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1 **Main Manuscript**

2 **IBEX – A versatile multi-plex optical imaging approach for deep phenotyping and spatial**
3 **analysis of cells in complex tissues**

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32 **Author Contributions**

33 A.J.R and R.N.G. wrote the manuscript. A.J.R, E.K., B.L, C.J.C., E.S., A.G., N.T., R.S., Z.R.Y,
34 R.T.B., and J.K designed, performed, and analyzed experiments. L.Y. and A.G. assisted with
35 display items. J.C., J.D., and J.M.H. provided technical insight, reagents, and tissues. All authors
36 helped write the manuscript.

37

38 **Abstract**

39

40 The diverse composition of mammalian tissues poses challenges for understanding the
41 cell-cell interactions required for organ homeostasis and how spatial relationships are perturbed
42 during disease. Existing methods such as single-cell genomics, lacking a spatial context, and
43 traditional immunofluorescence, capturing only 2-6 molecular features, cannot resolve these
44 issues. Imaging technologies have been developed to address these problems, but each
45 possesses limitations that constrain widespread use. Here we report a new method that overcomes
46 major impediments to highly multi-plex tissue imaging. Iterative Bleaching Extends multi-pleXity
47 (IBEX) uses an iterative staining and chemical bleaching method to enable high resolution imaging
48 of >65 parameters in the same tissue section without physical degradation. IBEX can be employed
49 with various types of conventional microscopes and permits use of both commercially available and
50 user-generated antibodies in an 'open' system to allow easy adjustment of staining panels based
51 on ongoing marker discovery efforts. We show how IBEX can also be used with amplified staining
52 methods for imaging strongly fixed tissues with limited epitope retention and with oligonucleotide-
53 based staining, allowing potential cross-referencing between flow cytometry, Cellular Indexing of
54 Transcriptomes and Epitopes by Sequencing (CITE-Seq), and IBEX analysis of the same tissue.
55 To facilitate data processing, we provide an open source platform for automated registration of
56 iterative images. IBEX thus represents a technology that can be rapidly integrated into most current
57 laboratory workflows to achieve high content imaging to reveal the complex cellular landscape of
58 diverse organs and tissues.

59 **Significance Statement**

60 Single cell flow cytometry and genomic methods are rapidly increasing our knowledge of
61 the diversity of cell types in metazoan tissues. However, suitably robust methods for placing these
62 cells in a spatial context that reveal how their localization and putative interactions contribute to
63 tissue physiology and pathology are still lacking. Here we provide a readily accessible pipeline
64 (IBEX) for highly multi-plex immunofluorescent imaging that enables a fine-grained analysis of cells
65 in their tissue context. Additionally, we describe extensions of the IBEX workflow to handle hard to
66 image tissue preparations and a method to facilitate direct integration of the imaging data with flow
67 cytometry and sequencing technologies.

68

69 **Introduction**

70 Mammalian tissues are composed of a wide variety of cell types, presenting a major
71 challenge to understanding the cell-cell interactions required for homeostasis as well as the
72 compositional changes associated with disease. To address this complexity, several multi-plexed

73 imaging methods utilizing conventional microscopes and commercially available antibodies have
74 been described to overcome the target detection limitations of conventional immunohistochemistry
75 (IHC) or immunofluorescence (IF) imaging (1-8). The majority of these methods generate high
76 dimensional datasets through an iterative, multi-step process (a cycle) that includes: 1)
77 immunolabeling with antibodies, 2) image acquisition, and 3) fluorophore inactivation or
78 antibody/chromogen removal. While these methods are capable of generating high dimensional
79 datasets, they are greatly limited by the number of markers visualized per cycle, length of time
80 required for each cycle, or involve special fluid-handling platforms not generally available to most
81 laboratories (1). Commercial systems based on the co-detection by indexing (CODEX) method (9)
82 have facilitated the acquisition of multi-plex imaging data by providing a fully automated instrument
83 for cyclic imaging. Despite this advancement, the proprietary nature of this method imposes
84 constraints on the reagents available for use as well as the number of markers to be imaged for
85 each round. Furthermore, cyclic imaging methods that employ a small number of markers per cycle
86 (<3) may result in tissue loss due to the stress of repeated fluid exchanges. To this end, novel
87 imaging techniques such as multi-plexed ion beam imaging (MIBI) (10) and imaging mass
88 cytometry (IMC) (11) enable the capture of multi-parameter data without cyclic imaging. However,
89 both of these methods require specialized instrumentation and consumables, with the latter often
90 again limited in breadth to choices made by the supplier, not the investigator. This constrains their
91 capacity for broadly analyzing human or experimental animal tissues with respect to lists of
92 validated antibodies, the ability to work across various established protocols for tissue processing,
93 and the capacity for real time changes to the epitope target list based on data emerging from high
94 content methods such as single cell RNA sequencing (scRNA-Seq).

