

# Robust phenotyping of highly multiplexed tissue imaging data using pixel-level clustering

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## 1 **Abstract**

2           While technologies for multiplexed imaging have provided an unprecedented  
3 understanding of tissue composition in health and disease, interpreting this data remains a  
4 significant computational challenge. To understand the spatial organization of tissue and how it  
5 relates to disease processes, imaging studies typically focus on cell-level phenotypes. However,  
6 images can capture biologically important objects that are outside of cells, such as the  
7 extracellular matrix. Here, we developed a pipeline, Pixie, that achieves robust and quantitative  
8 annotation of pixel-level features using unsupervised clustering and show its application across a  
9 variety of biological contexts and multiplexed imaging platforms. Furthermore, current cell  
10 phenotyping strategies that rely on unsupervised clustering can be labor intensive and require  
11 large amounts of manual adjustments. We demonstrate how pixel clusters that lie within cells can  
12 be used to improve cell annotations and decrease the amount of manual fine-tuning needed. We  
13 comprehensively evaluate pre-processing steps and parameter choices to optimize clustering  
14 performance and quantify the reproducibility of our method. Importantly, Pixie is open source and  
15 easily customizable through a user-friendly interface.

## 16 **Introduction**

17           The advancement of multiplexed tissue imaging technologies over the last few years has  
18 enabled the deep phenotyping of cells in their native tissue context.<sup>1-9</sup> Investigating the  
19 relationship between tissue structure and function using multiplexed imaging has led to important  
20 discoveries in many fields, including cancer, infectious disease, autoimmunity, and  
21 neurodegenerative disease.<sup>10-17</sup> As imaging studies continue to grow in number and size, so does  
22 the need for robust computational methods for analyzing these data. In most multiplexed imaging  
23 studies, cells are the objects of interest that are quantified and investigated in downstream  
24 analyses. As such, development of methods for accurate cell annotation is an active area of  
25 research.<sup>18-20</sup> Unlike assays that measure dissociated single cells such as CyTOF or single-cell  
26 RNA-sequencing, imaging data is not inherently measuring single cells and can capture  
27 substantial information content outside of cells. These extracellular features can have important  
28 biological functions. For example, the extracellular matrix is increasingly being recognized as an  
29 important modulator of the tissue microenvironment in cancer and other disease contexts.<sup>21-24</sup> In  
30 addition, protein aggregates can form as extracellular deposits and have been implicated in many  
31 neurological disorders.<sup>25</sup> These important acellular objects are captured in multiplexed images  
32 but are typically not the focus in multiplexed imaging studies.

33           One important consideration when analyzing imaging data is that the tissue sections that  
34 are captured in the images are two-dimensional cross sections of complex three-dimensional  
35 objects. Depending on the plane of tissue sectioning, what is observed in the images can be  
36 highly variable (Supplementary Fig. 1a). For example, depending on if the cell body or dendrites  
37 are in the plane of imaging, dendritic cell markers can take on a typical round cellular shape or  
38 might only be present as small spindle-like projections (Supplementary Fig. 1b). While cell  
39 segmentation methods for accurately defining the boundary of cells have recently been  
40 developed<sup>26-28</sup>, signal along the edges of cells can be misassigned to neighboring cells,  
41 particularly in dense tissues where cells are packed close together (Supplementary Fig. 1c,d).

42 Furthermore, cells that are elongated or shaped distinctly from spherical cells, or anucleated cells,  
43 are difficult to capture using cell segmentation (Supplementary Fig. 1e). Identifying phenotypes  
44 at the pixel-level can address many of these issues that confound high-dimensional image  
45 analysis.

46 Due to these challenges with analyzing multiplexed imaging data, we developed a  
47 pipeline, Pixie, for the quantitative annotation of pixel-level features that captures phenotypes  
48 independent of traditional cell segmentation masks. We perform extensive evaluation of pre-  
49 processing steps and parameter choices to optimize clustering performance, as well as  
50 comprehensively assess stochasticity, an often-ignored aspect of high-dimensional data analysis  
51 methods. In addition, we show the application of Pixie across various tissue contexts and imaging  
52 platforms, including mass-based, fluorescence-based, and label-free technologies. Finally, we  
53 show how pixel clusters can be utilized to improve the identification of cell phenotypes. Taken  
54 together, Pixie is a complete pipeline for generating both pixel and cell-level features that is  
55 scalable, cross-platform, and publicly accessible in Jupyter notebooks that include user-friendly  
56 graphical user interfaces (GUI) for cluster adjustment and annotation.

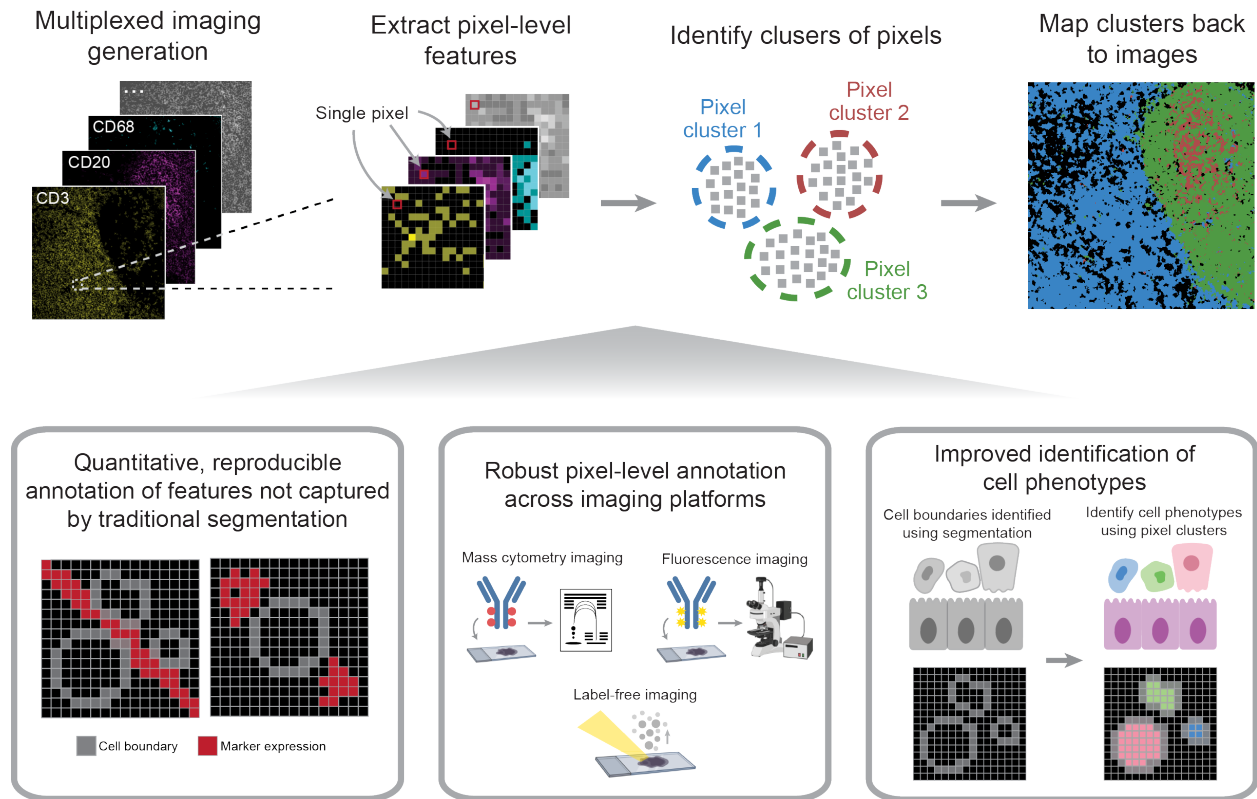
57

## 58 **Results**

### 59 **Overview of pixel clustering using Pixie**

60 We created a full pipeline, Pixie, for the generation of quantitative pixel-level features from  
61 multiplexed images, with the goal of designing a pragmatic workflow that balances automation  
62 and unbiased analysis with human curation (Fig. 1). We aimed to create a robust and scalable  
63 pipeline that is user-friendly and easily extensible. After a multiplexed imaging dataset has been  
64 generated, the output is a series of images, each corresponding to a different marker of interest.  
65 The first step is to decide which markers will be included in the clustering process. Typically,  
66 phenotypic markers are included, while functional markers that can be expressed across various  
67 cell types are excluded. After the user has specified the subset of markers relevant for

68 phenotyping, we perform a series of pre-processing steps, described in detail below (Fig. 2a,  
69 Supplementary Fig. 2a). A common practice in high-dimensional data analysis is to first cluster  
70 observations into a large number of clusters (such as 100), then metacluster these clusters into  
71 biologically relevant groups.<sup>10–12,15,17,29</sup> This allows for the capture of rare phenotypes and more  
72 precise clusters. In Pixie, we use a self-organizing map (SOM)<sup>30</sup>, an unsupervised clustering  
73 algorithm, to cluster all pixels into a large number of clusters (typically 100). We then combine  
74 these into metaclusters using consensus hierarchical clustering. If necessary, the final clusters  
75 can be manually refined and annotated with biologically relevant labels using a custom-built GUI  
76 in Pixie. These phenotypes can be mapped back to the original images and quantified in  
77 downstream analysis. Pixie is publicly available as user-friendly Jupyter notebooks that perform  
78 all pre-processing steps and clustering, starting from multiplexed images through generation of  
79 pixel phenotype maps, where the color of each pixel corresponds to its pixel cluster.

