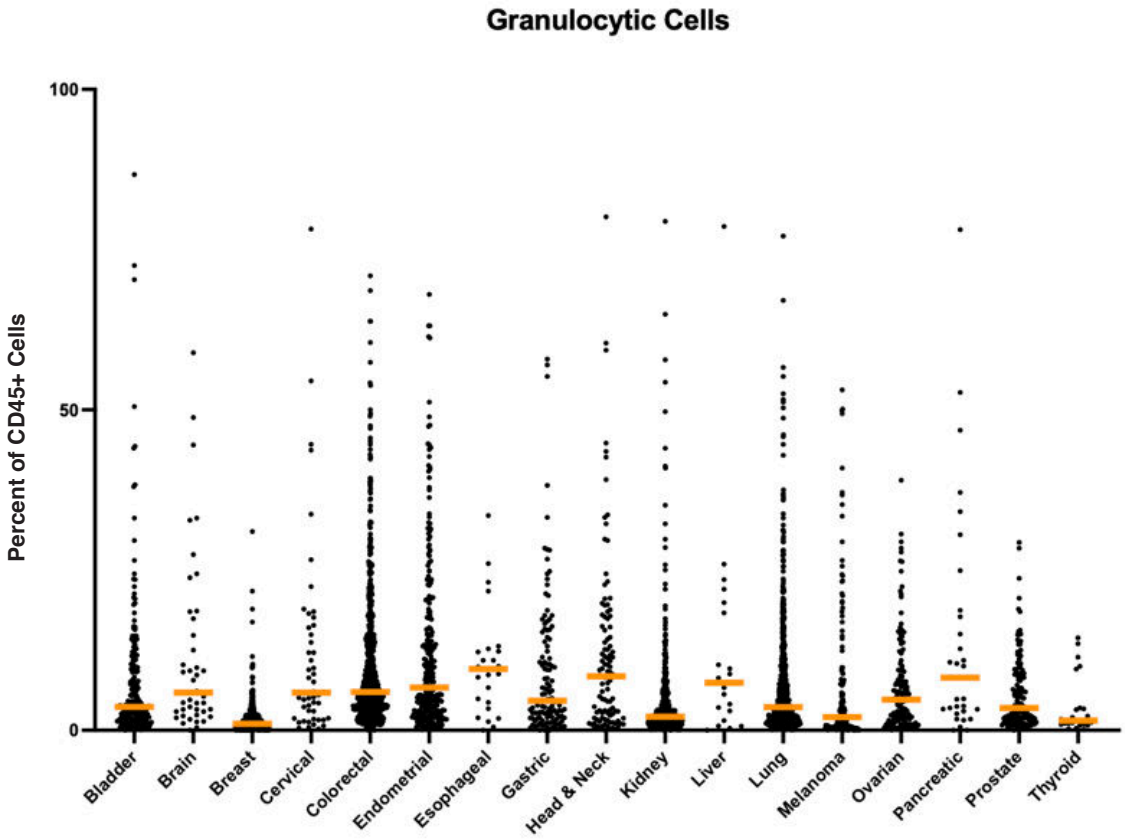
## FIGURE 3.8



## FIGURE 3.9



## FIGURE 3.9, CONTINUED



## Evaluation of Immunomodulatory Receptor Expression in Dissociated Tissue

Recent breakthroughs in checkpoint inhibitor and CAR-T cell therapies have accelerated research endeavors to refine current immunotherapies and develop the next-generation immunotherapeutic interventions.

Checkpoint inhibitor immunotherapies seek to directly modulate T cell function and enhance T-cell-mediated tumor cell killing by targeting inhibitory receptors expressed on T cells and their ligands expressed on tumor cells, among other intratumoral cell populations. Specifically, current therapies are targeting PD1 and CTLA4, inhibitory receptors that dampen T cell responses when they interact with their cognate ligands (CD80/CD86 or PDL1/PDL2, respectively) to suppress the immune response. Understanding the mechanism by which checkpoint inhibitor therapies work will be fundamental in not only improving their overall efficacy, but also in determining which patients will respond favorably, or unfavorably, to these treatment regimens. As a viable single cell suspension of the entire tumor microenvironment, dissociated tissue represents an excellent model system to evaluate immunomodulatory receptor expression and function.

