A Web-based Software Resource for Interactive Analysis of Multiplex Tissue Imaging Datasets

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Abstract

Highly multiplexed tissue imaging (MTI) is accomplished by applying powerful antibody-based spatial proteomics technologies that characterize tissues *in situ* at single-cell and potentially subcellular resolution and enable the creation of two-dimensional tissue maps. Example MTI methods include cyclic immunofluorescence (CyCIF), multiplex immunohistochemistry, Co-Detection by Indexing (CODEX), imaging mass cytometry, and multiplex ion beam imaging (MIBI). Analysis and visualization of MTI datasets is challenging and requires tens of analysis tools to be applied to datasets that are often hundreds of gigabytes in size. To make analysis of MTI datasets both broadly accessible and interactive, we have developed Galaxy-ME, a web-based software hub for interactive end-to-end analysis of MTI datasets. Galaxy-ME includes all tools and visualization platforms needed for analysis of MTI datasets, and the hub infrastructure enables both scalable and reproducible analyses. We have used the Galaxy-ME hub to process and visualize datasets from three different MTI assays to characterize both normal and cancerous tissue samples totaling 2.1 million cells. The Galaxy-ME hub is a publicly available open-source web resource that anyone can use to analyze their own MTI datasets.

Main

Highly multiplexed tissue imaging (MTI) technologies, such as cyclic immunofluorescence[1], multiplex immunohistochemistry[2], Co-Detection by Indexing (CODEX)[3], imaging mass cytometry[4], and multiplex ion beam imaging (MIBI)[5] are powerful and widely used *in situ* spatial proteomics

technologies for characterizing tissues at single-cell and potentially subcellular resolution. MTI generates rich antibody-based proteomics datasets that preserve the spatial context of the tissue and can be used to create detailed 2D tissue maps where cell phenotypes and spatial relationships can be investigated. Application of MTI is growing rapidly in both basic and translational research. MTI has been used to identify differences in tissue organization between healthy and diseased tissue[3] as well as to quantify the compositional and spatial features associated with many aspects of cancer and other diseases, including disease development[6], patient survival[7–9], and response to therapy[10]. MTI is also used extensively in many large tissue atlas consortia, including the Human Cell Atlas[11], the Human BioMolecular Atlas Program (HuBMAP)[12], and the Human Tumor Atlas Network[13].

With the substantial growth and use of MTI technologies in the biomedical research community, there is an acute need for robust analytical tools and visualizations of MTI datasets. A typical MTI dataset is tens or hundreds of gigabytes in size and includes images of a tissue sample from which each assayed marker protein is measured, yielding a stack of 30-100 images and tens or hundreds of thousands of cells analyzed per sample. A complete software analysis workflow for MTI must accomplish two large tasks: (1) provide primary image processing to produce single-cell feature tables that include marker abundance levels, morphological information, and spatial coordinates and (2) perform single-cell analysis to phenotype individual cells and quantify spatial relationships amongst cells. Because each of these large tasks requires running tens of individual analysis tools, computational workflows that execute all tools in sequence have been developed for particular MTI assays, including cyclic immunofluorescence[14], CODEX[15], multiplex immunohistochemistry[16], and imaging mass cytometry[17]. Recently, software workflows have been developed that can process datasets from several different MTI assays[18,19]. Some analysis workflows produce visualizations of raw image data or perform single-cell analyses, and many also connect with visualization platforms such as Napari[20] and OMERO[21]. General-purpose image analysis platforms such as CellProfiler[22], QuPath[23], and Fiji[24] also provide some functionality for processing MTI datasets.

Despite available tools, workflows, and visualizations, there remain challenges in accessibility, tool integration, and scalability that make it difficult to analyze MTI datasets. To address these challenges, we have developed Galaxy-MCMICRO Ecosystem (Galaxy-ME), a user-friendly and highly scalable web-based hub for interactive analysis of MTI datasets. Galaxy-ME makes analysis of MTI datasets widely accessible via a web-based graphical user interface (GUI) for analyses, and its underlying infrastructure ensures analyses are scalable and reproducible. We used Galaxy-ME to analyze two large MTI datasets from both healthy and diseased tissue, quantifying compositional and spatial features of the tissues and assessing differences between MTI platforms. Galaxy-ME is a public resource that anyone can use to analyze their own MTI datasets, or a private Galaxy-ME hub can be run on a local computer or computational cluster to analyze sensitive datasets such as those from patients.

Results

Motivation and Overview of Galaxy-ME

Two key challenges motivated the development of Galaxy-ME. First, high throughput MTI analysis workflows have many steps and are often executed using a command line text interface, requiring

significant computational expertise to use. Second, there are gaps between analysis and visualization when processing MTI datasets, making it difficult to iterate on analyses and easily visualize analysis results. While some desktop applications integrate analysis and visualizations together, these applications cannot easily be deployed on disparate infrastructures, such as cloud based resources, or at scale. Galaxy-ME addresses these issues by providing a web-based software workbench for MTI analyses that is accessible to all scientists, a comprehensive tool and visualization suite for analysis of MTI datasets, and infrastructure to ensure that all analyses are scalable and reproducible (Figure 1). Galaxy-ME is built on the Galaxy computational workbench (https://galaxyproject.org/)[25,26],