95 To facilitate the increasing need for high content analysis of tissues for projects such as
96 the Human Cell Atlas and others, the field needs a fully open and extensible method for multi-plex
97 imaging. Our laboratory has extensively characterized murine and human immune responses using
98 quantitative multi-parameter imaging of fixed frozen samples (12-18). Importantly, this method of
99 tissue fixation preserves tissue architecture and cellular morphology, is archivable, compatible with
100 large volume imaging (19), and in its optimal form, eliminates technical challenges posed by
101 formalin-fixed paraffin embedded (FFPE) samples. Leveraging this experience and our original
102 single cycle histo-cytometry method for multi-plex data acquisition (12), we have now developed
103 Iterative Bleaching Extends multi-pleXity (IBEX). This imaging technique reduces the time per
104 cycle, uses a high number of antibodies per cycle, employs widely available reagents and
105 instruments, provides open source software for image alignment, and minimizes physical damage
106 to the tissue during multiple imaging cycles. Beyond the basic IBEX workflow, we have developed
107 extensions to achieve multi-parameter imaging of heavily fixed tissues with limited retention of
108 target epitopes and have incorporated commercially available oligonucleotide-conjugated
109 antibodies to enable direct cross-comparisons to flow cytometry and scRNA-Seq data obtained by

110 the Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq) method (20). In
111 addition to describing the specifics of the IBEX method, we provide multiple examples of the use
112 of IBEX to analyze both immune and parenchymal cells in a diverse array of mouse and human
113 tissues to illustrate the general applicability of the method. The IBEX method described here can
114 be rapidly integrated into current laboratory workflows to obtain high dimensional imaging datasets
115 of a wide range of animal and human tissues.

116

117 **Results**

118

119 **IBEX builds and improves upon existing iterative imaging techniques**

120 Iterative imaging methods typically use either fluorophore bleaching or
121 antibody/chromogen removal to achieve multi-parameter datasets (1-8). Due to the harsh and
122 variable conditions required to remove chromogens and antibodies with diverse target affinities, we
123 pursued a strategy based on fluorophore bleaching. To achieve an efficient means to increase the
124 number of markers visualized on a single section, we sought a fluorophore inactivation method that
125 could bleach a wide range of fluorophores in minutes without epitope loss or tissue destruction.
126 While H₂O₂ in alkaline solution has been reported to inactivate Cy3- and Cy5-conjugated antibodies
127 in human FFPE samples (3), we observed significant tissue loss using this formulation over multiple
128 cycles with fixed frozen samples (Fig. S1A). Adams *et al.* demonstrated the initial feasibility of
129 borohydride derivatives to bleach fluorophores; however, their fluorophore quenching method
130 required 2 hours per cycle and comprised only 3 distinct imaging channels (6), making direct
131 application for highly multi-plex imaging impractical. To expand upon this method, we tested
132 antibodies directly conjugated to fluorophores with excitation and emission spectra spanning from
133 405 nm to 750 nm. We consistently found that the following fluorophores were inactivated within
134 15 minutes of exposure to 1 mg/ml of Lithium Borohydride (LiBH₄): Pacific Blue, Alexa Fluor
135 (AF)488, FITC, AF532, Phycoerythrin (PE), AF555, eFluor(eF)570, AF647, eF660, and AF700.
136 Brilliant Violet conjugates BV421 and BV510 bleached within 15 minutes of exposure to 1 mg/ml of
137 LiBH₄ in the presence of light. In contrast, AF594, eF615, and the nuclear markers JOJO-1 and
138 Hoechst required more than 120 minutes for significant loss of fluorescence signal (Table S1),
139 permitting these probes to be used as fiducials for alignment of images emerging from iterative
140 cycles.