The PD1/PDL1 pathway has been targeted by multiple different antibody-based immunotherapies, including Keytruda (pembrolizumab, anti-PD1), Opdivo (nivolumab, anti-PD1), Imfinzi (durvalumab, anti-PDL1), and Tecentriq (atezolizumab, anti-PDL1). Since the initial approval of Keytruda in the treatment of advanced melanoma in 2014, numerous oncological indications, including lung cancer, kidney cancer, head and neck cancer, and urothelial cancer, have been approved for anti-PD1 and anti-PDL1 therapies. The best-studied functions of PD1 have been elucidated on CD4+ and CD8+ T cells, where ligation of PD1 by its ligands PDL1 and PDL2 acts to dampen T cell responses. High percentages of PD1+ CD4+ and CD8+ T cells from DTCs generated from lung cancer, kidney cancer, head and neck cancer, urothelial cancer, colorectal cancer, and melanoma (**Figure 3.10**), ranging from 25% to 97% of cells. In addition to PD1+ T cells, tumor-infiltrating PD1+ B cells and PD1+ NK cells have been previously described. PD1+ B cells were observed from DTCs generated from all relevant indications, with the sole exception of kidney cancer, where PD1+ B cells were rare (**Figure 3.11**).

**Figure 3.10**

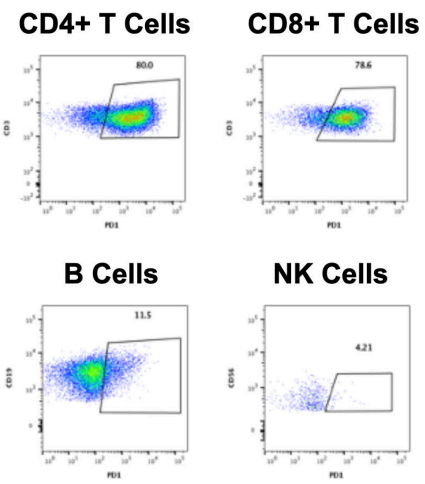
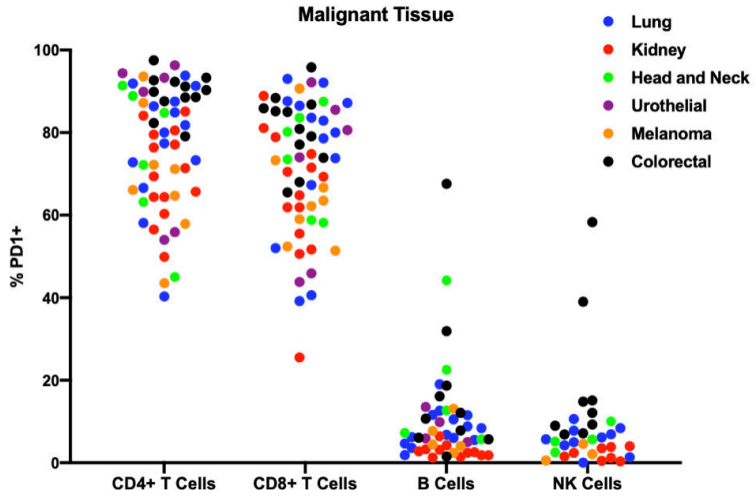



**Figure 3.11**



Surprisingly, two colorectal cancer and two head and neck cancer DTC samples were identified where the percentage of PD1+ B cells approached the percentage observed in the T cell compartment. Similarly to PD1+ B cells, PD1+ NK cells were observed in colorectal cancer, lung cancer, and head and neck cancer DTCs. NK cells were rare in the urothelial cancer DTCs analyzed, while melanoma and kidney cancer DTCs had very low percentages of PD1+ NK cells. Therefore, all major lymphocyte subsets present in the tumor microenvironment express PD1 and could be influenced by checkpoint inhibitor therapy.

Often checkpoint inhibitor therapy can have adverse effects, presumably due to the reactivation of tissue-resident T cells. To understand PD1 expression in non-malignant tissues, single cell suspensions from lung, kidney, tongue, and bladder tissues were generated from cadaveric donors, skin tissue was isolated from breast reduction procedures, and colon tissue was harvested from diverticulitis patients. With the exception of urothelial cancer/normal bladder, the immune cell composition of non-malignant tissues was lower than in non-malignant tissues when compared to malignant tissues from the same site (**Table 3.1**).

