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Single cell analysis of human tissues and solid tumors with mass cytometry

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Abstract

Background—Mass cytometry measures 36 or more markers per cell and is an appealing platform for comprehensive phenotyping of cells in human tissue and tumor biopsies. While tissue disaggregation and fluorescence cytometry protocols were pioneered decades ago, it is not known whether established protocols will be effective for mass cytometry and maintain cancer and stromal cell diversity.

Methods—Tissue preparation techniques were systematically compared for gliomas and melanomas, patient derived xenografts of small cell lung cancer, and tonsil tissue as a control. Enzymes assessed included DNase, HyQTase, TrypLE, collagenase (Col) II, Col IV, Col V, and Col XI. Fluorescence and mass cytometry were used to track cell subset abundance following different enzyme combinations and treatment times.

Results—Mechanical disaggregation paired with enzymatic dissociation by Col II, Col IV, Col V, or Col XI plus DNase for 1 hour produced the highest yield of viable cells per gram of tissue. Longer dissociation times led to increasing cell death and disproportionate loss of cell subsets. Key markers for establishing cell identity included CD45, CD3, CD4, CD8, CD19, CD64, HLA-DR, CD11c, CD56, CD44, GFAP, S100B, SOX2, nestin, vimentin, cytokeratin, and CD31. Mass and fluorescence cytometry identified comparable frequencies of cancer cell subsets, leukocytes, and endothelial cells in glioma ($R = 0.97$), and tonsil ($R = 0.98$).

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Conclusions—This investigation establishes standard procedures for preparing viable single cell suspensions that preserve the cellular diversity of human tissue microenvironments.

Keywords

Solid tumor; single cell biology; mass cytometry (CyTOF); human tissue; melanoma; glioma; tonsil; small cell lung cancer (SCLC)

Introduction

In preparing single cell suspensions of healthy and malignant tissue, a common goal is to preserve viability while maintaining cellular diversity and preserving rare subsets. Multidimensional cytometry is well suited to this challenge because it can simultaneously characterize known cell types and reveal novel cell subsets (1,2). Mass cytometry uses antibodies to quantify features of individual cells in primary tissues (3,4) and has been applied to characterize cell subsets in human bone marrow, blood, and germinal center tissues as well as diverse murine tissues (5-7). However, mass cytometry remains relatively untested in the context of solid tumors. Fluorescence flow cytometry and fluorescence activated cell sorting (FACS) have been used to prospectively isolate functionally distinct cell subsets and suggest that mass cytometry analysis could help to further characterize solid tumors (2). A key goal of this study was to evaluate the suitability of different cell preparation techniques for mass cytometry and to develop standard procedures and quality controls that do not require measuring light scatter. An additional goal was to use the multidimensionality of mass cytometry to characterize preservation of cellular diversity under different solid tumor cell preparation techniques.

In this study, mechanical and enzymatic dissociation protocols were systematically tested on multiple types of fresh human solid tumors and tissues to develop an efficient, reliable method for dissociation and singlecell analysis by mass cytometry. Human tonsils and lymphoma tumors reliably dissociate with mechanical force alone and we have previously established protocols for their study by fluorescence cytometry (8,9) and mass cytometry (10). Preparation techniques for tissue samples derived from intraoperative resections of gliomas (grades II-IV), melanomas, and patient derived xenografts (PDX) of small cell lung cancer (SCLC) were compared. As a control, the same techniques were applied to human tonsillar tissue. The abundance of different cell types, such as leukocytes, endothelial cells, epithelial cells, fibroblasts, and cancer cell subsets, was tested under these conditions. Established protein markers for expected cell types in tissues tested in this study were used in fluorescence cytometry (Table S1) and mass cytometry (Table S2, Table S4). The common markers were selected so that both rare and abundant cell types could be compared between mass and fluorescence cytometry. The additional markers in the mass cytometry panel provided a more comprehensive analysis of cell diversity.

Six enzymes for cell separation were selected to compare in solid tumor preparation protocols for mass cytometry analysis: HyQTase, TrypLE, collagenase (Col) II, Col IV, Col V, and Col XI. Enzyme choice was based in part on prior use in several solid tumor types and preparation of single cell suspensions containing cancer cell and immune subsets for

FACS (11-16). DNase was also tested to determine its ability to enhance live cell yield from dissociation. Dissociation kinetics for enzyme combinations in distinct tissue types were also characterized. Finally, specific enzymes and dissociation duration times were selected based on optimal viable cell yield and representation of expected cell populations.