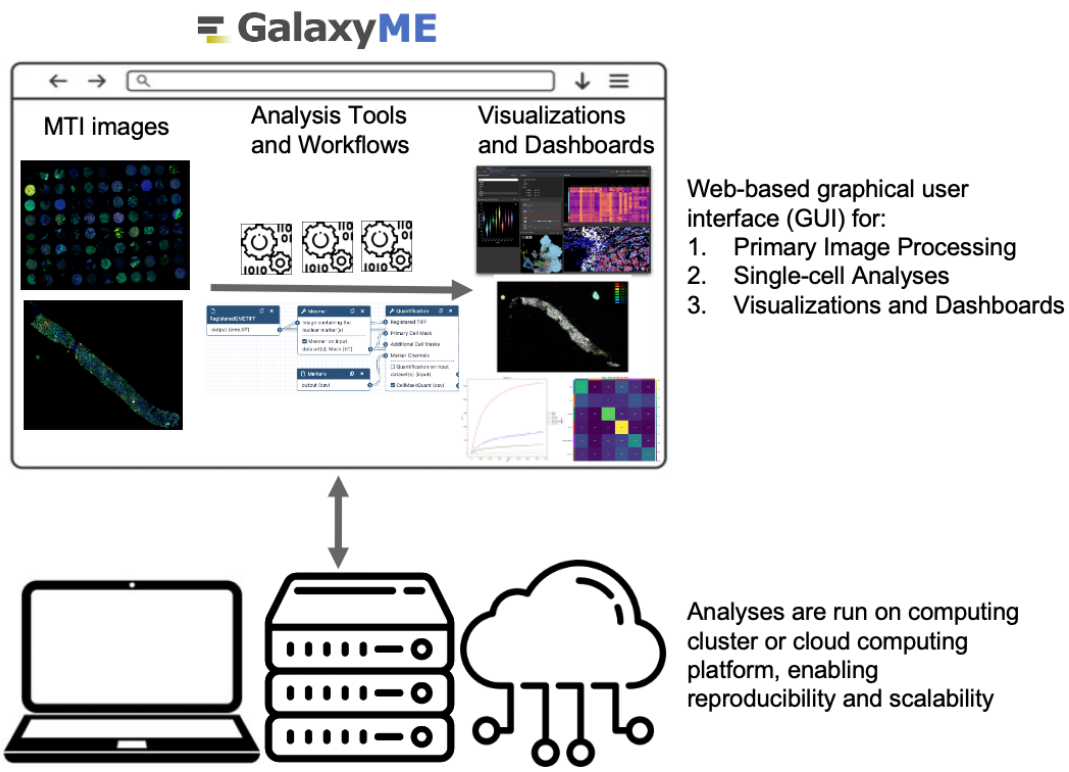

Figure 1: Galaxy-ME overview. top, Galaxy-ME provides a web-based user interface to perform all analysis and visualization tasks for multiplexed tissue imaging datasets: primary image analysis and visualization, marker quantification, single-cell analyses, and integrated dashboards for visual exploration of both primary and single-cell data. **bottom,** Galaxy-ME analyses can be run on a laptop, computing cluster, or a cloud computing platform. All analyses are completely reproducible, and running Galaxy-ME on a computing cluster or cloud computing platform makes it possible to complete large-scale analyses.

an open platform for user-friendly, reproducible, and collaborative biomedical data analyses. Galaxy is among the most popular software analysis platforms in the world and used by thousands of scientists daily across the world. Galaxy and Galaxy-ME are open-source and freely available for both academic and commercial use.

Galaxy-ME builds on prior work developing analyses for microscopy images in Galaxy. Early work applied Galaxy to primary image analysis of single-channel microscopy images[27]. More recently, we integrated the MCMICRO tool suite into Galaxy for primary image analysis of multiplexed imaging datasets[19]. Galaxy-ME is a substantial advance of this prior work in three ways. First, Galaxy-ME expands MCMICRO with many additional tools for analysis, visualization, and dashboarding of MTI datasets to create a tool suite of 15 tools that enables end-to-end analysis of highly multiplexed tissue imaging datasets (Figure 2 and Table 1). End-to-end analysis starts with primary image processing and proceeds to single-cell analyses, including phenotyping and spatial analyses.

The second advance that Galaxy-ME introduces is the leveraging of the full capabilities of Galaxy for analysis of multiplexed tissue imaging datasets. Using Galaxy's workflow editor and execution

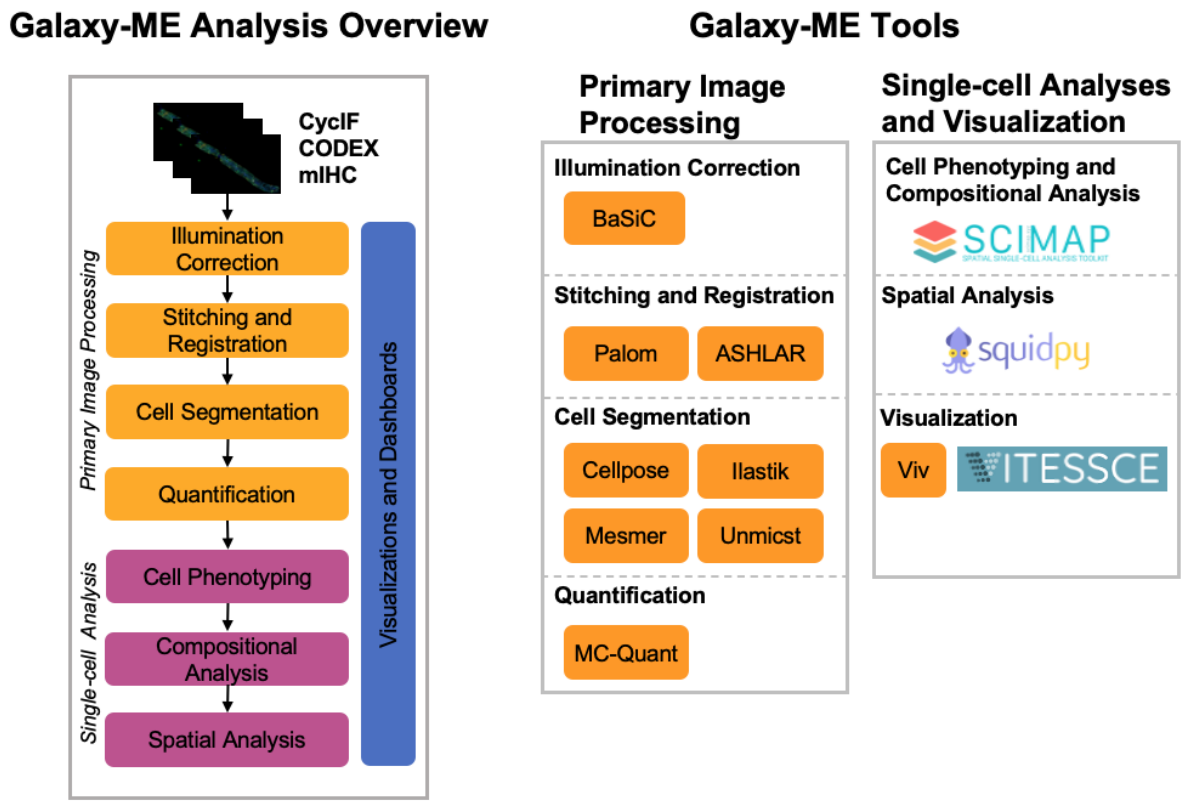

Figure 2: Galaxy-ME Tools and Visualizations. **left**, overview of multiplexed tissue imaging analyses that can be performed in Galaxy-ME. These include primary image processing, single-cell analyses, and visualizations plus dashboards. **right**, Individual software tools and packages integrated into Galaxy-ME that are available for each analysis step.

Tool Suite	Purpose	Number of Tools
MCMICRO[17]	Primary image processing	8
Viv[30]	Primary image viewing	1
Cellpose[26]	Cell segmentation	1
Mesmer[27]	Cell segmentation	1
Scimap[17]	Single cell phenotyping and spatial analysis	3
Squidpy[29]	Single cell spatial analysis	1
Vitessce[31]	Dashboard of interactive visualizations	2

Table 1. **Tools and visualizations in Galaxy-ME**.

support, Galaxy-ME includes a number of automated workflows that run multiple analysis tools to process an entire MTI dataset and generate all outputs. Analysis steps include image stitching and alignment, registration, cell segmentation, quantification, phenotyping using both prior knowledge and data-driven approaches, and spatial analyses. Key visualizations include interactive viewing of the registered image stack and dashboards of linked plots and images. The tools and visualizations in Galaxy-ME represents the current best-practice analysis approaches and integrates analysis tools and visualizations from multiple tissue atlas consortia including the Human Cell Atlas[11], the Human BioMolecular Atlas Program (HuBMAP)[12], and the Human Tumor Atlas Network[13]. Scientists can upload their own datasets and use Galaxy-ME to analyze them with available tools and workflows. When analyses are complete, all outputs can be downloaded from Galaxy-ME. Scientists can analyze datasets from several MTI platforms, including cyclic immunofluorescence, multiplex immunohistochemistry, and CODEX.