However, despite the lower percentages of immune cell percentages, CD4+ and CD8+ T cells isolated from non-malignant tissue displayed high expression of PD1, particularly in the case of tongue-resident T cells, which were >90% PD1+ (**Figure 3.12**). Interestingly, non-malignant colon and skin tissue had lower percentages of PD1+ CD4+ and CD8+ T cells. While B cells and NK cells were rare in non-malignant tissues, PD1+ B cells and PD1+ NK cells were present in lung and colon tissue. Collectively, these data demonstrate that PD1 is expressed by a majority of CD4+ and CD8+ T cells in both malignant and non-malignant tissues, with small, but distinct, populations of B cells and NK cells also expressing PD1. As PD1 expression is widespread amongst tissue and tumor-resident lymphocyte populations, it is crucial to understand the dynamics of PD1 functions on T cells within these environments when refining current checkpoint inhibitor therapies as well as developing the next generation of immunotherapeutic interventions.

Two ligands for PD1, PDL1 and PDL2, have been identified and currently PDL1 expression on tumor cells, as measured by immunohistochemistry, is evaluated on non-small cell lung cancer samples to identify patients to be prescribed Keytruda. To determine if PDL1 expression could be analyzed on DTCs, DTCs were generated from fresh tumor tissue from a subset of tumors with matched fixed tissues and analyzed these viable cells for PDL1 expression by flow cytometry. PDL1+ and PDL1- lung cancer tumor samples were identified that had been previously evaluated as 100% and 0% PDL1+ tumor expression by board-certified pathologists via immunohistochemistry (**Figure 3.13A**).

Furthermore, kidney cancer samples that had 25% and 28% PDL1+ tumor cells by IHC also had clearly identified populations of PDL1+ tumor cells by flow cytometry that matched the percentages observed by IHC. Overall, there was a high correlation between the percentage of PDL1+ tumor cells identified by either flow cytometry on viable, dissociated cells and immunohistochemistry on fixed tissue (**Figure 3.13B**).

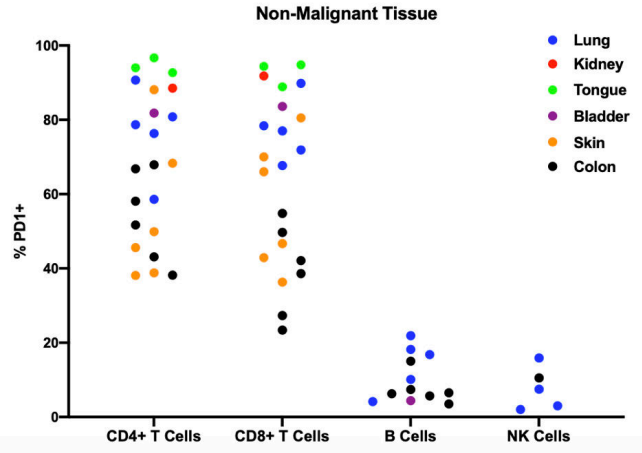
PDL1 expression was further analyzed on DTCs generated from lung cancer, kidney cancer, head and neck cancer, urothelial cancer, colorectal cancer, and melanoma patient samples. Similar to the results observed by IHC, the majority of these samples displayed low expression of PDL1 on tumor cells within these suspensions (**Figure 3.13C**).

However, there were multiple DTC samples, particularly those generated from lung cancer patients, that had a substantial percentage of tumor cells expressing PDL1. Therefore, PDL1 expression can be evaluated on tumor cells as assessed by both flow cytometry and immunohistochemistry, providing crucial information when determining the mechanism of action of anti-PDL1 therapies.

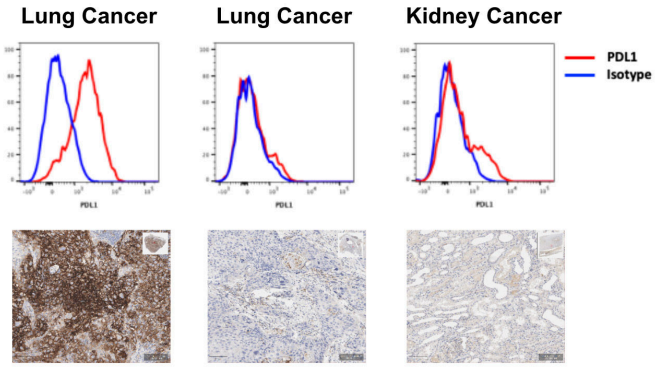
## Table 3.1

| Tissue | %CD45+ Malignant | %CD45+ Non-Malignant |
|---|---|---|
| Colorectal/Colon | 41.60% | 29.30% |
| Head and Neck/Tongue | 59.10% | 29.10% |
| Kidney | 67.10% | 23.40% |
| Lung | 75.30% | 68.30% |
| Melanoma/Skin | 32.80% | 19.90% |
| Urothelial/Blader | 37.70% | 41.90% |