Materials and Methods

Tissue Sample Collection

All samples were obtained with patient consent, with Vanderbilt institutional review board (IRB) approval, in accordance with the Declaration of Helsinki, and were de-identified. Gliomas were intraoperative specimens from WHO grade II, III, or IV tumors (IRB #131870), collected in sterile normal saline. Melanomas were cutaneous and lymph node resections (IRB #030220), collected in MEM (Corning/Mediatech, Corning, NY) with 10% FBS + 1X Pen/Strep (GE Healthcare, Pittsburgh, PA). Small-cell lung cancer (SCLC) patient derived xenograft (PDX) samples were obtained as a gift from the Rudin laboratory (LX-22, (17)) and propagated solely as patient-derived xenografts in female athymic nude mice (HSD:Athymic Nude-Foxn1^{nu/nu}) obtained from Envigo with Vanderbilt institutional animal care and use committee (IACUC) approval. SCLC PDX were collected in RPMI 1640 (Corning/Mediatech, Corning, NY) plus 10% FBS+ 1X Pen/Strep. Glioma, melanoma, and SCLC PDX samples were transported at room temperature without delay to the laboratory and processing began within 30 minutes of collection from patients. Human tonsillar tissue was obtained from routine tonsillectomies (IRB #121328), collected in RPMI 1640 (Corning/Mediatech, Corning, NY) plus 10% FBS+ 1X Pen/Strep, transported on ice, and processed within 4 hours of collection.

Mechanical and Enzymatic Dissociation

Sequential dissociation steps are described in detail in the main text. “Coarse mincing” indicates no additional mechanical dissociation of tissues (i.e. tissues were left as obtained intraoperatively). “Fine mincing” indicates additional mechanical dissociation using scalpels. Conventional mechanical dissociation of tonsils included fine mincing and immediate filtration of tissue through a 70µm cell strainer without additional enzymatic dissociation, as previously established (8-10). Dissociation enzymes were obtained from Sigma Aldrich (Darmstadt, Germany) (collagenase II, IV, V, and XI), ThermoFisher (Waltham, MA) (TrypLE-Express), and GE Healthcare (PA) (HyQTase). Collagenases were used at 1 mg/mL. HyQTase and TrypLE-Express were used at 1X according to the manufacturer’s recommendations. DNase I (Sigma Aldrich) was used at a final concentration of 0.25 mg/mL. For conditions involving collagenases and no enzyme, cells were resuspended in recommended media for specific tissue types prior to adding indicated enzymes (gliomas, DMEM/F12+Glutamax, (Gibco/Life Technologies, MA) with a defined hormone and salt mix (18) and 50 µg/mL gentamicin; melanomas, MEM with 10% FBS + 1X Pen/Strep; Tonsils and SCLC PDXs, RPMI 1640 + 10% FBS + 1X Pen/Strep. For dissociation conditions with HyQTase or TrypLE, tissues were dissociated in working concentrations of enzymes (with or without DNase), without addition of cell culture media, according to the manufacturer’s recommendations. Enzymatic dissociations were performed

in a 37°C incubator with 5% CO₂, with constant rocking on a nutating platform mixer at 18 rpm. Cells were then strained with 70 µm and 40 µm cell strainers prior to further analysis.

Quantification of cell viability

Cell suspensions obtained from different dissociation protocols were resuspended in corresponding cell culture media at volumes proportional to initial tissue weight (1 mL per 100 mg of tissue). Viable cells were quantified using Trypan Blue staining, normalized to the initial tissue weight, and reported as millions of live cells per gram of tissue.

Statistical testing

Enzyme conditions were compared as groups (horizontal lines) using a Student's t-test. The relationship between cell subset abundance measured by fluorescence or mass cytometry was compared using Pearson's correlation R and Spearman's rank correlation ρ (rho).

Cell line and cell culture

Jurkat cells were obtained from Utpal Dave at Vanderbilt, and were grown in RPMI 1640 + 10% FBS + 1X Pen/Strep as recommended. MeWo cells were obtained from Kimberly Dahlman and Jeffery Sosman with permission of Antoni Ribas (UCLA) and were grown in MEM + 10% FBS + 1X Pen/Strep, as recommended.