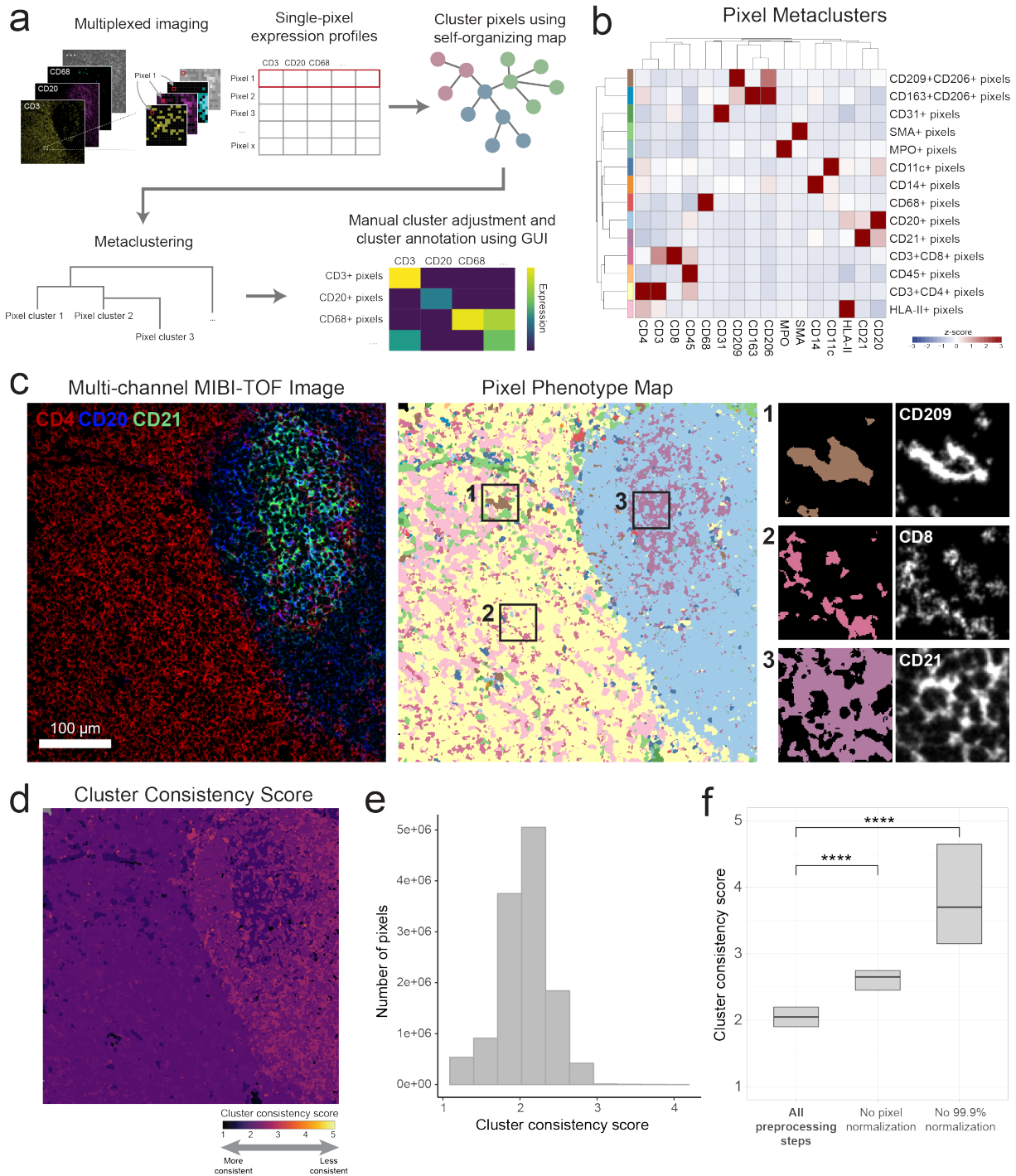


**Figure 1: Pixie robustly captures pixel-level and cell-level phenotypes in multiplexed imaging datasets.** After acquiring multiplexed images, single-pixel expression profiles are extracted from the multi-channel imaging dataset and clustered to identify pixel-level phenotypes. This method can be used to generate quantitative, reproducible annotations not captured by traditional cell segmentation, and is cross-platform and applicable across a variety of biological contexts. Finally, pixel-level phenotypes can be combined with traditional cell segmentation and be used to improve the annotation of cell-level phenotypes.

80           In Pixie, the user specifies the number of metaclusters for consensus hierarchical  
81 clustering and can manually adjust these metaclusters (Supplementary Fig. 2b). While there have  
82 been various methods developed to computationally determine what the algorithm predicts is the  
83 optimal number of metaclusters, we found that manual inspection of the cluster expression  
84 profiles is a fast and accurate method for determining the number of relevant phenotypes. The  
85 number of relevant phenotypes, although subjective, may vary based on the biological question  
86 of interest and is best understood by the researchers leading the study. For example, for a study  
87 that is focused on granular subsets of myeloid cells, it may be important to stratify populations  
88 that are expressing combinations of CD206, CD209, CD163, CD68, CD14, and CD16 within the  
89 monocyte/macrophage lineage. However, for another study that is interested in mapping the  
90 general immune landscape, these markers may be grouped together into the macrophage  
91 population. Analogous to “human-in-the-loop” approaches in the fields of machine learning and  
92 artificial intelligence, the manual annotation step allows us to utilize the biological expertise of the  
93 user to improve the results more quickly. Furthermore, one of the reasons that it has previously  
94 been difficult to manually adjust clustering results is that there was no good way to manually  
95 interact with the clustering outputs. Importantly, manual adjustments and annotations can be  
96 easily made using a custom-built GUI in our Jupyter notebooks (Supplementary Video 1).

### 97 **Pixie captures the major immune phenotypes in lymph node tissue**

98           To test pixel clustering in Pixie and optimize parameters, we used a dataset of lymph  
99 nodes stained with a panel of immune markers (Supplementary Table 1) and imaged using  
100 multiplexed ion beam imaging by time-of-flight (MIBI-TOF). In addition to T and B lymphocytes,  
101 lymph nodes contain many dendritic cells, follicular dendritic cells, and macrophages that have  
102 traditionally been difficult to capture using cell segmentation. Lymph nodes are also densely  
103 packed tissues, which can confound cell phenotyping. Therefore, we chose this lymph node MIBI-  
104 TOF dataset for proof of principle to evaluate our method.



**Figure 2: Pixie identifies accurate and consistent pixel-level features in lymph node tissue.** (A) Overview of pixel clustering in Pixie. Individual pixels are clustered using a self-organizing map (SOM) based on a set of phenotypic markers. The clusters output by the SOM are metaclustered using consensus hierarchical clustering. If necessary, users can manually adjust the metaclusters, then annotate each metacluster with its phenotype based on its expression profile using our easy-to-use GUI. (B) Heatmap of mean marker expression of pixel cluster phenotypes for an example dataset of lymph node samples. Expression values were z-scored for each marker. (C) Multi-channel MIBI-TOF image of a representative field-of-view (FOV) (left), the corresponding pixel phenotype map (middle), and representative insets (right). Colors in the pixel phenotype map correspond to the heatmap in B. (D) The FOV in C colored according to the cluster consistency score. (E) Distribution of cluster consistency score across all pixels in the dataset. (F) Comparison of cluster consistency score across different pre-processing steps. \*\*\*\* indicates  $p$ -value  $< 2e-16$  using a Wilcoxon test.

105           Using Pixie, we were able to capture the major immune phenotypes that would be  
106 expected in a lymph node (Fig. 2b). We found that automated metaclustering was able to generate  
107 accurate features and required only a small amount of manual adjustment (Supplementary Fig.  
108 2b). Importantly, we were able to capture more fine-grained features at the pixel-level that would  
109 likely be grouped together at the cell level. For example, CD209+CD206+ pixels clustered  
110 separately from CD163+CD206+ pixels (Fig. 2b), which would likely have both been assigned to  
111 macrophages at the cell level. Mapping these pixel clusters back to the original images, we can  
112 see that the pixel clusters accurately recapitulate underlying spatial trends in protein expression  
113 (Fig. 2c, Supplementary Fig. 3). We could clearly delineate the germinal center, B cell follicle, and  
114 surrounding T cell zone in the lymph node (Fig. 2c). Thus, we were able to quickly and accurately  
115 quantify high dimensional phenotypes at a pixel-level using Pixie.

#### 116 **Evaluating reproducibility**

117           Many algorithms commonly used to analyze high-dimensional datasets – including  
118 unsupervised clustering methods and dimensionality reduction techniques such as tSNE (t-  
119 distributed stochastic neighbor embedding) and UMAP (uniform manifold approximation and  
120 projection) – are stochastic, meaning that there is randomness inherent to the algorithms.  
121 Therefore, running the same method on the same dataset using a different random seed will  
122 generate distinct results. Good clustering results that reflect true biological phenotypes should be  
123 reproducible across different random initializations. Here, we evaluated the stochasticity of our  
124 pipeline and defined a metric, which we termed the “cluster consistency score”, for quantifying  
125 reproducibility (Methods). In an ideal situation, we would simply evaluate how consistently pixels  
126 were assigned to the same phenotype across replicates. However, like all unsupervised clustering  
127 approaches, Pixie relies on manual annotation using expert knowledge to assign each cluster to  
128 a phenotype. Given that this process would need to be repeated for each replicate in each  
129 experiment, direct evaluation of consistency in this manner would not be feasible for large

130 numbers of tests. The cluster consistency score allows us to measure reproducibility in an  
131 automated way and quantitatively compare different parameter choices.