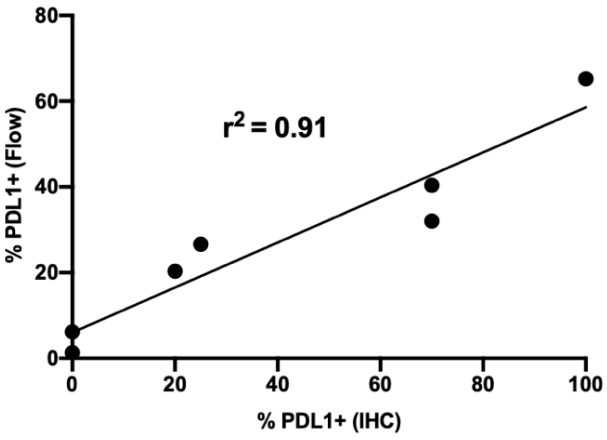
## Figure 3.12



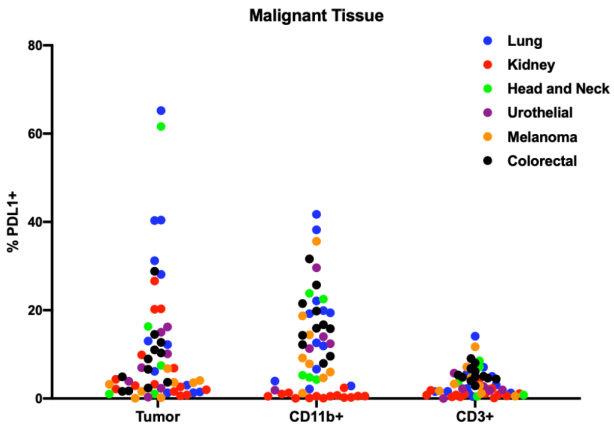

## Figure 3.13A




## Figure 3.13B




## Figure 3.13C



Additional cellular populations within the tumor microenvironment, including myeloid cells and T cells, express PDL1, potentially modifying and repressing the immune response against the tumor. Indeed, PDL1 expression was observed on both CD11b+ myeloid cells and CD3+ T cells from DTCs (**Figure 3.14**).

Overall, there was a higher percentage of CD11b+ myeloid cells that expressed PDL1 compared to CD3+ T cells, and PDL1+ myeloid cells and T cells were identified in most indications that were analyzed. Kidney cancer was the one exception, as very few PDL1+ myeloid and PDL1+ T cells were present. PDL1 expression was also examined on single cell suspensions generated from non-malignant lung, kidney, tongue, bladder, skin, and colon tissue. Unlike PD1 expression, which largely mirrored the expression observed in tumor-infiltrating lymphocytes, there were few PDL1+ epithelial cells identified from any tissue examined (**Figure 3.15**).

Only two non-malignant lung tissue samples had PDL1 expression on greater than 10% of the EpCAM+ epithelial cells, with the majority of non-malignant tissues displaying PDL1 on less than 5% of EpCAM+ epithelial cells. Furthermore, there were also very few PDL1+ CD11b+ myeloid cells or PDL1+ CD3+ T cells in non-malignant lung, colon, tongue, bladder, and kidney tissue examined. Surprisingly, there were abundant PDL1+ CD11b+ myeloid cells present in normal skin; CD11b+ cells in the skin include Langerhan's cells and dermal dendritic cells, both of which have been reported to express PDL1. Therefore, in contrast to PD1 expression of tissue- resident and tumor-infiltrating lymphocytes, PDL1 expression is largely restricted to tumor cells, tumor- infiltrating myeloid cells and tumor-infiltrating T cells, and is rare in epithelial cells and immune cells from non-malignant tissue.