Flow cytometry

Cell suspensions were evenly divided for parallel phenotyping with fluorescence and mass cytometry according to the protocols below. Conditions were identical between mass and fluorescence cytometry with the exception of an additional staining step including saponin for mass cytometry analyses of glioma and melanoma that include SOX2. This type of saponin step has been established to have no significant impact on subsequent mass cytometry staining (19).

Fluorescence flow cytometry

For fluorescence cytometry, live surface staining was performed for surface marker detection (Supplemental Table S1). After washing with PBS and pelleting twice (at $200 \times g$ for 5 min each time), cells were fixed with 1.6% paraformaldehyde (Electron Microscopy Services, Fort Washington, PA) for 10 min at room temperature, washed with PBS (HyClone Laboratories, Logan, UT), pelleted at $800 \times g$, and permeabilized with 100% ice-cold methanol (Fisher Scientific, Waltham, MA) at -20°C overnight following established protocols (9,20). Cells were washed twice with cell staining media composed of PBS plus 1% BSA (Fisher Scientific, Waltham, MA) and pelleted at $800 \times g$. For each comparison, cells were stained in 100 µL staining media for 30 minutes at room temperature. All antibodies are listed in Supplemental Tables. Note that some antibodies that detect cell surface antigens (CD45-BV786, CD44- PE, and CD31-PE-Cy7) were used after fixation and methanol permeabilization due to concerns for stabilization of fluorochromes after methanol exposure. After staining, cells were washed twice with PBS, pelleted at $800 \times g$, and resuspended in PBS for analysis on a 5-laser LSRII (BD Biosciences, San Jose, CA) at the Vanderbilt Flow Cytometry Shared Resource.

Mass cytometry

Solid tissue cells obtained from the same dissociation conditions as those analyzed by fluorescence flow cytometry were stained live for cell surface markers, fixed, permeabilized, and washed as for fluorescence flow cytometry above and in concordance with established mass cytometry protocols (4). Permeabilization with 0.02% Saponin (Millipore, Darmstadt, Germany) in PBS was also included before methanol permeabilization of gliomas and melanomas as part of an optimized multi-step protocol that included detecting SOX2, which was not included in the fluorescence panel. Metal-tagged antibodies were used to stain cells in 100 μ L cell staining media for 30 minutes at room temperature (Supplemental Table S4). After staining, cells were washed once with PBS, once with deionized water, pelleted at $800 \times g$, and resuspended in deionized water containing normalization beads (Fluidigm). Standard bead-based normalization was used as previously described (21). Cells were collected on a CyTOF 1.0 at the Vanderbilt Flow Cytometry Shared Resource. Original data were normalized with MATLAB normalization software prior to further analysis using Cytobank (22) and established mass cytometry analysis methods (23). viSNE analysis was performed using 60,000 cCasp3-HH3⁺ cells per sample. For glioma G-LC-15, the following markers were used for viSNE analysis: CD31, CD64, CD45RO, S100B, CD45, PDGFR α , SOX2, CD24, CD44, CD3, GFAP, α SMA, HLA-DR, and CD56. For tonsil T02-23, the following markers were used for viSNE analysis: CD4, IgD, CD16, CD45RO, CD45RA, CD45, CD27, CD86, CD33, CD11c, CD14, CD19, CD38, CD8, CD3, IgM, HLA-DR, and CD56. Samples of the same tissue type dissociated with different types of collagenase were analyzed simultaneously by viSNE.

Histone H3 testing

Healthy peripheral blood mononuclear cells (PBMCs) were used as controls in testing histone H3 as a nucleated cell marker for multiple flow cytometry platforms. PBMCs were stained live for detection of cell surface markers (Supplemental Table S2). After being washed twice with PBS, cells were then fixed with 1.6% paraformaldehyde and permeabilized with 100% ice-cold methanol for intracellular staining. Stained PBMCs were then evenly divided and half of the cells were stained with iridium at a final concentration of 0.25 μ M in PBS for 15 minutes at room temperature. Cells were then washed once with PBS, once with deionized water, pelleted at $800 \times g$, and resuspended in deionized water containing normalization beads. Cells were collected as described above.

Results

Tissue dissociation with collagenase and DNase improved live cell yield

A matrix of dissociation conditions was tested to identify optimal protocols for multiple solid tumor types and tonsil controls (Figure 1, Figure S1, and Figure S2). The mechanical dissociation protocol (see Materials and Methods) was first compared to fine mincing of tonsil tissue followed by a 2-hour enzymatic dissociation with combinations of collagenase and DNase. For tonsils, a combination of fine mincing, collagenase, and DNase resulted in superior live cell yield per gram of tissue compared to conventional dissociation methods (Figure S1, $p < 0.05$). Additionally, fine mincing of tonsils did not adversely affect cell viability (Figure S2) when compared to coarse mincing (left as obtained intraoperatively).