132 To calculate the cluster consistency score, we run pixel clustering on the same dataset  
133 and the same parameters five times, each time with a different random seed, then quantify how  
134 stable the cluster assignments are for each pixel across replicate runs (Methods). The cluster  
135 consistency score can be roughly interpreted as the number of different clusters a given pixel was  
136 assigned to across replicates. Lower scores indicate higher reproducibility, with a score of 1 being  
137 the best possible score. A score of 1 would indicate that in all the replicates, the same pixels were  
138 always grouped together in the same cluster. In contrast, a high score indicates bad  
139 reproducibility, meaning that the pixel was assigned to clusters that may have contained many  
140 other pixel types.

141 To benchmark the cluster consistency score using high-dimensional datasets that are  
142 commonly analyzed using stochastic methods, we used two publicly available single cell datasets,  
143 a CyTOF dataset of whole blood and single-cell RNA-sequencing dataset of peripheral blood  
144 mononuclear cells (PBMCs) (Supplementary Fig. 4c-e).<sup>31,32</sup> We would expect better  
145 reproducibility for single cell datasets than for pixel-level features, since as mentioned above,  
146 imaging data is not inherently single cell and is therefore more noisy. Pixel-level data is also  
147 noisier because it is only a fraction of the total cell volume. Even for these well-behaved single  
148 cell datasets, the cluster consistency score was above 1, showing that stochasticity is an inherent  
149 feature of multi-dimensional data analysis that should be taken into account.

150 We calculated this cluster consistency score for each pixel in the lymph node dataset (Fig.  
151 2d,e), which had an overall cluster consistency score of  $2.07 \pm 0.32$  (mean  $\pm$  SD). Despite the  
152 stochastic nature of the algorithm, by viewing the pixel phenotype maps across replicate runs, we  
153 can see that the majority of pixel cluster assignments were stable across replicates  
154 (Supplementary Fig. 4a), demonstrating the reproducible nature of this method. Here, we



155 developed a quantitative metric that can be used to assess the reproducibility of Pixie across  
156 different parameter choices.

### 157 **Optimization of Pixie for accurate pixel classification**

158 When developing Pixie, we optimized a series of pre-processing steps that leads to more  
159 accurate, reproducible pixel clustering results (Supplementary Fig. 2a). While these steps and  
160 parameters have been tested across a variety of datasets and platforms as described below,  
161 these are all tunable steps that can be easily modified in our Jupyter notebooks.

162 First, we apply a Gaussian blur to the data. Akin to dropout in single-cell RNA-sequencing  
163 data where genes are not detected due to low amounts of mRNA in individual cells and inefficient  
164 mRNA capture<sup>33</sup>, multiplexed images do not capture all of the protein expressed in the tissue.  
165 Analogous to compensating for dropouts, we use a Gaussian blur to smooth the signal to make  
166 the distribution more reflective of the true underlying data. We assessed four different standard  
167 deviations for the Gaussian blur and balanced resolution of features and cluster consistency by  
168 visualizing the clusters and evaluating the cluster consistency score (Supplementary Fig. 5). We  
169 determined that a standard deviation of 2 was optimal.

170 Next, we apply a pixel normalization step, in which for each individual pixel, we divide the  
171 signal of each marker by the total signal in that pixel, such that the sum total of that pixel is 1. The  
172 intuition behind this step is that when performing phenotyping, we are interested in the ratio  
173 between the phenotypic markers. The absolute intensity of pixels across images can be different,  
174 for example due to biological differences (such as downregulation of T cell receptors upon  
175 activation) or technical differences (drifts in instrument sensitivity, variations in tissue fixation and  
176 staining). While the underlying cause for the differences in intensity can be different, the resulting  
177 differences in absolute intensity of individual pixels confounds the phenotyping. The ratio of  
178 marker expression within each pixel contains the important phenotyping information. Without this  
179 key pre-processing step, the results contained one dominating pixel cluster that was poorly  
180 defined (low expressing for all the markers) (Supplementary Fig. 6). After pixel normalization, we

181 apply a 99.9% marker normalization step, where each marker is normalized by its 99.9<sup>th</sup> percentile  
182 value. When this step was excluded, the results contained poorly defined clusters that expressed  
183 many markers (Supplementary Fig. 7). Importantly, when either the pixel normalization or the  
184 99.9% marker normalization steps were excluded, the cluster consistency score was significantly  
185 worse (Fig. 2f), indicating that these pre-processing steps are vital for generating consistent  
186 clusters that are more likely reflective of true biology.

187         There are many additional parameters in the SOM that can be tuned. As described above,  
188 Pixie uses a SOM to first overcluster the pixels, then metaclusters. For the lymph node dataset,  
189 we tested using a SOM to directly cluster pixels into 15 clusters (Supplementary Fig. 8). This  
190 resulted in poor pixel cluster definition for some clusters, as well as a worse cluster consistency  
191 score, indicating that initial overclustering is a critical step for accurate results. Furthermore, the  
192 number of passes through the dataset that is used to train the SOM is another tunable parameter.  
193 For this dataset, we compared training the SOM using 1 pass, 10 passes, and 100 passes  
194 (Supplementary Fig. 9). We found that 10 passes were sufficient to achieve accurate clusters and  
195 that increasing the number of passes up to 100 did not change the clustering results.

196         Pixel-level data analysis can introduce significant computational demands. When training  
197 a SOM, all the pixels must be read into memory at the same time. For very large datasets, this is  
198 a computational bottleneck. To ensure that this approach is scalable, we wanted to see if a  
199 subsampling approach could yield equally valid results. We hypothesized that for large datasets,  
200 a random subset of pixels is a representative sample and provides the SOM with enough  
201 information to generate accurate clusters. Using a large dataset of around 800 million total  
202 pixels<sup>34</sup>, we trained the SOM using a random 10% subset of pixels (Supplementary Fig. 10). We  
203 found that the results of subsampling were highly concordant with the results from the whole  
204 dataset, and the cluster consistency score was consistent as well. We have shown that for large  
205 datasets, using a random subset of pixels to train the SOM is more computationally efficient and  
206 leads to highly accurate results. Therefore, this approach is scalable to large datasets.

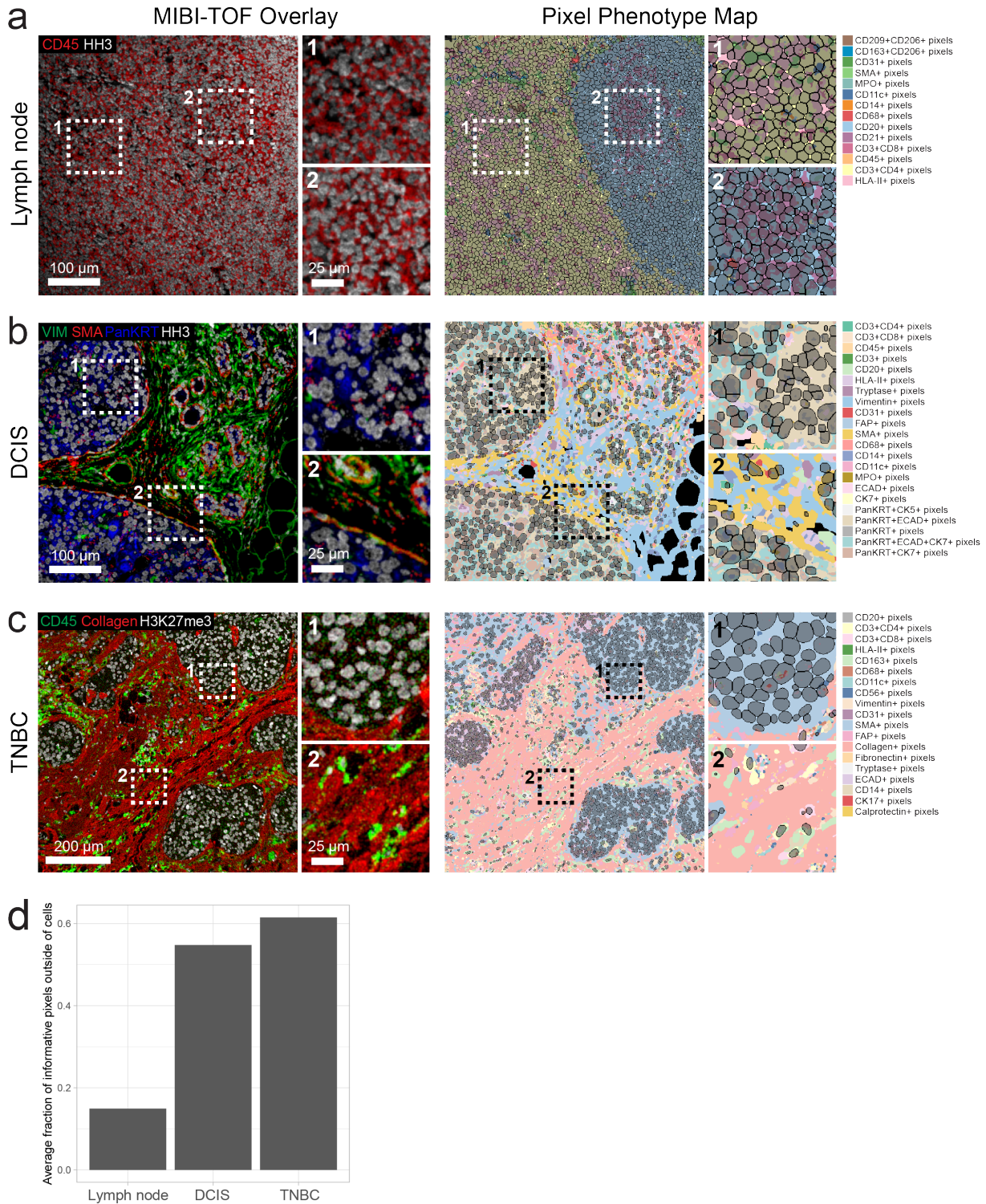
207 Here, we optimized a set of pre-processing steps and parameters for consistency and  
208 biological accuracy. While we have applied these steps and parameters across a variety of  
209 datasets, these parameters are easily modified in our user-friendly pipeline.