141 To prevent tissue destruction over multiple cycles, we evaluated several different tissue
142 adhesives and found that chrome gelatin alum securely adhered tissues to glass coverslips and
143 slides, permitting more than 15 cycles to be performed with no appreciable loss to the tissue (Fig.
144 S1B-C). We next reduced the antibody labeling time from 6-12 hours to 30-45 minutes by designing
145 programs for a non-heating microwave that facilitates rapid antibody penetration into the section.
146 Finally, although IBEX was designed to simply bleach the fluorophores, it was important to assess

147 whether LiBH₄ treatment physically removes antibodies from the tissue as this would have direct
148 bearing on both the design and order of imaging panels. To examine this issue, mouse lymph node
149 (LN) sections were immunolabeled with various primary antibodies, imaged, treated with LiBH₄,
150 and then incubated with a secondary antibody that would react with the primary antibody if it was
151 still present on the tissue. For most of the antibody isotypes tested, we found almost identical
152 staining patterns with the primary and secondary antibodies, indicating that LiBH₄ acts primarily by
153 fluorophore bleaching without stripping the fluorescently conjugated antibodies themselves (Fig.
154 S2).

155 The resulting method, IBEX, reduces the fluorophore inactivation and antibody labeling
156 steps to less than 1 hour (Fig. 1A). We first tested IBEX in practical use by examining the image
157 quality that could be obtained from a 3 cycle analysis of mouse LNs (Fig. 1B), a tissue with which
158 we had extensive experience using multi-parameter, single cycle staining and image collection.
159 This initial test used 6-8 markers per cycle and showed that all fluorophores, except for AF594 and
160 JOJO-1, bleached rapidly in the presence of LiBH₄ treatment +/- light with no appreciable signal
161 present after 10 minutes (Fig. 1C-D). These findings show that the IBEX pipeline performs as
162 designed and allows for the rapid capture of high quality, multi-plexed imaging data over multiple
163 cycles without tissue loss.

164

165 **A SimpleITK image registration pipeline**

166 The IBEX method yields a series of images that are collected separately. To properly
167 process these data and correctly assign markers to individual cells, it is essential that all the images
168 be aligned at high resolution. While various registration algorithms have been reported (21, 22), we
169 sought a method that could align large datasets, was flexible in terms of the repeated markers
170 (fiducials) utilized, and provided both a qualitative and quantitative metric for registration. For these
171 reasons, we developed a workflow using SimpleITK, a simplified open source interface to the
172 Insight Toolkit (ITK) that is compatible with multiple programming languages (23, 24). The
173 SimpleITK workflow is an image intensity-based form of image alignment that relies on a repeated
174 marker channel (fiducial) for registration. Due to the resistance of AF594, JOJO-1, and Hoechst to
175 bleaching, we utilized markers in these fluorophores as fiducials. For a multi-cycle IBEX
176 experiment, a 'fixed' image z-stack was selected and all other 'moving' images were resampled to
177 this image. A cross correlation matrix was generated on the repeated marker channels to provide
178 a quantitative means for assessing the quality of image registration (Fig. 2A). To test the fidelity of
179 this method, a 3 cycle IBEX experiment was performed on mouse spleen sections labeled with the
180 nuclear marker JOJO-1 and membrane label CD4 AF594. For these experiments, JOJO-1 was
181 selected as the fiducial used for image registration; however, CD4 (also repeated in these
182 experiments) showed pixel-to-pixel alignment as reflected in the images (Fig. 2B). Importantly, this
183 platform can readily scale to handle large datasets (>260 GB) comprised of 20 cycles of imaging

184 (Fig. S1B-D). The SimpleITK registration workflow thus provides the needed cell-cell registration
185 across x-y-z dimensions obtained via iterative imaging cycles using IBEX.

186

187 **IBEX is a versatile imaging method**

188 One obstacle to the wide adoption of existing multi-plex imaging methods is the need for
189 specialized instruments or custom imaging chambers, a luxury not afforded to all laboratories. In
190 contrast, IBEX is easily adaptable to diverse microscope systems and has no restrictions on the
191 substrate (slide, coverslip, etc.) used for imaging (Fig. S3A). As a proof-of-concept, immunized
192 mouse LN sections adhered to slides were visualized using an upright confocal microscope (4
193 cycles, 6 markers per cycle) or an inverted fluorescence microscope (4 cycles, 4 markers per cycle)
194 (Fig. S3B-C). These results demonstrate the compatibility of IBEX with a wide range of imaging
195 systems; however, it is worth noting that the microscope system (confocal versus widefield) and
196 configuration (light source, detectors, filter cubes) will dictate the image acquisition time, number
197 of markers per cycle, and sample type that can be effectively imaged (5 vs. 30 μm tissue thickness).