Since freshly resected tissues and tumors frequently differ in size, fine mincing was selected as an initial mechanical dissociation step for all tissue types. To determine the optimal enzymes for disaggregation of human gliomas, seven different enzymatic conditions were tested for their ability to yield live, single cells (Figure 1A, N = 3). Intraoperative samples of gliomas were finely minced and incubated with a cocktail of DNase plus one enzyme (either HyQTase, TrypLE, Col II, Col IV, Col V, or Col XI) or DNase alone for 2 hours at 37°C, with continuous rocking. Increased live cell yield per gram of tissue was seen in conditions containing collagenase and DNase as compared to other conditions ($p < 0.01$). Additionally, DNase plus collagenase improved live cell yield for glioma compared to collagenase alone ($p < 0.01$). No significant differences were observed in live cell yield per gram of glioma tissue between conditions using different types of collagenases plus DNase. High-resolution images of trypan blue stains are shown in Figure S3.

The same matrix of conditions was tested on intraoperative samples of human melanomas (Figure 1B, N = 3). As with glioma, no significant difference in live cell yield was observed between different types of collagenases, and viable cell yields were highest in conditions containing collagenases and DNase ($p < 0.01$). In freshly resected tonsils (Figure 1C, N = 4), collagenases with DNase gave a higher live cell yield than either DNase alone ($p < 0.05$) or TrypLE plus DNase ($p < 0.01$). However, collagenases with DNase did not significantly differ from HyQTase with DNase, and addition of DNase did not result in higher or lower live cell yield, in tonsil dissociation.

Enzymatic dissociation with collagenase and DNase for 1-2 hours provided superior live cell yields

While incubation in enzyme solutions enhanced tissue disaggregation (Figure 1 and Figure S1), excessive incubation might adversely affect cell viability. A dissociation time course was performed on intraoperative glioma specimens to determine the optimal time point for highest live single cell yield (Figure 2A). Gliomas were finely minced and incubated in collagenases plus DNase for 30 minutes, 1 hour, 2 hours, 4 hours, or 6 hours (Figure 2A, N = 3). Live cell yield per gram of glioma tissue significantly decreased after 4 hours of enzymatic dissociation with Col II, Col V, or Col XI plus DNase compared to earlier time points (Col II and Col XI, $p < 0.001$; Col V, $p < 0.05$), whereas it significantly decreased after 6 hours of dissociation with Col IV plus DNase (Figure 2A, $p < 0.001$).

Dissociation kinetics of tonsils were also characterized for time points ranging from 15 minutes to 24 hours (Figure 2C). Finely minced tonsils dissociated with Col II plus DNase for 1-2 hours gave higher live cell yield when compared to earlier time points ($p < 0.05$) as well as later time points ($p < 0.001$). Similarly, viable cell yield decreased significantly after 1-2 hours when tonsils were dissociated with either Col IV or Col XI plus DNase (IV, $p < 0.05$; IX, $p < 0.01$). Live cell yield from the combination of Col V and DNase also decreased after 6 hours ($p < 0.01$). Live cell yield from intraoperative melanoma specimens and SCLC patient-derived xenografts (PDXs) did not significantly decrease after 6 hours of dissociation, regardless of the type of collagenase (Figure 2B and 2D).

Testing histone H3 as a nucleated cell marker compatible with mass and fluorescence cytometry

An anti-Histone H3 (HH3) monoclonal antibody was next tested as a potential marker of nucleated cells that would function equivalently in fluorescence and mass cytometry. Jurkat T leukemia cells gated as intact cells were 98.9% positive for HH3 in fluorescence cytometry (Figure S4A). Similarly, when Jurkat cells were gated first as HH3+, they were observed to be >99.8% intact cells when gated using light scatter in fluorescence cytometry (Figure S4B). Peripheral blood mononuclear cells (PBMCs) were used to further test HH3 because PBMC have well-studied cell subsets that have been extensively characterized by both fluorescence and mass cytometry (4,24,25). PBMCs from a healthy donor were stained with a panel of 16 mass-tagged antibodies (Table S2). Frequencies of known cell subsets identified by biaxial gating were closely correlated in the same mass cytometry dataset gated using HH3 or established iridium-based gating (Figure S5, Pearson correlation $R = 1.00$, Spearman rank of subset abundance $\rho = 1.00$, Table S3), supporting the use of HH3 as nucleated cell marker across multiple flow cytometry platforms.