### 210 **Pixie captures extra-cellular information in multiplexed images**

211 While cells are important features to annotate in multiplexed images, a large amount of  
212 information is captured in multiplexed images outside of cells (Fig. 3). The extracellular matrix,  
213 blood vessels, and extracellular protein aggregates are examples of objects that can exist outside  
214 of the cellular space. The biological relevance of these objects can vary based on tissue type and  
215 disease context. For example, in immune tissue such as lymph nodes (Fig. 3a), cells are densely  
216 packed within the tissue and therefore most pixels fall within cells. However, for ductal carcinoma  
217 in situ (DCIS) or triple negative breast cancer (TNBC) that contain large amounts of extracellular  
218 matrix and structural proteins (Fig. 3b-3c), pixel clustering is useful for generating a quantifiable  
219 metric for extracellular features. For these tissue types, pixels that lie outside of cells that express  
220 phenotypic markers make up a majority of the total pixels in the image (Fig. 3d). Pixie assigns a  
221 phenotype to each of these pixels, which can then be quantified and analyzed in downstream  
222 analysis. Importantly, pixel clustering using Pixie allows us to utilize a larger percentage of the  
223 informative pixels captured using multiplexed imaging technologies than cell-level analysis only.

### 224 **Pixel-level features from Pixie are reproducible across replicate MIBI-TOF runs**

225 For multiplexed imaging approaches to be used in large translational studies and  
226 eventually in clinical diagnostics, not only must the imaging technology be robust, workflows for  
227 analyzing these data must be reproducible and accurate. In recent work by our group, we  
228 undertook a validation study to demonstrate the reproducibility of MIBI-TOF in which we assessed  
229 concordance across a dozen serial sections of a tissue microarray (TMA) of 21 cores that  
230 consisted of disease-free controls as well as multiple types of carcinomas, sarcomas, and central  
231 nervous system lesions.<sup>35</sup> Here, we demonstrate the reproducibility of Pixie on replicate serial  
232 sections (Supplementary Fig. 12a).

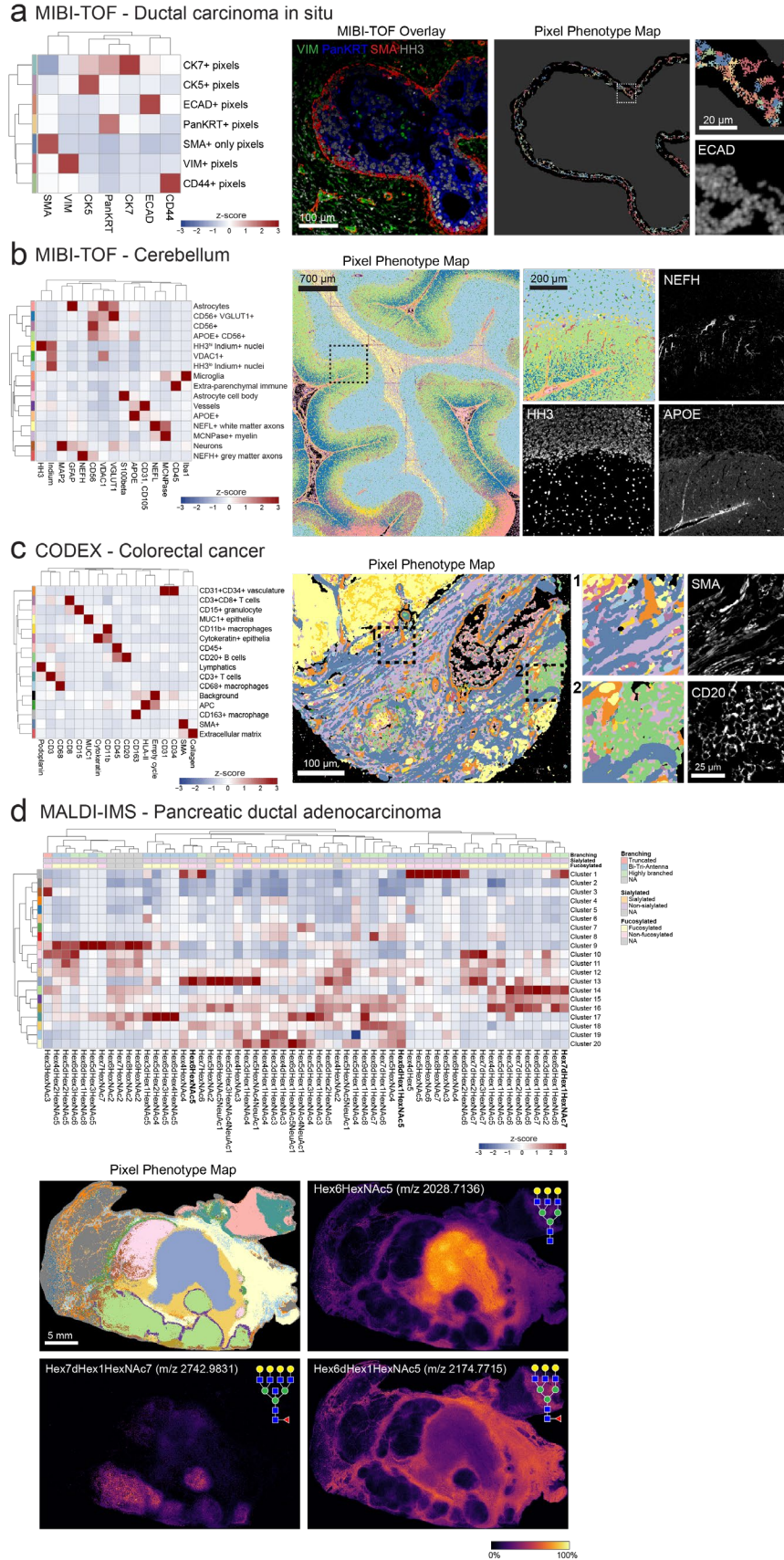


**Figure 3: Pixie captures more information in multiplexed images than cell segmentation masks alone.** Representative FOVs from (A) lymph node, (B) ductal carcinoma in situ (DCIS), and (C) triple negative breast cancer (TNBC), showing MIBI-TOF overlays (left) and pixel phenotype maps (right). Cells identified using cell segmentation are overlaid on the pixel phenotype maps in gray. Colors of the pixel phenotype maps correspond to the phenotypes indicated on the right. (D) Comparison of the average fraction of informative pixels (pixels that were included in pixel clustering) that were outside of the cell segmentation masks across the three datasets.

233           Using this pipeline, we clustered the pixels across all the images into 12 phenotypes  
234 (Supplementary Fig. 12b). Because we had six serial sections per tissue core in the TMA, we  
235 could assess the reproducibility of Pixie by finding the correlation between serial sections of the  
236 same tissue core. Because true biological replicates are not possible, we compared serial  
237 sections of each tissue core as a proxy. As a result, we would expect some true biological  
238 differences between serial sections. Despite these differences, the overall Spearman correlation  
239 was high, with  $R^2$  of  $0.92 \pm 0.03$  (mean  $\pm$  SD) (Supplementary Fig. 12c), demonstrating the  
240 reproducibility of pixel clustering using Pixie. This high reproducibility was obtained despite the  
241 intensity differences across experiments, showing that our normalization pipeline is robust to  
242 technical variation and batch effects (Supplementary Fig. 12d). Here, we show that despite  
243 differences in absolute pixel intensity, Pixie is able to capture reproducible pixel phenotypes,  
244 demonstrating that pixel clustering can generate biologically meaningful annotations across entire  
245 cohorts.