**Figure 3.14**

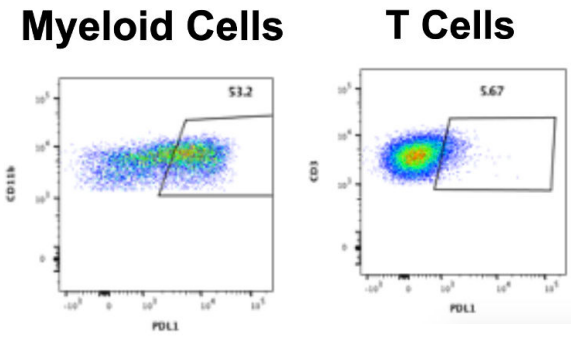
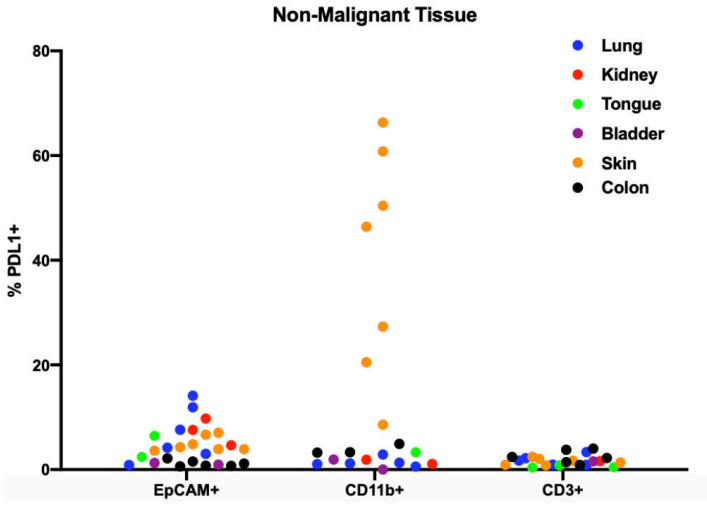



**Figure 3.15**



# PURIFICATION OF DISSOCIATED TISSUE FOR DOWNSTREAM ANALYTICAL WORKFLOWS

Dissociated tissue represents the entire cellular composition of the tumor microenvironment. Oftentimes, analytical evaluation of the dissociated tissue requires either clean up of the sample to remove dead cells and cellular debris or the isolation of specific cell subsets from the complex cellular mixture of the parent sample. DTCs are amenable to both immunomagnetic methods for the purification of cellular subsets, as well as fluorescence-activated cell sorting for either dead cell/debris removal or the isolation of very specific cell populations, and Discovery Life Sciences has implemented best practices for dissociated tissue clean up and processing.

## Dead Cell and Debris Removal from Dissociated Tissue

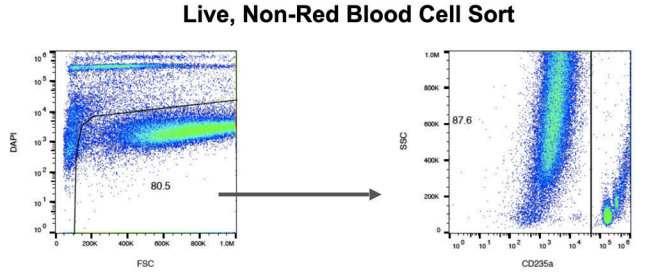
The process of dissociation of primary tissue generates both dead cells and cellular debris. The dead cells and debris can be problematic for very sensitive downstream analytical workflows, particularly complex cell culture and single cell genomics technologies, and efficient removal of dead cells and debris is crucial for these techniques. Discovery has designed two workflows for eliminating dead cells and debris from DTCs, providing viable, quality specimens for downstream assays using cell sorting. Both of these strategies utilized a live/dead discriminator to eliminate dead cells and debris. In the first strategy, CD235a is used to remove any remaining red blood cells present in the dissociated tumor preparations (**Figure 4.1A**).

### BEST PRACTICES FOR DTC PURIFICATION

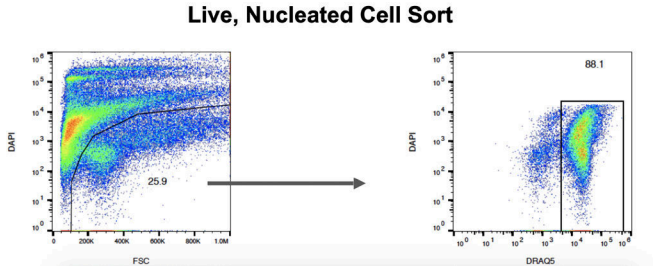
- For Highly Sensitive Applications, Cell Sorting Should be Utilized to Remove Dead Cells and Debris
- CD235a can also be added to Remove Red Blood Cells
- Magnetic Enrichments Can Be Utilized to Purify Cell Populations
- Tumor Cells: Miltenyi Tumor Cell Isolation Kit
- CD45+ TILs: Miltenyi CD45 (TIL) Microbeads
- CD3+ T Cells: Miltenyi REAlease CD3 (TIL) Microbead Kit

While rare following cryopreservation, in some samples, these red blood cells are a substantial proportion of the live cell fraction and can complicate downstream genomics applications, including 10X Genomics single cell workflows. The use of CD235a also allows for the co-staining of additional cell surface receptors to simultaneously remove dead cells and debris for the sample while collecting valuable information of the cellular composition and biomarker expression within the sampe. For the second strategy, DRAQ5 is used to identify all nucleated cells within the tumor cell mixture (**Figure 4.1B**). In this workflow, only live, nucleated cells are selected, with dead cells, debris, and red blood cells being excluded. While very efficiently identifying live nucleated cells, the wide emission spectrum for DRAQ5 precludes the use of the majority of other fluorophores at the same time. Regardless, both of these strategies are streamlined and scalable for the optimal removal of dead cells and debris from dissociated tissue for downstream assays.