The third advance of Galaxy-ME is the introduction of a fully-featured and web-based platform for analysis of multiplexed tissue imaging datasets. Galaxy-ME uses Galaxy's web-based interface to create a graphical user interface (GUI) that all scientists, regardless of their computational expertise, can use to run MTI analyses and visualize results. The Galaxy-ME GUI enables individual analysis tools or entire computational workflows to be run using a standard web interface where inputs are selected, parameters are set, and tools are executed on remote computing resources. Visualization of both imaging data and single-cell analyses is also done in a web browser. Galaxy-ME's GUI makes it simple to move between analysis and visualization to support visual analysis of datasets, such as inspecting the output of individual tools and changing parameters based on observations made from visualizations.

Building on top of the Galaxy platform enables Galaxy-ME to take advantage of the scalability and reproducibility features that Galaxy offers. Galaxy-ME tools and visualizations are orchestrated by the Galaxy server and executed on computing resources that are configured for the server, such as an institutional computing cluster, a cloud computing platform, or even a laptop computer. Using these large resources enables analyses to scale to large imaging datasets and process imaging datasets in parallel. At the same time, scientists can use Galaxy's GUI to organize and analyze thousands of files. To ensure reproducibility, Galaxy records all analysis steps and parameters, ensuring that all Galaxy-ME analyses can be completely reproduced. Finally, there are >8,500 analysis tools integrated into Galaxy and available via its Tool Shed[28], making it possible to combine analysis of MTI datasets with a large variety of other biomedical data analyses, including genomics, transcriptomics, drug discovery, and machine learning.

Public web services for Galaxy-ME are available at https://cancer.usegalaxy.org/ and at https://spatialomics.usegalaxy.eu/. Galaxy-ME can also be downloaded and run locally on a laptop or in connection with a computing cluster by following the instructions to download and install Galaxy followed by installation of Galaxy-ME.

Primary Image Processing

Multiplex tissue images can be generated by a diverse set of microscopy and mass spectrometry-based approaches. For example, with cyclic immunofluorescence microscopy, images are collected through sequential cycling using fluorophore-conjugated antibodies that label individual proteins in a

biological tissue sample. Despite the variety of MTI methods, they all generate a stack of individual image channels containing intensity values that act as a proxy for the abundance of each protein in the assay. Prior to single-cell or spatial analyses, multiplex image datasets require extensive primary processing to go from a stack of raw, un-aligned images to tabular data with protein marker intensities and morphological features for every cell in the tissue.

Primary image processing in Galaxy-ME is done largely with the MCMICRO[19] suite of tools, in addition to supplemental tools for key steps (Figure 2b). Image processing steps include: (1) illumination correction between microscopy tiles (with the BaSiC[29] tool); (2) stitching of tiles into channel mosaics (ASHLAR); (3) registration of channel mosaics into a multi-channel OME-TIFF pyramid (ASHLAR, PALOM); (4) single-cell segmentation (UnMICST and S3segmenter[30], Ilastik[31], Cellpose[32], Mesmer[33]); and (5) quantification of protein marker intensities for every cell (MCQuant). Processing tissue microarrays requires an additional step to dearray sample cores into separate images (UNet-Coreograph), which is performed after illumination correction and stitching. Single-cell segmentation—the process of creating an image mask that assigns pixels into individual indexed cells—is a challenging but critical image processing step that can vary dramatically in performance between assays and tissues. For this reason, several segmentation tools have been integrated into Galaxy-ME so that the best segmentation method can be applied to each dataset. Final outputs from primary image processing are (1) an OME-TIFF file that includes all image channels and (2) a cell feature table with mean protein marker intensities and morphological features (area, eccentricity, orientation, and solidity) for each cell identified in the segmentation mask.

Single-Cell Analysis and Visualization

Single-cell analysis tools in Galaxy-ME use the cell feature table produced by primary image processing for cell phenotyping, compositional analysis, and spatial analysis (Figure 2). Galaxy-ME supports single-cell phenotyping using two complementary approaches. First, cells can be gated using a biologically-driven, semi-automated hierarchical gating approach using the SciMap[34] package from MCMICRO. The output of this approach is a distinct phenotype for each cell, such as *CD8+ T-cell* or *luminal neoplastic tumor cell*. The second approach for phenotyping cells is a data-driven approach where cells are clustered using community metrics[35,36] (e.g. Louvain, Leiden). This approach produces clusters that can then be annotated based on the markers enriched in each cluster. Galaxy-ME uses spatial analysis methods in SciMap and SquidPy[37] to quantify spatial interactions, neighbor enrichment between cell types, spatial neighborhoods, and other metrics of tissue spatial organization. Galaxy-ME uses the common ANNData format (https://anndata.readthedocs.io) for storing single-cell data.

There are three primary visualizations available in Galaxy-ME. Avivator is a light-weight and web-based image viewer built with the Viv[38] library that enables viewing multi-channel OME-TIFF images hosted on a web server. With Avivator, different image channels can be selected and visualized, and it is possible to pan and zoom around images. For additional data viewing, Galaxy-ME provides a tool to create Vitessce[39] interactive dashboards that include multiple connected visualizations. These visualizations include multiplex images augmented with labeled segmentation masks as well as phenotype information, UMAP plots, phenotype marker enrichment, and single-cell heatmaps.

Analysis of Multiplex Tissue Imaging Datasets from Different Assays

We used Galaxy-ME to perform fully automated analysis of datasets from three distinct multiplex tissue imaging assays: cyclic immunofluorescence (CycIF)[1], multiplex immunohistochemistry (mIHC)[2], and Co-Detection by Indexing (CODEX)[3]. A supplementary webpage linking to all Galaxy histories for this work is available at https://bit.ly/GalaxyME-Histories.

Our first MTI analysis was completed on three datasets obtained by imaging consecutive tissue sections, taken from the same resection of human tonsil tissue, that were assayed with the CycIF, mIHC, or CODEX platforms (Figure 3a). This dataset was generated by the Human Tumor Atlas Network[13] and was previously analyzed by MCMICRO[19]. The MCMICRO analysis focused on primary image process and stopped at single-cell protein abundance levels. Our analysis re-processed the images using Mesmer for segmentation and extended what was done with MCMICRO to include cell phenotyping as well as compositional and spatial analyses. A fully automated analysis was performed using a Galaxy-ME workflow (Figure 3b) that produced several text outputs as well as dashboards for interactive analysis (Figure 3c).