#### 246 **Applications of Pixie across biological contexts and imaging platforms**

247           In one example, we used pixel-level analysis to capture biologically meaningful  
248 phenotypes in the myoepithelial layer in ductal carcinoma in situ (DCIS). In previous work, our  
249 group used MIBI-TOF to characterize the transition from DCIS to invasive breast cancer (IBC)  
250 using a 37-plex panel.<sup>11</sup> DCIS is a pre-invasive lesion that is itself not life-threatening, but if left  
251 untreated, will progress to IBC in up to 50% of cases.<sup>36</sup> The myoepithelial layer surrounding the  
252 ductal cells is an important histological feature that is known to undergo transformations during  
253 the progression to IBC. Normal breast myoepithelium is a thick, highly cellular layer between the  
254 stroma and ductal cells. In DCIS, the myoepithelium becomes stretched out in a thin layer with  
255 few, elongated cell bodies. In IBC, complete loss of this layer is accompanied by local invasion of  
256 tumor cells. Therefore, understanding the changes in ductal myoepithelium may shed light on  
257 what drives the progression of DCIS to IBC. Classical cell phenotyping strategies, which rely on  
258 detecting cells with a strong nuclear signal and are often optimized for conventional cell shapes,



**Figure 4: Applications of Pixie across imaging platforms and biological contexts.** (A) Pixel-level phenotyping using Pixie of the myoepithelial layer in ductal carcinoma in situ (DCIS) imaged using MIBI-TOF.<sup>11</sup> Heatmap of mean marker expression of the pixel clusters (left), MIBI-TOF overlay of a representative FOV (middle), and corresponding pixel phenotype map and inset (right). In the pixel phenotype map, the black region represents the myoepithelial layer. (B) Identification of pixel-level neuronal and immune features in MIBI-TOF images of the human cerebellum. Heatmap of mean marker expression of the pixel clusters (left), pixel phenotype map of a tiled image of the cerebellum (middle), and comparison of an inset with single-marker images (right). (C) Pixel clustering in a CODEX dataset of colorectal cancer using Pixie.<sup>17</sup> Heatmap of mean marker expression of the pixel clusters (left), pixel phenotype map of a CODEX image (middle), and comparison of insets with single-marker images (right). (D) Pixel-level annotation of a MALDI-IMS dataset of pancreatic ductal adenocarcinoma using Pixie.<sup>37</sup> Heatmap of mean glycan expression of the pixel clusters (top). The rows correspond to pixel clusters and columns correspond to glycans. Pixel phenotype map (colors correspond to the heatmap) and comparison with selected glycans (bottom). Expression values were z-scored for each marker.

259 fail to capture the myoepithelial phenotype. To be able to quantify features of this important  
260 histological region, we used Pixie to identify discrete myoepithelial phenotypes (Fig. 4a). We  
261 identified seven myoepithelial phenotypes that could be quantified and compared across clinical  
262 subgroups: CK7+, CK5+, ECAD+, PanKRT+, VIM+, CD44+, SMA+. Here, we used Pixie to  
263 quantify distinct phenotypes in a small, but clinically relevant histological region that would not  
264 be captured using classical cell segmentation.

265 Another setting in which analyzing high-dimensional images has been challenging is in  
266 studies of the human brain. Studying the brain has historically been difficult due to the strong  
267 inherent autofluorescence of brain tissue, limiting the use of traditional fluorescence-based  
268 imaging techniques. We used MIBI-TOF to image neuronal and immune protein targets in the  
269 human brain. Due to the abnormal shapes of neuronal objects and the complex spatial  
270 conformations of features such as dendrites, cell bodies, and axons, classical cell segmentation  
271 techniques have limited efficacy, and detection of neuronal objects is an ongoing area of  
272 research.<sup>13</sup> Using Pixie, we were able to map the full neuronal landscape of the human  
273 cerebellum, including neurons, axons, vessels, astrocytes, and microglia (Fig. 4b). For example,  
274 NEFH (neurofilament heavy chain)-expressing grey matter axons could clearly be identified as a  
275 pixel cluster but would likely be missed in cell segmentation due to their irregular shape.  
276 Therefore, pixel clustering using Pixie allows for the retention and classification of informative

277 pixels that are independent of cell segmentation masks and can be used in downstream analysis,  
278 such as spatial analysis.

279 To test the cross-platform compatibility of Pixie, we applied it to a publicly available  
280 CODEX (co-detection by indexing) multiplexed imaging dataset of colorectal cancer.<sup>17</sup> In contrast  
281 to MIBI-TOF which uses metal-labelled antibodies, CODEX is a fluorescence-based method and  
282 achieves multiplexing by using DNA-barcoded antibodies and multiple cycles of imaging  
283 fluorescent nucleotides.<sup>1</sup> Pixel clustering using Pixie enabled us to capture the major structural  
284 and phenotypic features in the tissue, including vasculature, epithelia, lymphatics, and immune  
285 cells (Fig. 4c). Because CODEX uses fluorescence imaging, there are significant levels of  
286 autofluorescence in the images. By including images of the empty cycles in the pixel clustering,  
287 we were able to ameliorate the effect of autofluorescence on the phenotyping by defining an  
288 autofluorescence-specific cluster. Here, we have shown the applicability of Pixie to fluorescence-  
289 based imaging approaches.

290 Furthermore, we applied Pixie to a previously published MALDI-IMS (matrix-assisted laser  
291 desorption ionization-imaging mass spectrometry) dataset of pancreatic ductal  
292 adenocarcinoma.<sup>37</sup> MALDI-IMS is a label-free imaging approach, meaning antibodies are not  
293 used to target specific epitopes, and can be used to assess the distribution of complex  
294 carbohydrates in tissue, typified by N-linked glycans *de novo*. MALDI-IMS has been used to map  
295 N-glycan distribution across multiple cancer types.<sup>38-40</sup> Unlike MIBI-TOF, CODEX, and other  
296 antibody-based approaches, MALDI-IMS was used to map glycosylation patterns and not protein  
297 expression in this dataset, so traditional image analysis techniques that rely on detecting cellular  
298 objects are not applicable. Here, we used pixel clustering in Pixie to annotate discrete phenotypes  
299 in the tissue based on glycosylation patterns (Fig. 4d). Importantly, the pixel clusters identified  
300 here were reflective of the tissue features that were manually identified in the original publication.<sup>37</sup>  
301 For example, the pixel cluster localized to the center of the tissue, cluster 13 (purple-blue cluster),  
302 corresponded to the necrotic region of the tissue as determined by H&E in the original publication.



303 One of the defining glycans in this pixel cluster was Hex6HexNAc5 (m/z 2028.7136), which was  
304 identified in the original publication as being localized to necrotic tissue. Similarly,  
305 adenocarcinoma and normal pancreatic regions were captured using pixel clustering and were  
306 defined by glycans identified in the original publication, such as Hex7dHex1HexNAc7 (m/z  
307 2742.9831) and Hex6dHex1HexNAc5 (m/z 2174.7715), respectively. Therefore, despite the fact  
308 that MALDI-IMS is a completely different type of imaging technology that quantifies a different  
309 type of molecule on different feature scales, Pixie was able to identify histologically relevant  
310 features in an automated fashion, as well as identify glycans that were commonly co-occurring.  
311 Through these four case studies, we have shown that pixel clustering can be useful across  
312 biological contexts and imaging platforms to capture informative pixel-level features independent  
313 of cell segmentation masks.

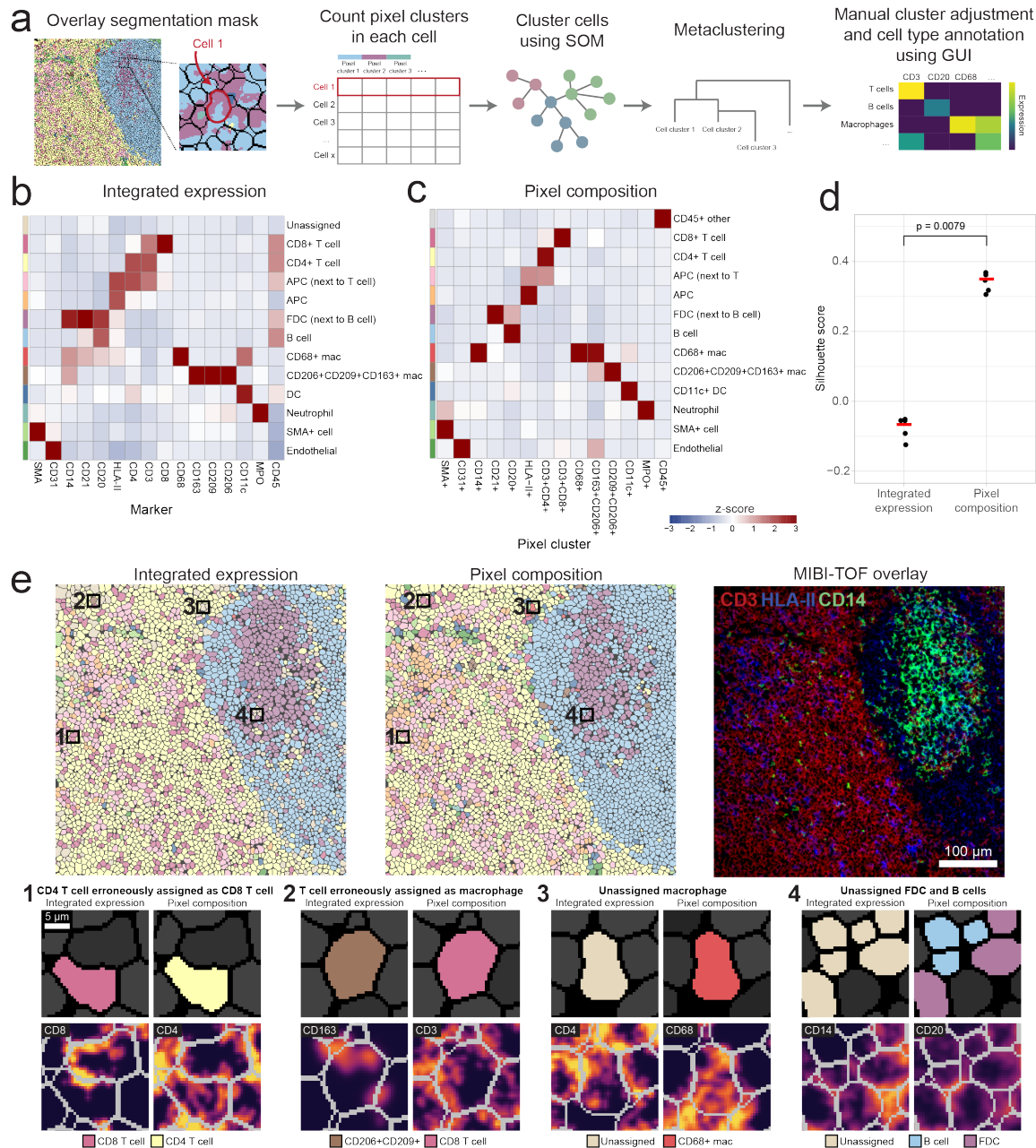
#### 314 **Using pixel clusters to improve cell-level annotations in Pixie**