## Figure 4.1A



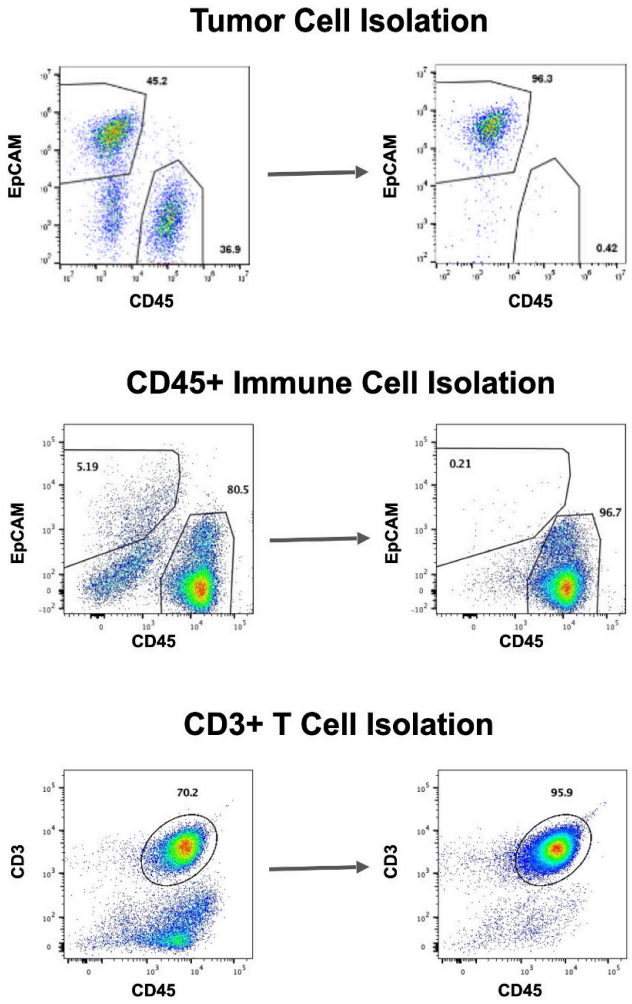

## Figure 4.1B



## Immunomagnetic Isolation of Cell Populations from Dissociated Tissue

The complex cellular composition of dissociated tissue represents the opportunity to isolate specific subsets for downstream analysis. DTCs are amenable to immunomagnetic isolation kits for the purification of tumor cell, immune cell, and T cell populations from the heterogeneous cell population from the parent tissue. The Tumor Cell Isolation Kit from Miltenyi Biotec selectively targets the immune cells, endothelial cells, and fibroblasts for positive selection, leaving a negative cell fraction composed of tumor cells (**Figure 4.2, top**). Importantly, this strategy does not require expression of a specific cell surface receptor on the tumor cells, allowing for utility in numerous indications. Tumor cells were isolated from over 100 unique DTC samples across nine indications, with an average post selection purity of 94.6% (as defined by EpCAM+ cells) and an average yield of 50.5% (**Table 4.1**). In addition to tumor cells, CD45+ immune cells, and specifically CD3+ T cells, can be positively selected from dissociated tissue. For total immune cells, CD45 (TIL) Microbeads from Miltenyi Biotec provides efficient selection and high purity of CD45+ immune cells from dissociated tissue (**Figure 4.2, middle**). For isolating CD3+ T cells, the REAlease CD3 (TIL) Microbead kit from Miltenyi Biotec positively selects CD3+ T cells from the heterogeneous cell population, but also removes the microbead complexes, resulting in an unlabeled, purified T cell fraction (**Figure 4.2, bottom**). Collectively, these immunomagnetic selection kits provide cost-effective and efficient enrichment of target cell populations.