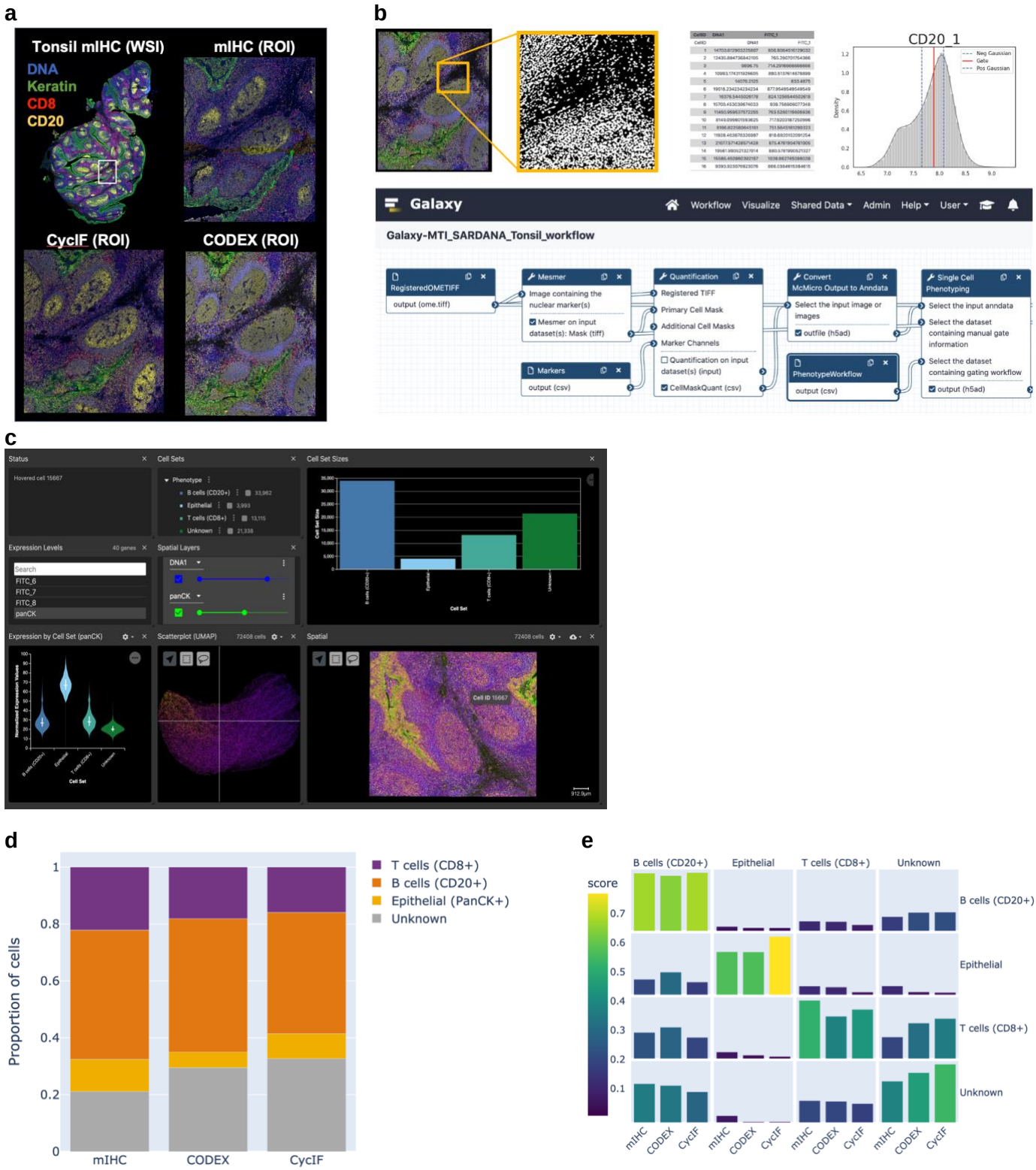

Figure 3: Cross-platform comparison of a tonsil specimen. **a**, an overview of the tonsil images analyzed. Shown is the mIHC whole slide image (top left), and the analyzed region of interest imaged using mIHC (top right), CycIF (bottom left), and CODEX (bottom right). The platforms shared 5 common markers, including DNA (blue), Keratin/PanCK (green), CD8 (red), and CD20 (yellow). **b**, the Galaxy-ME workflow and history used for the CODEX tonsil image analysis. Processing began with the registered OME-TIFF image of the ~70,000 cell region of interest. The images were segmented and single-cell mean marker intensities, spatial coordinates, and morphological features were computed. Markers were automatically thresholded, and cells were assigned phenotypes using Scimap. **c**, a Vitessce dashboard, which can be launched out of the CODEX analysis history in Galaxy, showing the registered image overlaid with a phenotype-labeled segmentation mask, compositional barplots, UMAP representations, and marker expression violin plots. Individual cells can be highlighted using a cursor and data representing highlighted cells will be spotlighted in each of the plots. Vitessce also includes heatmaps (not pictured). **d**, stacked barplots showing the proportion of phenotypes in the tonsil ROI between each platform. **e**, a neighborhood enrichment matrix calculated using Squidpy for each platform. Each bar shows the normalized count of neighbor phenotypes (columns) for each query phenotype (rows).

The same region of interest was analyzed across the three datasets, and each underwent cell segmentation using Mesmer. The mean cell count across the three platforms was 69,329 cells with a standard deviation of 3152 cells. Differences in total cell counts are likely due to spatial separation in the resection as they were non-adjacent slides and from platform-specific performance by Mesmer. The three platforms each had a nuclear marker and shared four common protein markers: Pan Cytokeratin, CD45, CD20, and CD8. Cells were labeled as positive or negative for each protein using a threshold for marker positivity that was determined automatically using Scimap and validated with manual gating. Of the four markers, only CD45 showed poor concordance in the proportion of positive cells between the platforms. This was likely due to CD45 antibody quality control issues for the CODEX data (Figure S1). Phenotypes were assigned to cells based on marker positivity/negativity using Scimap and were highly concordant across the platforms (Figure 3d). Spatial patterns were quantified for each platform by calculating a normalized spatial interaction score between each phenotype using a spatial neighborhood graph from Squidpy (Figure 3e). Spatial interaction scores between cell types were similar across all platforms, showing spatial organization is also concordant across platforms.