198 In the case of animal studies, it is also very useful to be able to integrate antibody staining
199 with imaging of fluorescent marker proteins expressed by engineered cells transferred into animals
200 or expressed *in situ*. We therefore next investigated whether the IBEX method could be used to
201 image tissues from animals expressing fluorescent proteins (25). To this end, high dimensional
202 imaging was performed on thymic tissues from transgenic animals expressing the following
203 fluorescent proteins (FPs): cyan (CFP), green (GFP), yellow (YFP), and red (RFP) (26). No
204 appreciable loss in signal was observed after 10 IBEX cycles for any of the FPs examined (Fig.
205 S4A-B). The photostable FPs were used as fiducials for a 4 cycle IBEX experiment that
206 incorporated the bleachable fluorophores AF647 and AF700, yielding a dataset that provided
207 information on clonality (CFP, GFP, YFP, RFP) of T cells (CD4, CD8, Foxp3) and myeloid cells
208 (CD11c, MHC II) in the thymus (Fig. S4C).

209 To determine how IBEX performs using sections from a variety of tissues, we performed
210 3-5 cycle IBEX experiments on murine spleen, thymus, lung, small intestine, and liver tissue
211 sections (Fig. 3A-B, Movies S1-S5, Table S2). It is important to note that the cycle and marker
212 numbers described here are provided as a proof-of-concept and do not reflect technical limitations
213 of the method. The antibody panels were designed to capture the major cellular populations and
214 structures present in each organ and fluorophores were chosen to avoid native tissue
215 autofluorescence. Organ-specific fiducials were selected based on expression throughout the
216 tissue, e.g., EpCAM to mark the epithelium of the small intestine and laminin for the liver sinusoids.

217 Collectively, these data confirm the ability to use IBEX to obtain high quality, multi-plexed imaging
218 datasets from a wide range of tissues.

219

220 **IBEX enables highly multi-plex, quantitative imaging**

221 The design principles of IBEX were chosen to enable a very high number of parameters to
222 be attained in the analysis of an individual tissue sample. To determine how extensively the multi-
223 plexing capacity of IBEX can be pushed, we first performed 10 cycle 41 parameter IBEX
224 experiments on LNs obtained from naïve and sheep red blood cell (SRBC)-immunized mice (Fig.
225 4A, Table S2, Movie S6). While epitope loss has been described for other iterative imaging
226 techniques (5), we minimized this problem by increasing the number of markers per cycle and
227 grouping markers present on the same cell in the same cycle. We observed qualitatively similar
228 staining patterns when antibody panels were applied on individual sections alone (serial) versus
229 on the same section iteratively (IBEX) (Fig. S5A and Movie S7). Therefore, quantitative differences
230 observed between the two methods likely reflect biological differences resulting from variations in
231 the magnitude of the immune response in individual LNs and not technical differences associated
232 with epitope loss or steric hindrance (Fig. S5B and Movie S7).

233 To assess the quality of data generated by the IBEX method, we employed the open
234 source, computational histology topography cytometry analysis toolbox (histoCAT) to quantify
235 differences in LN organization resulting from immunization (27). Individual cells were segmented
236 based on membrane and nuclear labels with Ilastik (28) and CellProfiler (29) and then analyzed
237 using the histoCAT graphical user interface (GUI) (Fig. S6A). The unsupervised clustering
238 algorithm Phenograph (30) identified 29 phenotype clusters shared across the naïve and
239 immunized LNs that could be visualized using the data dimensionality reduction method t-SNE (31)
240 in histoCAT (Fig. 4B). As a testament to the fidelity of cell-cell alignment, phenotype clusters were
241 often characterized by the expression of several different markers present in distinct imaging cycles
242 (Fig. S6B-C). The abundance of these cell phenotypes varied from naïve and immunized LNs (Fig.
243 S6D) and could be manually annotated based on marker expression to reveal an increase in
244 plasma cells (PCs) (cluster 10) and germinal center (GC) B cells (cluster 6) in immunized LNs (Fig.
245 4C). As histoCAT relies on nuclear and membrane-based cell segmentation, it suffers from
246 limitations frequently encountered with this approach: miscalling of phenotypes due to spatial
247 overlap and improper segmentation of non-lymphocyte populations (32). The former is evident in
248 the identification of the B cell specific transcription factor pax5 (33) on CD3⁺CD4⁺PD-1⁺Bcl6⁺ T
249 follicular helper (Tfh) cells (cluster 19), an artifact due to the close proximity of these cells within

250 the GC (Fig. S6C). Nevertheless, the results presented here demonstrate that IBEX-generated
251 images are compatible with established methods for analyzing high dimensional imaging data.