Assessment of cell subset diversity in solid tumor following collagenase and DNase treatment

Two- to seven-dimensional fluorescence flow cytometry has been used extensively to characterize presence and abundance of cell subsets in patient-derived tissues. Glioma cell subsets consistent with those documented in prior studies were present after a 1-hour dissociation with DNase plus Col II using fluorescence flow cytometry (Figure 3A, Col II). In glioma sample G-RT-06, 55.4% of all events were identifiable as intact nucleated cells based on HH3 staining. CD45⁺ immune cells comprised 59.7% of live intact cells, which included CD3⁺ T cells (26.7%) as well as other immune cell types (71.8%). Presence of immune cell subsets was confirmed with immunohistochemistry (IHC) staining of formalin-fixed paraffin-embedded (FFPE) sections of the same sample (Figure S6). Additionally, CD31⁺ endothelial cells were detected (5.1% of non-immune cells), as were cell subsets that differentially expressed CD56 (NCAM) and GFAP. The abundance of nucleated cells and other known cell subsets was similar between different collagenase types (Figure S7).

To determine if cells derived from dissociations using collagenase and DNase were suitable for mass cytometry analysis, cells obtained from intraoperative glioma resections (G-RT-06) were stained with 16 isotope-labelled antibodies (Table S4). Histone H3 was used to identify intact nucleated cells. A biaxial analysis sequence similar to that used for fluorescence flow cytometry analysis was used for comparison of subset abundance identified by these two cytometry platforms (Figure 3B). A strong correlation of cell subset abundance between the two methods was observed and quantified (Table 1; Pearson's $R = 0.97$, Spearman's rank $\rho = 0.93$). Similar comparisons were performed in tonsils (Figure 3D). Strong correlations of subset abundance between the two different cytometry platforms was also observed in tonsil (Table 1; Pearson's $R = 0.98$, Spearman's rank $\rho = 0.90$).

Subsets of immune cells in tonsils were also identified by fluorescence flow cytometry, including CD3⁺ T cells, CD44⁺ antigen-presenting cells (APCs), CD44⁻ APCs, and additional immune and non-immune cell types, as expected (Figure 3C, Col II). Abundance

of tonsil cell subsets was similar between dissociations using different collagenase types (Figure S8). Single cells obtained from resected melanomas (MP-04) and a melanoma cell line, MeWo, were analyzed by fluorescence flow cytometry and were observed to have intrinsic auto-fluorescence on some channels, whereas glioma and tonsil samples studied here showed no auto-fluorescence (Figure S9). Mass cytometry was next used to study melanoma tumors (Figure S10). CD45⁺ immune subsets, including CD45⁺HLA-DR⁺ antigen-presenting cells, CD45⁺CD3⁺ T cells (CD8⁺ and CD8⁻, and CD45RO⁺ memory and CD45RO⁻ non-memory), as well as CD31⁺ endothelial cells were identified in melanoma. Additionally, among the non-immune, non-endothelial cells, other cell subsets were identifiable by nestin, SOX2, CD44, HLA-ABC, vimentin, and cytokeratin.

To characterize the effects of different types of collagenase on the presence of cell subsets, mass cytometry analysis of cells derived from glioma dissociation at one hour with DNase plus either Col II, Col IV, Col V, or Col XI was performed (Figure 4). This time point was selected based on its highest live cell yield across multiple tissue types, shown above. viSNE analysis (7) was used to compare cell subsets in the different dissociation conditions. Known cell subsets in gliomas were present in all conditions, including CD45⁺ immune cells (CD3⁺ T cells, and CD64⁺ microglia), CD45⁻CD31⁺ endothelial cells, GFAP⁺ glial cells, S100B⁺ astrocyte-like cells, and SOX2⁺ stem-like cells. Established cell subsets were also observed in tonsil specimens dissociated for one hour in all types of collagenase (Figure S11). As expected, the majority of cells were CD45⁺ immune cells. Additionally, known immune subsets, including CD3⁺CD4⁺ helper T cells, CD3⁺CD8⁺ cytotoxic T cells, CD19⁺IgD⁺ naïve B cells, and CD19⁺CD27⁺ memory B cells, were identified. These findings suggest that both mass cytometry and fluorescence cytometry identify key cell subsets in glioma and tonsil dissociated with collagenase plus DNase.