Our second analysis focused on matched datasets imaged using two marker panels for CycIF and one marker panel for mIHC applied to three adjacent slides of a custom breast cancer tissue microarray (TMA), also generated by the Human Tumor Atlas Network. This TMA includes 88 cores, with representation of numerous control tissues, normal breast tissue, and all major breast cancer subtypes (Figure 4a). This analysis demonstrates how Galaxy-ME supports batch processing and provenance tracking of the many samples on a TMA (Figure 4b). For the CycIF datasets, whole slide images were subdivided into separate images for each tissue core using UNetCoreograph; the mIHC imaging data had been previously dearrayed. After dearraying, batch processing was performed on the 88 core images: we first performed cell segmentation via Mesmer followed by quantification of cell features using MCMICRO's MCQuant module. Cell feature tables from each separate core were then pooled and assigned phenotypes using Scimap automatic gating (Figure 4c). Across the cores ~650,000 total cells were found, totaling almost 2 million cells across the three slides for the entire analysis.

Per-core total cell counts across the three MTI panels were highly concordant ($r = 0.99$ CycIF-CycIF, $r = 0.85$ CycIF-mIHC), suggesting that cell segmentation performed similarly between the different imaging modalities (Figure 4d). High per-core concordance was observed across the MTI panels in the proportion of positive cells for PanCK ($r = 0.66$ CycIF-CycIF, $r = 0.77$ CycIF-mIHC), aSMA ($r = 0.77$ CycIF-CycIF, $r = 0.72$ CycIF-mIHC), and Ki67 ($r = 0.70$ CycIF-CycIF, $r = 0.85$ CycIF-mIHC) (Figure S2). However, less concordance ($0.2 < r < 0.6$) across platforms was observed for low-abundance immune populations. Despite limited concordance across immune populations, the mean phenotype distributions by clinical subtype were concordant between the CycIF immune panel and mIHC panel (Figure 4e), demonstrating that cellular phenotype analyses between adjacent slides from different MTI platforms yield concordant results.

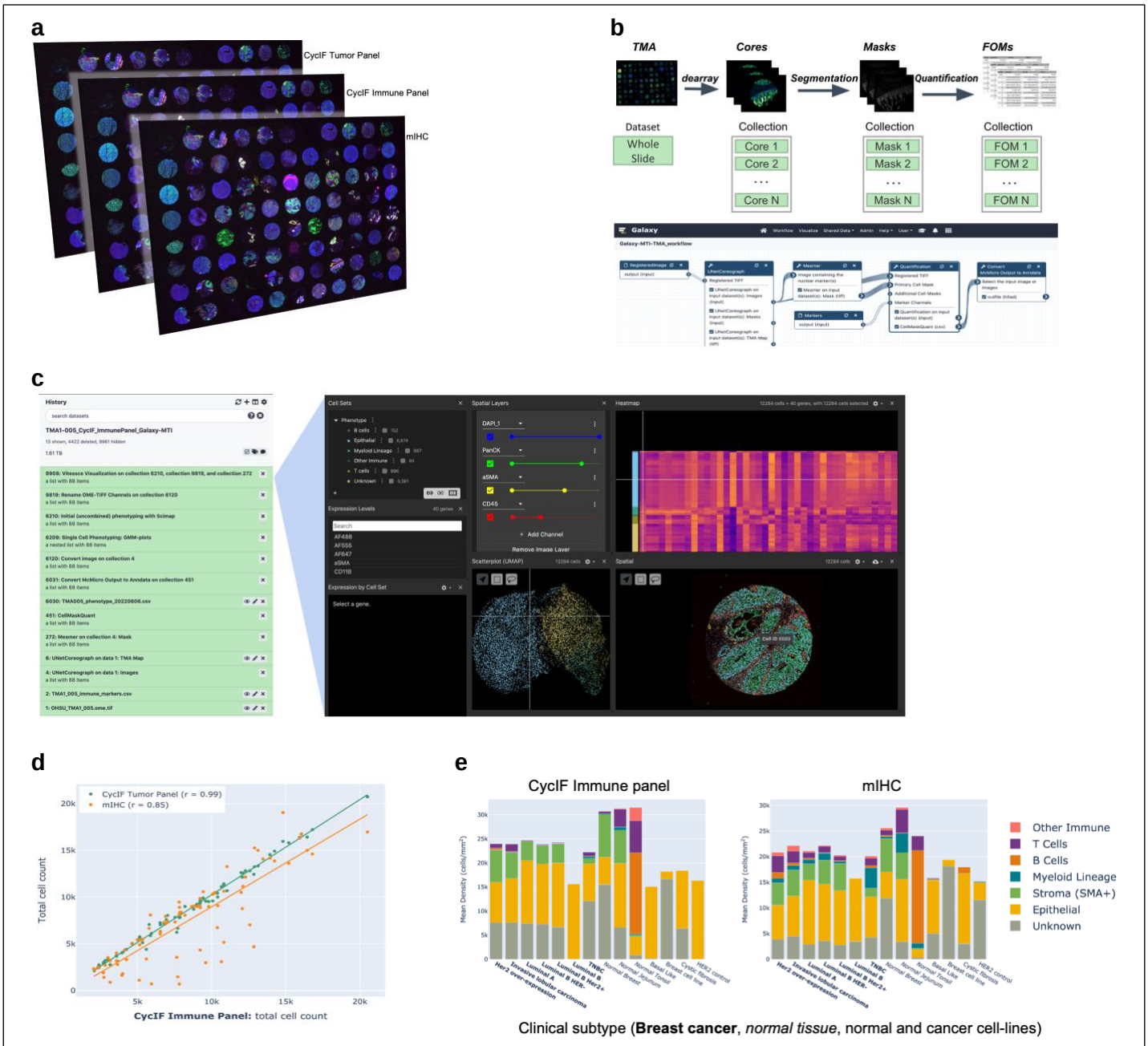

Figure 4: Cross-platform comparison of a breast cancer tumor microarray. **a**, three image datasets were obtained from multiplexed tissue imaging using cyclic immunofluorescence (CycIF) with a panel of tumor markers and another panel of immune markers as well as multiplexed immunohistochemistry (mIHC); **b**, analysis overview (top) and Galaxy workflow used for analysis (bottom); **c**, Galaxy analysis history showing datasets generated from analysis and dashboard for a single core; **d**, concordance of cell counts between datasets; **e**, mean densities for cell types for CycIF immune panel and mIHC.

Similar to the spatial analysis conducted for the tonsil images, spatial neighborhood graphs were constructed for each core using Squidpy and used to calculate closeness centrality scores for cell populations. Closeness centrality is a measure of how clustered a group of nodes in a graph are, and was highly concordant across slides for the epithelial (r = 0.73 CycIF-CycIF, r = 0.91 CycIF-mIHC) and stroma (r = 0.80 CycIF-CycIF, r = 0.75 CycIF-mIHC) cell populations (Figure 5a), but less concordant for the immune population (r = 0.51 CycIF-CycIF, r = 0.56 CycIF-mIHC) (Figure 5b). To explore the spatial organization of the TMA, Latent Dirichlet Allocation (LDA) was used to identify recurrent cellular neighborhoods (RCNs) in the CycIF Immune panel and mIHC. Following LDA, the

single-cell latent space weights were used for K-means clustering (k=25; Figure S3). Subsequent hierarchical clustering identified 12 and 10 RCNs for the mIHC and CycIF platforms, respectively. Phenotype composition of the RCNs shows the CycIF (Figure 5c) had more epithelial dominated RNCs while mIHC (Figure 5d) had more immune dominated RCNs. Overall, most RCNs were concordant in the phenotype composition across the RNCs based on cosine similarity (Figure 5e), and both technologies' RCNs captured common distinct spatial structures such as epithelial-stromal interface and mixed populations of immune cells (Figure 5f).