315 Since cells are the building blocks of tissue, it is important to generate accurate  
316 annotations of cells, in addition to the pixel features described above. Development of methods  
317 for accurate cell annotations is an active area of research.<sup>18,19</sup> The current paradigm for annotating  
318 cells in images is to use unsupervised clustering, where the input features are the sum of the  
319 expression of each marker for each cell, in a manner similar to analysis of flow or mass cytometry  
320 data.<sup>1,10–12,15,17,41</sup> We rely on cell segmentation to generate accurate cell masks, then integrate the  
321 expression of each marker within each cell mask to generate the expression profile for each cell.  
322 However, as discussed above, imaging data is not measuring dissociated single cells. Bright  
323 signal present along the perimeter of a cell can be inaccurately assigned to its neighboring cells,  
324 particularly in dense tissue where the cells are packed close together (Supplementary Figure  
325 1c,d). Therefore, cell clusters using integrated expression values can have poor cluster definition  
326 due to noisy signal. In this paradigm, clusters with poor definition are usually manually inspected  
327 and compared against the images, which is a time-consuming process. Often, manual gating  
328 steps are needed to identify cells that cannot be clustered using this method. Therefore, cell

329 phenotyping using the integrated expression of each cell requires a significant amount of manual  
330 work to visually inspect the images and adjust the clustering.

331 In Pixie, we use the pixel clusters resulting from the workflow described above to improve  
332 cell classification. After generating single cell masks using cell segmentation, instead of  
333 integrating the expression of each marker, we tabulate the number of pixels belong to each pixel  
334 cluster in each cell. The number of pixel clusters in each cell is then used as the feature vector  
335 into a SOM, followed by consensus hierarchical clustering and manual cluster adjustment and  
336 annotation, as described above for pixel clustering (Fig. 5a). We hypothesized that this method  
337 would improve cell annotation because it quantifies discretized pixel phenotypes. When simply  
338 integrating expression over the cell, misassigned pixels could have a large impact on clustering,  
339 if their expression is very bright. Even though there may be bright pixels from a neighboring cell  
340 or from features such as dendrites that seem like they are protruding into the cell in a 2D image,  
341 the number of those pixels should be low. Because there should be few of these pixels that are  
342 misassigned to the cell of interest, the real signal of that cell should drive the clustering in this  
343 method. Furthermore, this method also quantifies the degree of protein co-expression at a pixel-  
344 level, which is information that is lost when integrating expression for each cell.

345 To test cell clustering using Pixie, we used the lymph node dataset described above. We  
346 compared the cell expression profiles from using integrated expression and pixel composition for  
347 cell clustering (Fig. 5b,c). When using integrated expression, while the major immune phenotypes  
348 could be identified, there was one cluster that was unassigned, which was also the largest cluster  
349 (Supplementary Fig. 13b). Under this paradigm, this unassigned cluster would usually require  
350 manual comparisons with the images to determine the true phenotypes of these cells. In  
351 comparison, there was no unassigned cluster when using pixel composition to perform cell  
352 clustering. Therefore, by using pixel clusters to perform cell clustering in Pixie, we obtained  
353 accurate cell clusters with better cluster definition and less amount of manual work, saving the  
354 researcher considerable time. Importantly, using pixel composition for cell clustering also resulted



**Figure 5: Pixel clusters can be used to improve cell annotations in Pixie.** (A) Overview of cell-level phenotyping using pixel clusters. After pixel clusters have been identified and annotated, the frequency of each pixel cluster within each cell boundary (identified using cell segmentation) is used as the feature vector for cell clustering. Cells are clustered using a SOM and clusters are metaclustered using consensus hierarchical clustering. The metaclusters are then manually adjusted and annotated by the user. (B) Using the same dataset as shown in Fig. 2, heatmap of mean marker expression of cell phenotypes obtained from cell clustering using integrated marker expression for each cell. Clusters were manually adjusted and annotated. (C) Using the same dataset as displayed in Fig. 2 and in B, heatmap of mean pixel cluster frequency of cell phenotypes obtained from cell clustering using pixel cluster composition for each cell in Pixie, as outlined in A. Clusters were manually adjusted and annotated. Expression values were z-scored for each marker. (D) Comparison of Silhouette score for cell clusters obtained using integrated expression or pixel composition. Five replicates were performed for each method. Red bar indicates the average Silhouette score. P-value was determined using a Wilcoxon test. (E) Comparison of cell phenotype maps colored according to cell phenotypes obtained using either integrated expression (left) or using pixel composition (middle), and a MIBI-TOF overlay (right) for one representative FOV. Colors in the cell phenotype maps correspond to the heatmaps in B and C, respectively. Four representative examples of the advantage of using pixel composition over integrated expression for cell clustering (bottom).

355 in significantly higher Silhouette scores, which measures how well cells are clustered with other  
356 cells that are similar to each other, a commonly used metric for evaluating clustering performance  
357 (Fig. 5d). Furthermore, the cluster consistency score was lower when using pixel composition to  
358 perform cell clustering (Supplementary Fig. 13e,f).

359       Upon closer inspection of the images, we can identify various examples where using pixel  
360 composition to cluster cells in Pixie was advantageous to using integrated expression (Fig. 5e).  
361 In the first example, a cell was erroneously assigned as a CD8 T cell. The neighboring cell had  
362 clear CD8 expression, and the CD8 signal from this neighboring cell was confounding cell  
363 annotation. By using pixel composition, the cell was correctly assigned as a CD4 T cell. In the  
364 second example, a cell that had clear CD3 signal and was likely a T cell was being misassigned  
365 as a CD163+ macrophage, due to sparse CD163 signal that could be due to a neighboring  
366 macrophage being sectioned in 2D. Using pixel composition, this cell was assigned as a T cell.  
367 In the third example, a cell that was unassigned when using integrated expression, possibly due  
368 to noisy expression from neighboring cells, was correctly assigned as a CD68+ macrophage using  
369 pixel composition. Finally, in the fourth example, cells that were unassigned when using integrated  
370 expression were assigned as B cells and FDCs using pixel composition. Because B cells and  
371 FDCs are closely packed and often interacting in a lymph node follicle, it can be difficult to  
372 correctly assign phenotypes to these cells, emphasizing the ability of Pixie to perform well on  
373 traditionally challenging phenotypes.

374       In addition, we used Pixie to perform cell annotation of the TMA dataset described in  
375 Supplementary Fig. 12, where we assessed the concordance between serial sections of a TMA  
376 that was randomized with respect to staining and imaging day. Using the pixel clusters shown in  
377 Supplementary Fig. 12b, we classified cells into 10 cell phenotypes, then assessed concordance  
378 between serial sections by calculating the average Spearman correlation between serial sections  
379 of the same tissue core (Supplementary Fig. 14). Overall, the Spearman correlation was high,  
380 with  $R^2$  of  $0.93 \pm 0.05$  (mean  $\pm$  SD). Similar to pixel-level features, Pixie was able to capture

381 reproducible cell phenotypes despite differences in absolute pixel intensity between imaging runs  
382 (Supplementary Fig. 14d).

383 Here, we have shown the utility of pixels clusters for improving cell phenotyping. By using  
384 pixel cluster composition to perform cell clustering in Pixie, we obtain accurate cell clusters that  
385 require fewer manual adjustments than when using integrated expression.

386

## 387 **Discussion**

388 Here, we present Pixie, a complete pipeline for identifying pixel-level and cell-level  
389 features from multiplexed imaging datasets and demonstrate its utility across a variety of tissue  
390 types and imaging platforms. Using an example dataset of lymph nodes imaged using MIBI-TOF,  
391 we demonstrate the robustness and accuracy of our method. We also show applications of Pixie  
392 in DCIS and brain MIBI-TOF datasets, as well as CODEX and MALDI-IMS datasets. Finally, we  
393 show how pixel clusters can be used to improve cell classification. Importantly, Pixie is available  
394 as user-friendly Jupyter notebooks that perform all steps of the pipeline and includes a GUI for  
395 manual adjustment and annotation of clusters. Our notebooks are open source can be easily  
396 customized to each user's requirements.

397 Using pixel clusters to annotate features in images has been previously performed in  
398 various contexts in multiplexed imaging as well as spatial transcriptomics. In Gut et al., the authors  
399 also use a SOM to cluster pixels in iterative indirect immunofluorescence imaging (4i) images and  
400 relate these pixel features to cells, terming them Multiplexed Cell Units (MCUs).<sup>6,42</sup> In spatial  
401 transcriptomics, pixel-level features have been used to perform cell annotation and infer tissue  
402 substructures.<sup>43-46</sup> These methods demonstrate the utility of pixel-level analysis. Here, we build-  
403 upon this previous work by providing a comprehensive evaluation of the pre-processing steps and  
404 parameter choices that optimize clustering performance, show use cases across imaging  
405 platforms and biological questions, and present a user-friendly pipeline for running this method.