### Figure 4.2




### Table 4.1

| | Pre Selection EpCAM+ % | Post Selection EpCAM+ % | Post Selection Viability | Yield |
|---|---|---|---|---|
| **Indication** | **Average** | | | |
| Bladder | 59.70% | 96.40% | 80.10% | 62.70% |
| Breast | 65.20% | 93.90% | 86.00% | 65.70% |
| Colorectal | 53.30% | 94.90% | 86.00% | 35.70% |
| Endometrial | 55.50% | 95.00% | 81.00% | 38% |
| Gastric | 36.30% | 92.60% | 86.70% | 46.90% |
| Kidney | 27.40% | 89.20% | 76.70% | 52.80% |
| Lung | 22.80% | 96.20% | 83.60% | 59.90% |
| Ovarian | 58.10% | 96.30% | 82.60% | 41.70% |
| Pancreatic | 47.30% | 96.30% | 94.60% | 100% |
| **Total** | 49.20% | 94.60% | 83.60% | 50.50% |

# TISSUE CULTURE APPLICATIONS FOR DISSOCIATED TISSUE

Dissociated tissue contains the entire cellular heterogeneity of the parent tissue, including tumor/epithelial cells, immune cells, endothelial cells, and fibroblasts. Culture of these cellular components, either individually or combined, provides the opportunity to understand responses to current and next generation therapeutic agents. Tumor cells from DTCs are amenable to both short-term, spheroid cultures as well as long-term, organoid cultures. These systems provide the opportunity to treat with both short term and long term agents to understand drug responses.

**BEST PRACTICES FOR SHORT TERM DTC CULTURE**

- Ultra-Low Attachment Plates
- Serum-Free Media
- Antibiotics/Antifungals

*For tumors derived from organs with normal flora, high levels of antibiotics/antifungals may be required.

## Short-Term, Spheroid Cultures

As single cell suspensions from primary tumor tissue, DTCs can be utilized for short-term, spheroid cultures. Discovery has evaluated culture conditions for five indications - colorectal cancer, kidney cancer, lung cancer, melanoma, and ovarian cancer (**Table 5.1**). For these cultures, it is recommended that ultra-low attachment plates be used to allow for sphere formation.

## Table 5.1

| | Colorectal Cancer (1) | Kidney Cancer (2) | Lung Cancer (3) | Melanoma (4) | Ovarian Cancer (5) |
|---|---|---|---|---|---|
| Culture Media | DMEM/F12 | DMEM/F12 | DMEM/F12 | DMEM/F12 | DMEM/F12 |
| Insulin | 10 µg/ml | 10 µg/ml | 10 µg/ml | 10 µg/ml | 10 µg/ml |
| Transferrin | 5.5 µg/ml | 5.5 µg/ml | 5.5 µg/ml | 5.5 µg/ml | 5.5 µg/ml |
| Sodium Selenite | 0.03 µM | 0.03 µM | 0.03 µM | 0.03 µM | 0.03 µM |
| HEPES | 5 mM | 5 mM | 5 mM | 5 mM | 5 mM |
| BSA | 0.40% | 0.40% | 0.40% | 0.40% | 0.40% |
| Glucose | 0.60% | 0.60% | 0.60% | 0.60% | 0.60% |
| Sodium Bicarbonate | 0.10% | 0.10% | 0.10% | 0.10% | 0.10% |
| Gentamycin | 50 µg/ml | 50 µg/ml | 50 µg/ml | 50 µg/ml | 50 µg/ml |
| Amphotericin B | 25 µg/ml | 4 µg/ml | 4 µg/ml | 4 µg/ml | 4 µg/ml |
| Penicillin | 100 U/ml | 100 U/ml | 100 U/ml | 100 U/ml | 100 U/ml |
| Streptomycin | 100 µg/ml | 100 µg/ml | 100 µg/ml | 100 µg/ml | 100 µg/ml |
| Glutamine | 2 mM | 2 mM | 2 mM | 2 mM | 2 mM |
| B27 Supplement | -- | 1X | -- | 1X | -- |
| Basic Fibroblast Growth Factor | 10 ng/ml | 20 ng/ml | 10 ng/ml | 20 ng/ml | 10 ng/ml |
| Epidermal Growth Factor | 20 ng/ml | 10 ng/ml | 20 ng/ml | 10 ng/ml | 20 ng/ml |
| Leukemia Inhibitory Factor | -- | -- | -- | 10 ng/ml | -- |