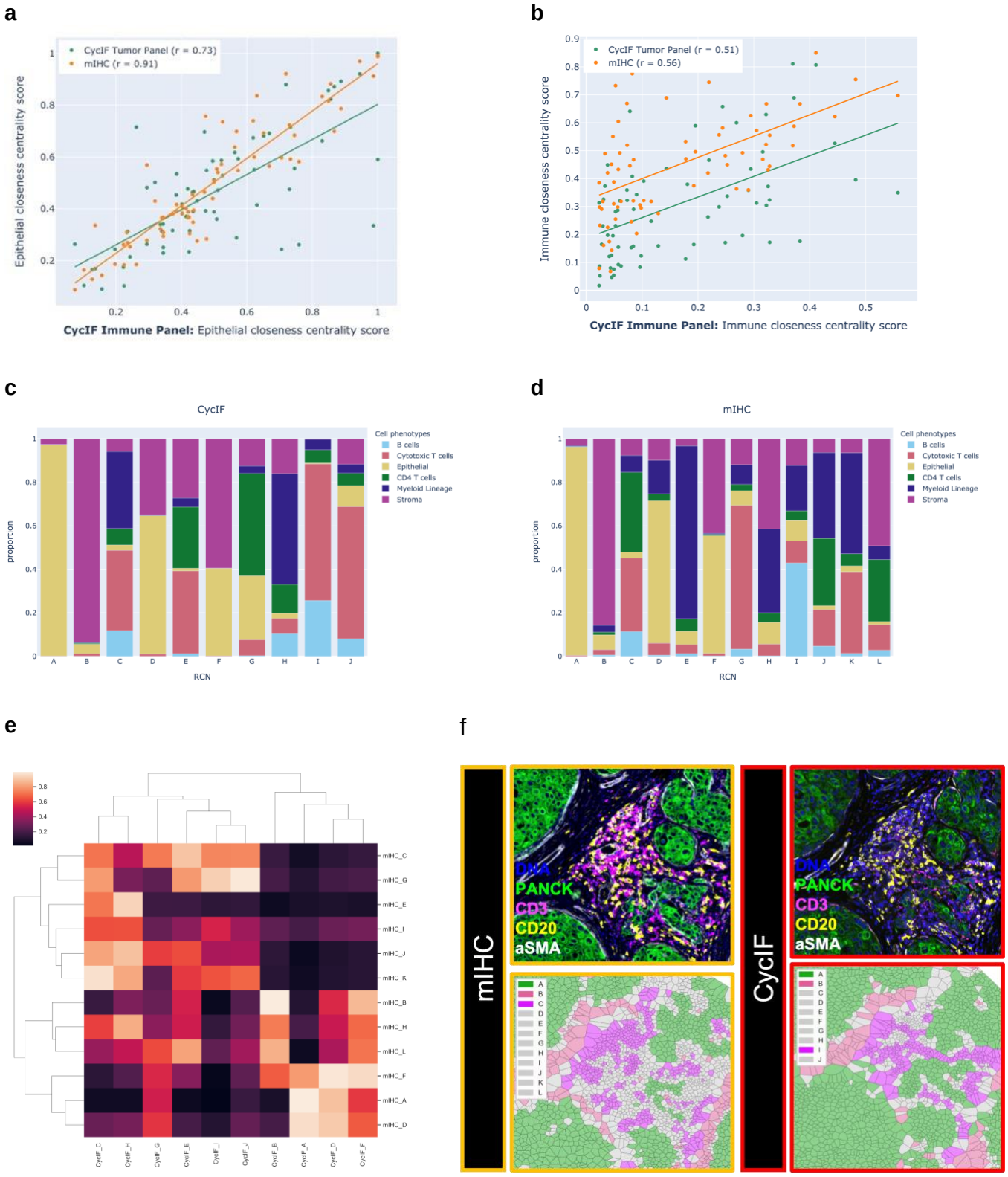

Figure 5. Cross-platform analysis of spatial organization. Concordance compared across the CycIF Immune panel, tumor panel, and mIHC platform of **a**, epithelial closeness centrality score across datasets; and **b**, immune closeness centrality score across datasets. Phenotype composition across recurrent cellular neighborhoods identified in the **c**, CycIF immune panel and **d**, mIHC platform. **e**, Cosine similarity matrix of the RCNs across the platforms. **f,** Example RCNs identified in both technologies: (i) the interface between epithelial (RCN A, green) and stromal (C, dark pink) is clear and (ii) mixed immune cell populations are colored dark pink and identified by RCN C in mIHC and RCN I in CycIF.

Together, these results demonstrate that automated analysis of datasets from several MTI technologies can be performed in Galaxy-ME with a high degree of concordant across technologies. The observed concordance across marker intensity levels, phenotypes and single-cell composition, and spatial organization suggests multiple MTI platforms produce high-quality datasets. Given imaging datasets from multiple MTI technologies, Galaxy-ME analysis tools and workflows accurately process these datasets to produce high-quality single-cell compositional and spatial results.

Discussion

Analysis and visualization of multiplexed tissue imaging datasets remains a challenging problem because these datasets are very large and require using many analysis tools and visualizations together, in an iterative and interactive manner. Galaxy-ME is a software hub and tool suite for comprehensive and flexible visual analysis of MTI datasets. Galaxy-ME provides a web-based graphical user interface for running individual analysis tools, executing entire analysis workflows, and interactive visualization of images and single-cell analysis results. The set of analysis tools and visualizations for Galaxy-ME makes it possible to perform entire analyses of MTI datasets, including all steps of primary image processing as well as single-cell analyses. Primary image analysis steps include illumination correction, stitching, registration, cell segmentation, and marker quantification. Single-cell analysis steps include cell phenotyping by hierarchical gating or clustering and a variety of spatial analyses. Visualizations include a multichannel image viewer and interactive dashboards of connected plots that summarize all aspects of MTI analysis. There are two public web services for using Galaxy-ME, https://cancer.usegalaxy.org/ and https://spatialomics.usegalaxy.eu/, and Galaxy-ME can also be downloaded and run locally as well. Galaxy-ME enables analysis of datasets from a wide variety of multiplexed tissue imaging assays and uses standard formats to enable compatibility with as many assays as possible.