Longer dissociation times led to disproportionate cell death and loss of cellular diversity

To determine if the abundance of cell subsets changed over time with enzymatic dissociation, time course dissociations of glioma sample, G-LC-15 (Figure 5), and tonsil sample, T02-23 (Figure S13), with DNase plus Col II were performed. Cell subsets were identified using sequential biaxial analysis and given the indicated labels following expert review. Apoptotic cells, defined by high cCasp3 signal, were excluded from subsequent cell subset quantification (Figure 5A). Within the population of HH3⁺ nucleated cells, marker analysis identified CD45⁺ immune cells and CD31⁺ endothelial cells. Known subsets of immune cells were present within the CD45⁺ population, including microglia (HLA-DR⁺CD64⁺), memory T cells (CD3⁺CD45RO⁺), and non-memory T cells (CD3⁺CD45RO⁻) (Figure 5B). Within the CD45⁻CD31⁻ population, pericytes (α SMA⁺) and ependymal cells (CD24⁺) were seen, as well as rare SOX2⁺ stem-like cells, GFAP⁺ glial cells, PDGFR α ⁺ cells, and S100B⁺ astrocyte-like cells (Figure 5C). Quantification of these cell subsets was performed in samples obtained from different dissociation durations to characterize maintenance and enrichment of cell subsets over time (Figure 5D). Among immune cells, a decrease in microglia (after 1 hour) and memory T cells (after 4 hours) was noted, whereas the proportion of nonmemory T cells appeared to remain constant over the full range of times tested. SOX2⁺ stem-like cells were most abundant after 1 hour of dissociation and decreased thereafter. Even though the proportion of SOX2⁺ stem-like cells increased at 24

hours after dissociation, the overall decrease in viable cells after 4-6 hours of glioma dissociation (Figure 2A) suggested an overall loss in total viable stem-like cells at later time points. Additionally, the abundance of GFAP⁺ glial-like cells (known to be present in most gliomas, Figure S12) remained constant during the initial 10 hours of dissociation and showed a decrease after 16 hours. This suggested that longer dissociation depletes key cell subsets in glioma. Most of the nucleated, non-apoptotic cells that remained after 24 hours of dissociation lacked expression of the key cell identity markers used in this study. Moreover, the abundance of cCasp3⁺ apoptotic events also increased over time (Figure 5E).

A similar time course strategy was applied to tonsil specimen dissociation (Figure S13A). A decrease in the abundance of most immune cell subsets was observed at all time points greater than 1 hour of dissociation with Col II plus DNase (Figure S13B). This decrease affected all T cell subsets, plasma cells/blasts, germinal center B cells, class-switched memory B cells, and unswitched memory B cells. Notably, abundance of naïve B cells remained constant during the initial 6 hours of dissociation and only decreased after 10 hours. CD27-IgD⁻ B cells increased in abundance at time points extending to 6 hours, followed by a decrease at 10 hours. Dendritic cells were the only immune cell subsets that continued to increase in abundance at 24 hours of dissociation. As expected, longer dissociation times likewise led to an increase in apoptotic cells (Figure S13C).

Discussion

A common protocol of collagenase II plus DNase for 1 hour was identified as effective for preparing viable and mass cytometry compatible single cell suspensions of all tested human solid tumors and healthy tissues. Multiple types and combinations of enzymes and dissociation kinetics were compared in freshly resected patient-derived tissues and patient-derived xenografts. Unexpectedly, collagenase also resulted in greater viable cell yield from tonsils when compared to the conventional dissociation method (Figure 1 and Figure S1), indicating that the protocol for preparation of tonsil and lymphoma tumors could be further refined. DNase clearly improved live cell yield from gliomas and melanomas and is strongly recommended for tissues where there may be ongoing cell death. Even though DNase was not observed to improve tonsil dissociation, DNase also did not adversely affect tonsil cell viability. Live cell yield from glioma dissociation began to decrease after 4-6 hours. However, live cell yields from melanoma and SCLC PDX were constant throughout the dissociation duration tested (6 hours) for all types of collagenase. In contrast, live cell yield from tonsils was maximal during the initial 2 hours of dissociation, except for collagenase V, which significantly decreased only after 6 hours.