406 First, we illustrate the beneficial effect of each of the pre-processing steps in the Pixie  
407 pipeline – Gaussian blurring, pixel normalization, and 99.9% marker normalization. While we have  
408 determined an optimal parameter space for each of these pre-processing steps using multiple  
409 datasets, these parameters may need to be tuned for each individual dataset. For example, we  
410 determined a lower Gaussian blur was appropriate when analyzing the smaller myoepithelial area  
411 in DCIS and that no Gaussian blur was needed when analyzing MALDI-IMS data. There are many  
412 other parameters of the SOM that could be tuned, such as the learning rate, initialization of the  
413 cluster centers, and distance function. We found that the default parameters of the SOM (see  
414 Methods) worked well for our use cases; however, these could all be easily changed in our  
415 pipeline. We encourage users to visualize the resulting pixel clusters alongside marker expression  
416 images to assess the best parameter choices for their own datasets.

417 While we used a SOM as the clustering algorithm in our pipeline, there are many other  
418 unsupervised clustering algorithms that have been developed for a similar purpose.<sup>47–50</sup> We chose  
419 to use a SOM because it is accurate, fast, and scalable, which is particularly important for this  
420 method because we are clustering a large number of pixels in which the number of observations  
421 can approach 1 billion. In contrast, the number of cells from single-cell RNA-sequencing or CyTOF  
422 experiments are usually on the order of thousands or millions. One popular clustering algorithm  
423 is the Leiden algorithm, which is built upon the Louvain algorithm, both often used in  
424 transcriptomic analysis and implemented in the popular Seurat package.<sup>51,52</sup> PhenoGraph,  
425 another popular clustering algorithm, is a graph-based method that identifies communities using  
426 Louvain. We performed a time comparison of a SOM (implemented in FlowSOM), Leiden  
427 (implemented in Seurat), and PhenoGraph (implemented in Rphenograph) and observed that a  
428 SOM has the fastest runtime (Supplementary Fig. 15). If the user wishes to use another clustering  
429 algorithm, the modular nature of our code allows the clustering algorithm to be easily replaced.

430 Additionally, we have shown that Pixie can quantify biologically meaningful features that  
431 are not captured by traditional cell segmentation across disease contexts and imaging platforms.

432 In one example, we used Pixie to define a clinically meaningful feature in DCIS that could stratify  
433 patient groups. One of the defining features of DCIS is that the myoepithelium becomes stretched  
434 out as the tumor cells proliferate and expand. We found that normal breast myoepithelium exists  
435 in a luminal, E-cadherin (ECAD)-positive phenotypic state, which transitions to a more  
436 mesenchymal, vimentin-positive state in DCIS, which aligns with an analogous shift in tumor cell  
437 differentiation. In previous work from our group, we built a random forest classifier using 433  
438 parameters for predicting which DCIS patients would progress to IBC, including the pixel-level  
439 features.<sup>11</sup> Importantly, a high abundance of the ECAD+ myoepithelium pixel cluster was the  
440 number one predictor of IBC recurrence in this study, highlighting the utility of this pixel clustering  
441 method.

442 Lastly, we demonstrate the utility of using pixel clusters to annotate cell-level features and  
443 show its improvement over using integrated marker expression for cell annotation. While we offer  
444 one improvement to the traditional cell clustering methodology, there are many other algorithms  
445 that have been developed to address similar problems. REDSEA (Reinforcement Dynamic  
446 Spillover Elimination) improves cell assignments by correcting for spillover signal at cell  
447 boundaries.<sup>20</sup> While the pixel clustering method described here similarly accounts for spillover  
448 signal from neighboring cells, Pixie also accounts for pixels that may not be at the cell boundary  
449 that are confounding accurate classification, such as noisy pixels or pixels from objects actually  
450 not associated with the cell of interest, such as dendrites from nearby cells or the extracellular  
451 matrix. Furthermore, while Pixie relies on unsupervised clustering, another class of algorithms  
452 that can perform cell type assignment relies on feeding the algorithm prior knowledge.<sup>18,19,44,53</sup>  
453 Astir is a probabilistic model that uses prior knowledge of marker proteins to assign cells to cell  
454 types in multiplexed imaging datasets.<sup>18</sup> A recently published algorithm, CELESTA, identifies cell  
455 types in multiplexed images by utilizing spatial information.<sup>19</sup> One of the inputs of CELESTA is a  
456 user-defined cell type signature matrix. Methods that rely on a priori knowledge limit the potential  
457 for discovery of cell states and relies of an accurate reference list of marker expression. Although

458 we currently do not make use of the spatial location of pixels or cells for performing phenotype  
459 annotation, this represents an exciting new avenue for future work.

460 As the amount of multiplexed imaging datasets continues to grow, automated, fast, and  
461 scalable approaches for analyzing these data are needed. Pixie is a simple, fast method that can  
462 generate quantitative annotations of features both independently and in conjunction with cell  
463 segmentation that will enable the comprehensive profiling of various tissues across health and  
464 disease.

465

## 466 **Methods**

### 467 **Pixel clustering methodology**

468 The pixel clustering method in Pixie is illustrated in Supplementary Fig. 2a and described  
469 above. Single pixel expression profiles were extracted from single-channel TIFs, Gaussian  
470 blurred with a standard deviation of 2 (unless otherwise noted), pixel normalized by dividing by  
471 the total pixel expression, and 99.9% marker normalized by dividing by each marker's 99.9<sup>th</sup>  
472 percentile. The 99.9% normalization step is necessary because markers that are systematically  
473 brighter would otherwise likely drive the clustering. By normalizing all markers to their 99.9<sup>th</sup>  
474 percentile, markers have a more even contribution to the clustering results. Next, we used the  
475 FlowSOM<sup>30</sup> implementation of a SOM to cluster all pixels into 100 clusters. Unless otherwise  
476 noted in the manuscript, we used the following parameters of the SOM for all clustering: grid  
477 size = 10 x 10, start learning rate = 0.05, end learning rate = 0.01, initialization function =  
478 random, distance function = Euclidean, training passes = 10. Next, the mean expression profile  
479 of each of the 100 clusters was determined, z-scored for each marker, then z-scores were  
480 capped to a maximum value of 3. The clusters were then metaclustered using the  
481 ConsensusClusterPlus<sup>54</sup> implementation of consensus hierarchical clustering using the z-scored  
482 expression values. Metaclusters were manually adjusted and annotated using a custom-built  
483 GUI (Supplementary Video 1). These final phenotypes were mapped back to the original images



484 to generate pixel phenotype maps. To generate expression heatmaps, we calculated the mean  
485 expression for each cluster and found the z-score for each marker.

486 All processing was performed on a Google Cloud Compute Engine instance. The machine  
487 type, number of cores, and available memory were adjusted based on the size of the dataset.

#### 488 **Cell clustering methodology**

489 Cell segmentation for all datasets was performed using the pre-trained Mesmer  
490 segmentation model.<sup>26</sup> For each cell in the image that was identified using Mesmer, we counted  
491 the number of each pixel cluster in each cell. We normalized these values by the total cell size  
492 and applied a 99.9% feature normalization (features here were the pixel clusters). Cells were then  
493 clustered using a SOM, and metaclustered using consensus hierarchical clustering, analogously  
494 to pixel clustering as described above.

#### 495 **Cluster consistency score**

496 To assess the stochasticity of Pixie, we created the “cluster consistency score” metric.  
497 Across different replicate runs of the same input data, the same phenotype may be output as a  
498 different cluster number, so assessing reproducibility by comparing the number of pixels  
499 belonging to the same phenotype is not easily automated and instead requires significant  
500 amounts of manual annotation. For example, the pixel cluster that is defined by CD20 may be  
501 pixel cluster 1 in the first run and pixel cluster 2 in the second run. Manual annotation of each  
502 cluster is infeasible for large numbers of tests when assessing pre-processing steps and different  
503 parameter choices. To measure reproducibility quickly and quantitatively, we created the cluster  
504 consistency score. Cluster consistency score is calculated as follows:

- 505 1. For one set of parameters, we run the entire pipeline using the same input data five times,  
506 each time with a different random seed. We call these replicates 1-5.
- 507 2. For each replicate, for each cluster, we quantify the minimum number of clusters in another  
508 run that it takes to get to 95% of the pixels in that cluster. For example, if there are 1000  
509 pixels belonging to cluster 1 of replicate 1, for these pixels, we count the number of pixels

510 in each cluster of replicate 2. We then rank this count table and determine the minimum  
511 number of clusters in replicate 2 it takes to get to 950 pixels in cluster 1 of replicate 1.

512 3. For a single replicate, we calculate this number in a pairwise manner with all other  
513 replicates. For example, for replicate 1, we calculate this number for replicate 1-replicate  
514 2, replicate 1-replicate 3, replicate 1-replicate 4, replicate 1-replicate 5. These numbers  
515 are averaged.

516 4. Steps 2 and 3 are repeated for each cluster in each replicate. The result is that each pixel  
517 cluster in each replicate has a score associated with it.

518 5. These scores are mapped back to the pixel assignments. For example, if a pixel was  
519 assigned as pixel cluster 1 in replicate 1, the corresponding score determined in the  
520 previous step is assigned to that pixel. Each pixel is assigned 5 features, corresponding  
521 to the score from each replicate. These 5 features are averaged for each pixel, resulting  
522 in one score for each pixel.

523 See code for full implementation. A low score indicates good reproducibility while a high  
524 score indicates bad reproducibility, meaning that the pixel was assigned to clusters that may have  
525 contained many other pixel types. At the cell level, the same paradigm was used, but instead of  
526 pixel clusters, we assessed cell clusters.