Galaxy-ME uniquely centralizes all MTI analyses and visualizations into a single web browser platform. This overcomes several barriers other analysis approaches often encounter, such as requiring use of a text command line interface, manually connecting multiple desktop-based tools and visualizations, downloading and managing very large datasets, and setting up configurations for making use of large computing resources. By streamlining analysis and visualization of MTI datasets into a single web-based graphical user interface, Galaxy-ME democratizes access, analysis, and visualization of MTI datasets so that any scientist, regardless of their informatics expertise, can work with MTI datasets.

Building an MTI analysis hub on the Galaxy computational workbench has numerous advantages. Galaxy workflows are both automated and easily modified with different tools for the same tasks, making it possible to develop benchmarking results that compare performance of tools on different analysis tasks such as cell segmentation and phenotyping. Whereas many MTI and general-purpose analysis platforms are focused specifically on image analysis, Galaxy-ME tools can be connected with more than 8,500 other tools available in Galaxy to create analyses that extend far beyond imaging. For instance, Galaxy machine learning tools[40] can be used to develop predictive models that use features derived from MTI datasets to predict clinical attributes such as overall survival or response to therapy. The Galaxy community has developed tool suites and workflows for processing

bulk and single cell omics data[41–44], enabling integration of Galaxy-ME tools with these other tool suites to do sophisticated multimodal analyses. The Galaxy framework ensures precise reproducibility of all analyses, including all MTI analyses, by recording the details of all analysis steps. Galaxy-ME can scale to analysis of MTI datasets that are large in size, number, or both by taking advantage of Galaxy's capabilities for large-scale analysis. Galaxy's user interface enables organizing hundreds or thousands of datasets into collections that can be processed together. A Galaxy server can be configured to use all kinds of computational resources from local compute and data storage to computing clusters and cloud computing platforms. Limitations in computational power and data storage arise from the underlying computational platform and not Galaxy or the Galaxy-ME tool suite.

To understand how different MTI assays compare with each other, we used Galaxy-ME to analyze datasets from cyclic immunofluorescence, multiplex immunohistochemistry, and CODEX from both normal tonsil and breast cancers tissues. This analysis demonstrates that a fully automated approach for MTI analysis is feasible and produces concordant results for both single-cell composition and spatial quantifications. These results are a key step forward in demonstrating that MTI assays and automated analyses of MTI datasets are consistent, reproducible, and do not suffer from significant technical artifacts or analysis challenges. However, our analysis revealed that automated phenotyping methods for markers associated with low-abundance cell populations is less accurate than for markers associated with high-abundance populations. Additional automated data-driven methods that phenotype markers for low-abundance populations or manual validation may be needed to prevent low-quality phenotyping results for these markers.

Galaxy-ME is a unique software resource for the analysis of multiplexed tissue imaging datasets. It includes all analysis tools and visualizations for end-to-end analysis, and it is user-friendly, free to use, accessible to scientists regardless of their informatics expertise, and is available via a Web browser. Looking forward, there are opportunities to add additional tools and visualizations that extend Galaxy-ME beyond single-cell analysis to focus on quantification and organization of both larger and smaller units of biological organization. Larger units of biological organization may include cellular neighborhoods composed of similar cell types and organized in a similar way or functional tissue units[45]. Smaller units of organization would focus on subcellular characteristics such as epithelial polarity and protein or RNA localization within cells.

Data Availability

The tonsil image datasets analyzed are available at https://www.synapse.org/MCMICRO_images, and the breast cancer tissue microarray images are available at https://doi.org/10.7303/syn32627552.1.

Code Availability

The Galaxy-ME code repository, including galaxy tool wrappers and Docker files, are available at: https://github.com/goeckslab/tools-mti.

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Figure S1.

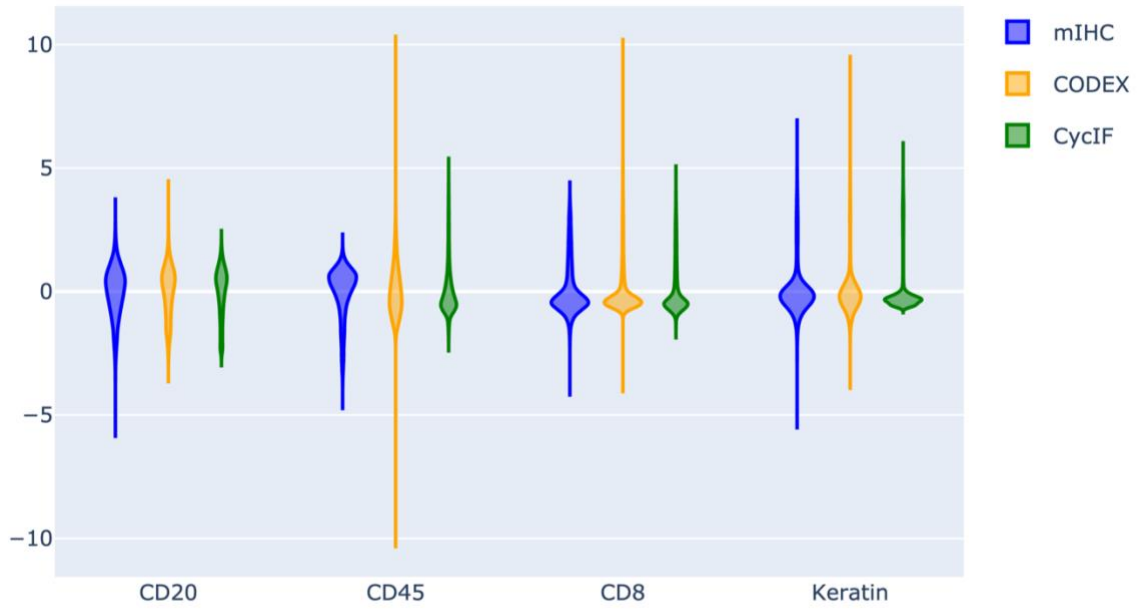

Figure S1. Tonsil rescaled marker intensity distributions by platform. Rescaled intensity distributions for the lineage markers shared between the three imaging platforms: mIHC (blue), CODEX (yellow), and CycIF (green). For CD20, CD8, and Keratin, the distribution peaks are concordant; however, CD45 shows discordance that may be explained by antibody quality control issues in the CODEX data. For this reason, CD45 was excluded from phenotype classification.

Figure S2.