Critically, dissociation of tissue using combined collagenase and DNase preserved cellular diversity, as seen by mass cytometry and standard fluorescence flow cytometry (Figure 3 and Figure 4). At one hour after dissociation, known cell subsets were present as expected in each of the tested tissue types. These included immune cells in tonsil, infiltrating immune cells in glioma and melanoma, and tissue-specific cell subsets, such as cancer cell subsets, endothelial cells, glial cells, pericytes, and stem-like cells in gliomas. A difference in abundance of T cells observed between fluorescence and mass cytometry was determined to be due to use of different anti-CD3 antibody clones, as has been previously reported (25,26).

While immune cells and GFAP⁺ cells in glioma were confirmed with IHC stains and observed to be in relatively close agreement between IHC and flow cytometry, small tissue sections and sections that do not sample all tumor regions may over- or under-represent cell subsets or overlook rare cells. The quantitative analysis of a large number of whole cells by multidimensional flow cytometry (10^5 to 10^7) provides a strong complement to the location information provided by imaging cytometry (27).

Longer dissociation times led to increased cell death and disproportionate depletion of cell subsets in both gliomas and healthy tonsil. Additionally, the abundance of glial/astrocyte-like cells, as well as rare stemlike cells in glioma, decreased over time. Even though the proportions of some cell subsets increased at later time points (endothelial cells, pericytes, SOX2⁺ stem-like cells in gliomas, and dendritic cells and CD27⁺ IgD⁺ B cells in tonsils), the significant increase in cell death over a long period of dissociation would result in an overall decrease in total yield of those cell types. Comparison of the results from gliomas, melanomas, SCLC xenografts, and tonsil tissue indicates that different tissues may be sensitive to prolonged enzymatic digestion. Dissociation conditions should be evaluated closely and carefully matched to tissue type and study goals. However, based on the results here, no more than 1 hour of dissociation is recommended unless the protocol is being optimized for a specific purpose. In future single-cell-level studies of other complex solid tissues, it will be critical to identify conditions that efficiently generate single-cell suspensions while preserving rare subpopulations of interest. Additionally, cell viability stains such as Cisplatin can be included in future mass cytometry experiments that aim to test cell functions like signaling, proliferation, viability, or cytokine production (28).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Collagenase plus DNase treatment provides better yield of live cells from three human tissues than no enzyme, TrypLE, HyQTase, or collagenase treatment alone

Graphs show millions of viable cells per gram yielded by different tissue preparation conditions following fine mincing for (A) gliomas, (B) melanomas, and (C) tonsil tissue. In addition to DNase (closed symbols), preparation enzymes tested included no additional enzyme (No enz), recombinant trypsin TrypLE (Tryp), HyQTase (HyQ), and collagenase (Col) II, IV, V, or XI. Average live cell yield is indicated for each condition by the thick horizontal line. Individual tissues or tumors are represented by different symbols. Representative trypan blue stained images are depicted under each condition. Scale bars =

100 μm . Symbols denote not significant (n.s.), $p < 0.05$ (◆), or $p < 0.01$ (◆◆). N indicates number of separate individual sample donors tested under each condition for each tissue type.

Figure 2. Collagenase and DNase treatment for 1 or 2 hours provided better overall live cell yield than other times

(A) Gliomas (N = 3) were finely minced and treated for varying times with DNase and either Col II, Col IV, Col V, or Col XI. Yield of live single cells ($\times 10^6$) per gram was quantified from Trypan blue images after 30 minutes ('), 1 hour (h), 2h, 4h, and 6h (filled symbols).

Individual tissues or tumors are represented by different symbols. Grey circles mark average yield and are connected with dashed lines to indicate dissociation kinetics. Dissociation kinetics were similarly assessed for (B) melanomas (N = 3), (C) tonsil tissue (N = 4, except for 10h, 16h, 24h where N = 2), and (D) SCLC PDX tumors (N = 3) (D). Symbols denote not significant (n.s.), $p < 0.05$ (◆), $p < 0.01$ (◆◆), or $p < 0.001$ (◆◆◆).