527 We selected 95% when calculating the cluster consistency score because we determined  
528 that it was a good benchmark value. As expected, lowering this threshold resulted in lower cluster  
529 consistency scores, and raising this threshold resulted in higher cluster consistency scores  
530 (Supplementary Fig. 4b).

### 531 **Benchmarking cluster consistency score with reference cell datasets**

532 To assess the cluster consistency score, we used two benchmark datasets, a CyTOF  
533 dataset<sup>31</sup> and single-cell RNA-sequencing dataset downloaded from the Seurat tutorial website.<sup>32</sup>  
534 The CyTOF dataset contained 1,140,035 cells from whole blood and was downloaded from:  
535 <https://doi.org/10.5281/zenodo.3951613>. We randomly subsampled 5000 cells from the dataset

536 and clustered the cells into 100 clusters using FlowSOM and metaclustered into 15 metaclusters  
537 using consensus hierarchical clustering. The Seurat dataset contained 2,700 peripheral blood  
538 mononuclear cells (PBMCs) and was downloaded from:  
539 [https://cf.10xgenomics.com/samples/cell/pbmc3k/pbmc3k\\_filtered\\_gene\\_bc\\_matrices.tar.gz](https://cf.10xgenomics.com/samples/cell/pbmc3k/pbmc3k_filtered_gene_bc_matrices.tar.gz).  
540 The data was processed as outlined here:  
541 [https://satijalab.org/seurat/articles/pbmc3k\\_tutorial.html](https://satijalab.org/seurat/articles/pbmc3k_tutorial.html). The data was log normalized and the  
542 first 10 PCs from PCA were used as the input features. We constructed a KNN graph based on  
543 the Euclidean distance in PCA space and used the Leiden algorithm to cluster cells. For both  
544 CyTOF and RNA-seq datasets, the cluster consistency score was calculated as outlined above.

#### 545 **Lymph node MIBI-TOF dataset**

546 Six lymph nodes were imaged using a MIBI-TOF instrument with a Hyperion ion source  
547 using 37 markers (Supplementary Table 1). FOVs were imaged at a field size of 500  $\mu\text{m}$  x 500  
548  $\mu\text{m}$  at 1,024 x 1,024 pixels.

#### 549 **Replicate serial section TMA dataset**

550 The dataset assessing the reproducibility of TMA serial sections was previously published  
551 in Liu and Bosse et al.<sup>35</sup> The processed imaging data is available at  
552 <https://doi.org/10.5281/zenodo.5945388>. Imaging parameters and pre-processing methodology  
553 (background subtraction, denoising) are described in the manuscript. Pixel clustering and cell  
554 clustering were performed as described above. Markers included in the clustering are indicated  
555 in Supplementary Fig. 12b.

#### 556 **Decidua MIBI-TOF dataset**

557 The decidua MIBI-TOF dataset was previously described in Greenbaum, Averbukh, Soon  
558 et al.<sup>34</sup> Imaging parameters and pre-processing methodology (background subtraction, denoising)  
559 are described in the manuscript. To compare the full dataset against a subset dataset, we  
560 randomly subsampled 10% of the total number of pixels for each replicate run. Subsequent steps

561 (Gaussian blur, pixel normalization, 99.9% marker normalization) were performed as described  
562 above. Markers included in the clustering are indicated in Supplementary Fig. 10a,b.

### 563 **DCIS MIBI-TOF dataset**

564 The DCIS MIBI-TOF dataset was previously published in Risom et al.<sup>11</sup> The processed  
565 imaging data is available at <https://data.mendeley.com/datasets/d87vg86zd8/3>. Imaging  
566 parameters and pre-processing methodology (background subtraction, denoising) are described  
567 in the manuscript. Processing steps (Gaussian blur, pixel normalization, 99.9% normalization)  
568 were performed as described above. Markers included in the clustering are indicated in  
569 Supplementary Fig. 11a.

570 For the myoepithelial analysis in Fig. 4a, masks of just the myoepithelial zone were  
571 generated as described in the manuscript. Images were first subset for pixels within the  
572 myoepithelial masks, then pixels within the myoepithelium mask were further subset for pixels  
573 with SMA expression  $> 0$ . Upon inspecting clustering results for a few different standard deviations  
574 (sigma) for the Gaussian blur, we determined that a Gaussian blur of 1.5 was more appropriate  
575 for this use case, since we were interested in discrete pixel features in a small histological region  
576 of the full image. Subsequent steps (pixel normalization, 99.9% marker normalization) were  
577 performed as described above.

### 578 **TNBC MIBI-TOF dataset**

579 This dataset will be published in a forthcoming publication, currently under preparation.  
580 TNBC samples were imaged using a MIBI-TOF instrument. Each FOV was 800  $\mu\text{m}$  x 800  $\mu\text{m}$  at  
581 2,048 x 2,048 pixels. Processing steps (Gaussian blur, pixel normalization, 99.9% normalization)  
582 were performed as described above. Markers included in the clustering are indicated in  
583 Supplementary Fig. 11b.

### 584 **Cerebellum MIBI-TOF dataset**

585 This dataset will be published in a forthcoming publication, currently under preparation.  
586 Human cerebellum samples were imaged using a MIBI-TOF instrument. Each FOV was 700  $\mu\text{m}$

587 x 700  $\mu\text{m}$  at 1,024 x 1,024 pixels, and FOVs were tiled to generate the final cerebellum image.  
588 Processing steps (Gaussian blur, pixel normalization, 99.9% normalization) were performed as  
589 described above. Markers included in the clustering are indicated in Fig. 4b.

#### 590 **Colorectal cancer CODEX dataset**

591 The CODEX dataset was previously published in Schürch et al.<sup>17</sup> The processed imaging  
592 data was obtained from The Cancer Imaging Archive at [https://doi.org/10.7937/tcia.2020.fqn0-](https://doi.org/10.7937/tcia.2020.fqn0-0326)  
593 0326. We selected 20 representative FOVs from CRC\_TMA\_A: reg012\_X01\_Y01\_Z09,  
594 reg039\_X01\_Y01\_Z08, reg059\_X01\_Y01\_Z11, reg046\_X01\_Y01\_Z09, reg015\_X01\_Y01\_Z08,  
595 reg052\_X01\_Y01\_Z09, reg047\_X01\_Y01\_Z08, reg027\_X01\_Y01\_Z09, reg035\_X01\_Y01\_Z09,  
596 reg018\_X01\_Y01\_Z09, reg042\_X01\_Y01\_Z08, reg041\_X01\_Y01\_Z09, reg069\_X01\_Y01\_Z09,  
597 reg063\_X01\_Y01\_Z08, reg068\_X01\_Y01\_Z09, reg024\_X01\_Y01\_Z09, reg019\_X01\_Y01\_Z09,  
598 reg064\_X01\_Y01\_Z10, reg061\_X01\_Y01\_Z10, reg045\_X01\_Y01\_Z10. Processing steps  
599 (Gaussian blur, pixel normalization, 99.9% normalization) were performed as described above. In  
600 addition to the markers indicated in Fig. 4c, empty cycle TIFs were also included in the clustering.

#### 601 **Pancreatic ductal adenocarcinoma MALDI-IMS dataset**

602 The MALDI-IMS dataset was previously published in McDowell et al.<sup>37</sup> Raw MALDI-IMS  
603 data (corresponding to Figure 3 in the original publication) was provided upon request by Dr.  
604 Richard Drake. Data was provided as mis, bak and tsf files, which were imported into SCiLs Lab  
605 2022a imaging software. In SCiLs Lab, N-glycan spectra were normalized by total ion count and  
606 converted to vendor-neutral imzML format.<sup>55</sup> The imzML and ibd files were parsed using pyimzML  
607 in Python, and the expression at each m/z peak was extracted as single-channel TIF images  
608 corresponding to each extracted m/z peak. These m/z peaks were then mapped to glycans by  
609 accurate mass as annotated in the original paper. These single-channel TIFs were then  
610 processed and clustered as described above for single-marker MIBI-TOF images. Upon  
611 inspecting clustering results for a few different standard deviations (sigma) for the Gaussian blur,  
612 we determined that no Gaussian blur was necessary for pixel clustering of MALDI-IMS data. This

613 is expected because MALDI-IMS data is lower resolution than MIBI-TOF data. Subsequent steps  
614 were performed as described above.

## 615 **Visualization**

616 Plots were created using the ggplot2 and pheatmap R packages and the matplotlib Python  
617 package. Schematic representations were created with biorender (<https://biorender.io/>). Figures  
618 were prepared in Adobe Photoshop and Adobe Illustrator.

619

## 620 **Data availability**

621 User-friendly Jupyter notebooks for running Pixie are available at:  
622 <https://github.com/angelolab/ark-analysis>. The code used in this study is available at  
623 [https://github.com/angelolab/publications/tree/master/2022-Liu\\_etal\\_Pixie](https://github.com/angelolab/publications/tree/master/2022-Liu_etal_Pixie).

624

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639

640 **Ethics declaration**

641 M.A. is an inventor on patents related to MIBI technology. M.A. is a consultant, board member,  
642 and shareholder in Ionpath Inc.

643

644 **Author Contributions**

645 C.C.L., N.F.G., and M.A. developed the methodology. C.C.L. and A.K. developed the software.

646 E.F.M. and D.M. stained and acquired the lymph node and brain MIBI-TOF data, respectively.

647 K.X.L. and D.M. assisted in analyzing the MALDI-IMS and MIBI-TOF brain images, respectively.

648 C.C.L. wrote the manuscript. All authors reviewed the manuscript and provided feedback. M.A.

649 supervised the work.

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