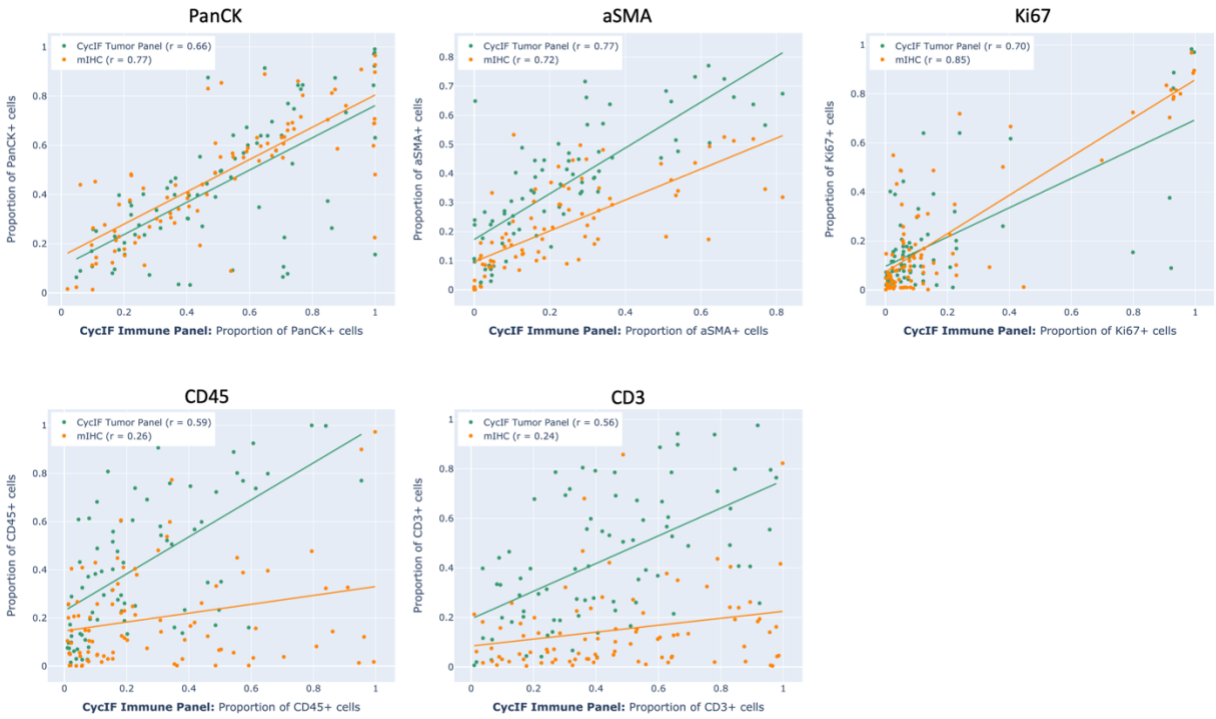

Figure S2. Per-core marker positivity correlations between adjacent slides in a TMA. For each plot, the CycIF tumor panel (green) and mIHC (orange) slides are plotted against the CycIF immune panel where each point is a core in the TMA. The three panels show high concordance for PanCK, aSMA, and Ki67; however, concordance is lower for the immune markers, CD45 and CD3, particularly between mIHC and CycIF.

Figure S3.

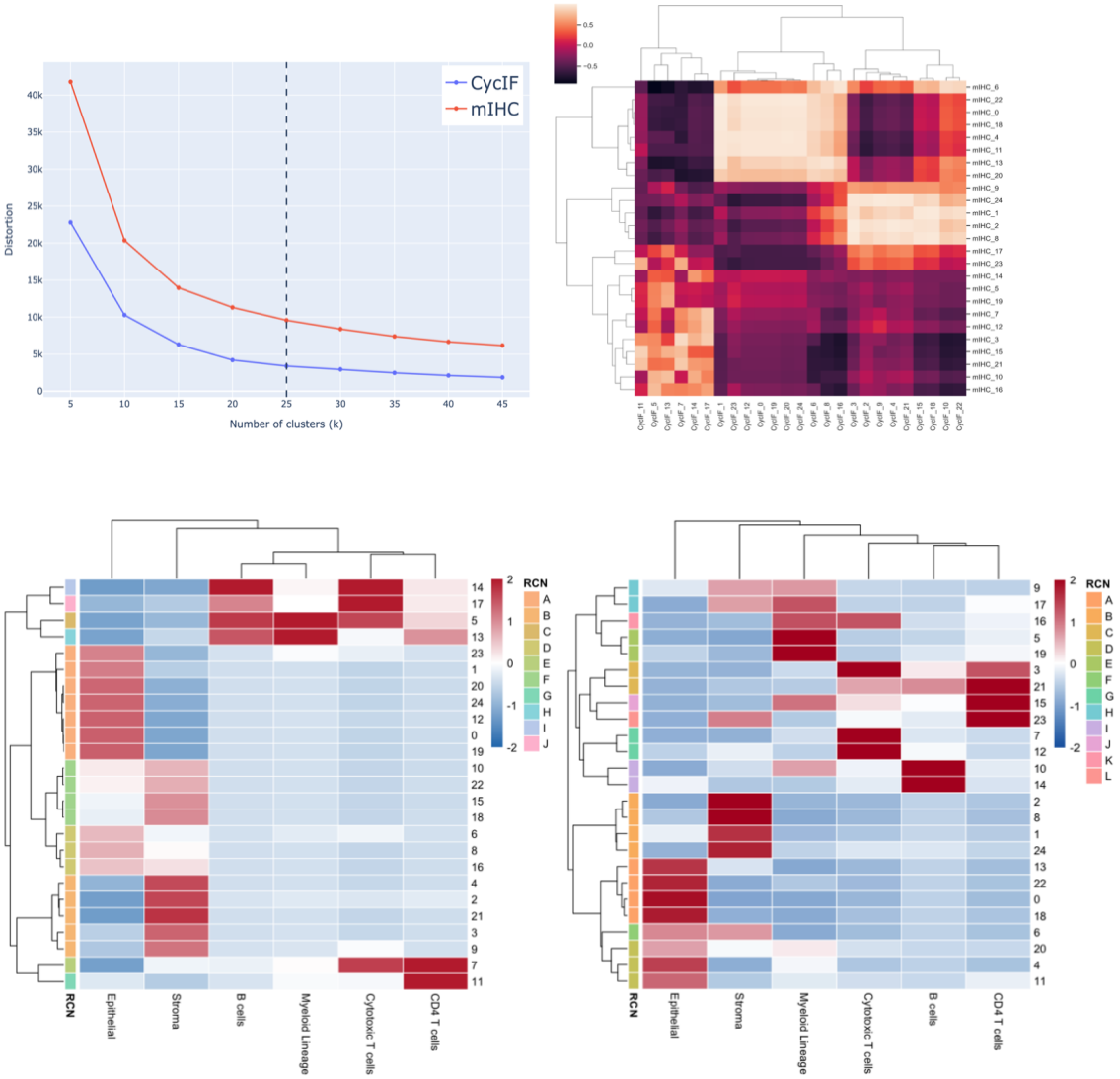

Figure S3. Recurrent cellular neighborhood analysis using LDA. Elbow plot to identify the optimal k for K means clustering. Heatmap of cosine concordance of cluster phenotype composition across the CycIF and mIHC technologies. Heatmap of clusters for CycIF and mIHC to define recurrent cellular neighborhoods.