Figure 3. Frequency of cell types in glioma, and tonsil tissue quantified by fluorescence and mass cytometry

Biaxial plots show gating for established cell types in human tumors and tissues prepared using Col II plus DNase for 1 hour. Nucleated cells (HH3⁺) were identified. Immune cells (CD45⁺), T cells (CD45⁺ CD3⁺), APCs (CD45⁺ CD3⁻ HLA-DR⁺), endothelial cells (CD31⁺ CD45⁻), and non-immune nonendothelial cells (CD45⁻ CD31⁻) were also found. (A) In fluorescence cytometry analysis of glioma from an individual patient (G-RT-06), CD56 (NCAM) and GFAP expression are shown for CD45⁻ CD31⁻ cells. (B) A similar gating scheme was applied to mass cytometry data from G-RT-06. In tonsil tissue from donor T02-23, CD44 and HLA-DR are shown for CD45⁺ CD3⁻ HLA-DR⁺ cells, for both

fluorescence (C) and mass cytometry analysis (D). Frequency of terminal populations (dashed gates) was compared between fluorescence and mass cytometry in Table 1.

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Figure 4. Treatment of a glioma with different collagenases yielded comparable cell subset frequencies

viSNE plots show non-apoptotic nucleated cells (cCasp3⁺HH3⁺) from glioma G-LC-15 obtained following 1-hour treatment with DNase plus either Col II, VI, V, or XI. Heat plots indicate cell density (first column) or expression of 8 proteins indicating cell type (CD45, CD3, CD64, CD31, GFAP, CD56, S100B, and SOX2). viSNE mapping was run together. Color-coded inserts next to the complete map highlight cell subsets (grey = CD45⁺CD3⁺ T cell, $0.9 \pm 0.1\%$; red = CD45⁺CD64⁺ microglia, $3.9 \pm 1.0\%$; green = CD45⁻CD31⁺ endothelial cells, $0.7 \pm 0.2\%$; fuchsia = SOX2⁺ stem-like cells, $1.2 \pm 0.5\%$).

Figure 5. Enzymatic treatment times longer than one hour differentially impact glioma tumor cell subsets

Biaxial plots and bar graphs quantify cell subsets measured in mass cytometry analysis of glioma G-LC-15 after varying treatment times with collagenase II and DNase. (A) Gating for apoptotic cells (cCasp3⁺) and live immune cells (cCasp3⁻CD45⁺), endothelial cells (cCasp3⁻CD31⁺), and non-immune, nonendothelial cells (cCasp3⁻CD45⁻CD31⁻). (B) Subsets of glioma tumor-infiltrating immune cells were identified, including microglia (HLA-DR⁺CD64⁺), CD45RO⁺ and CD45RO⁻ subsets of CD3⁺ T cells, and other immune cells. (C) Pericytes (CD45⁻CD31⁻αSMA⁺), ependymal cells (CD45⁻CD31⁻CD24⁺), SOX2⁺ stem-like cells (CD45⁻CD31⁻SOX2⁺), GFAP⁺ cells (CD45⁻CD31⁻GFAP⁺), and astrocyte-like cells (CD45⁻CD31⁻S100B⁺) were quantified as subsets of G-LC-15. (D) Gating for cell types as in (A-C) was applied to mass cytometry analysis of cells from G-LC-15 treated with collagenase II plus DNase for 1, 2, 4, 6, 10, 16, or 24 hours. (E) Percentage of apoptotic cells as in (A) was measured for each dissociation time, as in (D).

Table 1
Mass and fluorescence cytometry detect comparable frequencies of cell types in glioma, melanoma, and tonsil tissue

Tissue	Subsets	Percent [‡]		R [*]	Rank		$\rho^{\S}$
		MC	FC		MC	FC	
Glioma G-RT-06	CD56 ⁺ GFAP ⁻	0.7	0.1		1	1	
	Endothelial cells	1.0	0.8		2	3	
	CD56 ⁺ GFAP ⁺	1.1	0.2		3	2	
	CD56 ⁻ GFAP ⁻	11.4	6.1	0.97	4	4	0.93
	CD56 ⁻ GFAP ⁺	18.1	15.4		5	6	
	T cells	27.8	15.1		6	5	
Tonsil T02-23	Other immune cells	39.3	30.9		7	7	
	Non-APCs	2.1	2.5		1	2	
	Non-immune	5.2	2.3		2	1	
	CD44 ⁺ APCs	16.2	17.7	0.98	3	3	0.90
	T cells	27.1	33.8		4	4	
	CD44 ⁺ APCs	40.3	39.1		5	5	

[‡] Frequency of terminal populations (dashed gates in Figure 3, percent) was measured by mass cytometry (MC) or fluorescence cytometry (FC)

^{*} Pearson's correlation coefficient R

[§] Spearman's ranked ρ