Additionally, as these tissues are resected in a non-sterile environment and, in the case of colorectal cancer tumors, derive from anatomical locations with normal flora, it is recommended that antibiotics/antifungals such as penicillin, streptomycin, gentamycin, and amphotericin B. For colorectal cancer samples, high doses of amphotericin B, as previously described, may be required to control fungal growth (Kondo et al, PNAS, 2011). Finally, as fetal bovine serum is undefined and can have unwanted physiological responses, serum-free media is recommended. For initial evaluation of these short-term spheroid cultures, tumor cells were purified from DTCs using the Miltenyi Tumor Cell Isolation Kit and cultured for 3-5 days (**Figure 5.1**). After 4 days in culture, spheroids were visible by brightfield microscopy, and flow cytometric analysis revealed maintenance of the EpCAM+ tumor fraction (or CD146+ tumor fraction for melanoma). As the entire cellular composition of the tumor is present in DTCs, T cells can also be co-cultured with these tumor spheres through the addition of high-dose IL-2 (**Figure 5.2**), resulting in the maintenance of EpCAM+ tumor cells and CD45+ immune cells after 5 days in culture. These short term spheroid cultures provide the opportunity for acute treatment to evaluate drug responses.

## Media References

*1. Kondo et al.* "Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer" PNAS, 2011.

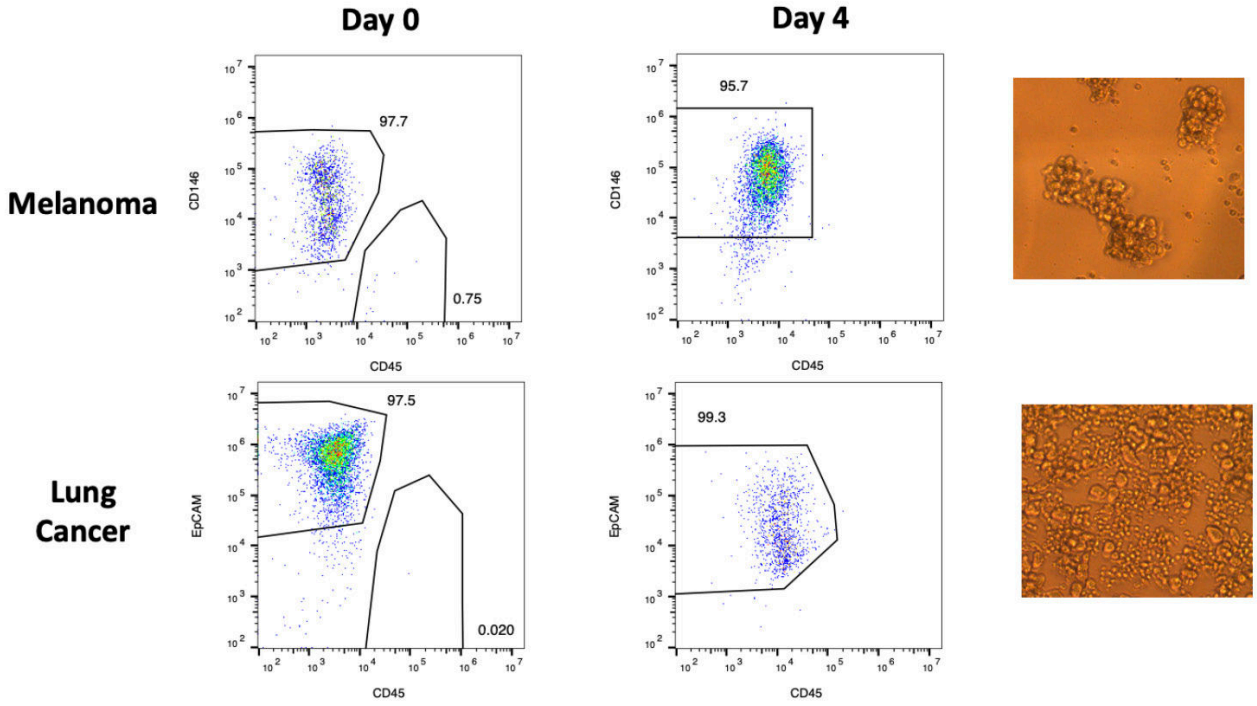
*2. Zhang et al.* "Spheres derived from the human SN12C renal cell carcinoma cell line are enriched in tumor initiating cells" Journal of Experimental & Clinical Cancer Research, 2016.

*3. Eramo et al.* "Identification and expansion of the tumorigenic lung cancer stem cell population" Cell Death and Differentiation, 2008.

*4. Wong et al.* "Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin" Journal of Cell Biology, 2006.

*5. Zhang et al.* "Identification and characterization of ovarian cancer-initiating cells from primary human tumors" Cancer Research, 2008.

## Figure 5.1



## Figure 5.2