252

253 **IBEX scales to capture ultra-high content data from large areas of human tissues**

254 In addition to capturing the cellular landscape of a diverse range of murine tissues, the
255 IBEX method scales to enable high content imaging of human tissues. To this end, we present an
256 application of the IBEX method to visualize tumor-immune interactions in a human pancreatic LN
257 with metastatic lesions as shown by CD138+EpCAM+ cells in the LN capsule and sinuses (Fig. 5A,
258 Table S3). Interestingly, cancer cells were segregated from lymphocytes by extensive collagen
259 remodeling and recruitment of myeloid cells expressing the secreted protein acidic and rich in
260 cysteine (SPARC), a matricellular glycoprotein involved in extracellular matrix (ECM) deposition
261 and implicated in metastasis (34). To move to even deeper analysis of a much larger sample, we
262 used a total of 66 antibodies for the analysis of a > 3mm² human mesenteric LN section. Using this
263 approach, we were able to deeply phenotype a wide range of immune cells while observing no
264 tissue degradation over 20 cycles (Fig. 5B, Movie S8, Table S3). Additionally, we observed
265 subcellular resolution for PC-specific markers (membrane: CD138, nuclear: IRF4, cytoplasmic:
266 IgA1, IgA2) present in distinct cycles with no epitope loss, as evidenced by our ability to label the
267 immune marker CD45 with two different antibody clones present in cycles 9 and 19 (Movie S8).
268 The utility of this method is further exemplified by our ability to characterize the complex stroma of
269 the LN, shown to contain 9 distinct clusters in scRNA-Seq experiments from mouse LNs (35), using
270 a wide range of antibodies visualized *in situ*: α -SMA, CD21, CD23, CD34, CD35, CD49a, CXCL12,
271 CXCL13, desmin, fibronectin, and vimentin. These data highlight the capacity of IBEX to identify a
272 large number of distinct cell types in clinically relevant samples, while also placing these
273 components in a spatial setting missed by methods employing dissociated single cells.

274

275 **Extensions of IBEX workflow**

276 Given the inherent versatility of the IBEX method, we sought to extend our workflow to
277 develop two unique protocols, one that enables detection of low abundance epitopes and another
278 that permits iterative imaging with oligonucleotide-conjugated antibodies corresponding to those
279 used in scRNA-Seq experiments. Opal IHC, a method of signal amplification that employs
280 incubation with an unconjugated primary, followed by a horseradish peroxidase (HRP)-conjugated
281 secondary, and deposition of Opal fluorophore in the tissue, is an attractive method for detection
282 of very low levels of specific proteins (36). We first tested this method by staining for endogenous
283 levels of the chemokine CXCL9 in the liver sinusoids of mice (Fig. S7A), which showed a signal not
284 readily detected with direct or indirect staining methods. Further, because Opal IHC is well
285 described for the imaging of fixed (FFPE) human tissues (37, 38), we next evaluated whether this
286 method could be expanded upon to achieve multi-parameter imaging of tissues from high

287 containment facilities. Due to the extreme fixation conditions required to inactivate select agents
288 such as the Ebola virus (10% formalin for 8 days), the majority of stainable epitopes are lost in
289 these tissues (39, 40). To overcome this significant technical limitation, we developed the Opal-
290 plex method that is based on the IBEX pipeline. Opal-plex extends the usual fluorophore limitations
291 of Opal by combining multi-plex Opal IHC with cycles of IBEX-based bleaching to eliminate signal
292 from the following LiBH₄-sensitive dyes (Opal 570, 650, and 690) while utilizing the LiBH₄-resistant
293 dye (Opal 540) as a fiducial (Fig. 6A, Fig. S7B). Using this approach, we achieved single cell
294 resolution of 10 unique markers in heavily fixed mouse LNs (Fig. 6B, Movie S9).

295 We next evaluated whether oligonucleotide-conjugated antibodies, including those used
296 for CITE-Seq, are compatible with our IBEX workflow. While immunolabeling with oligonucleotide-
297 conjugated antibodies is well established (9), the use of a large number of commercially available
298 TotalSeqA™ antibodies with publicly available oligo-tag sequences, the employment of non-
299 proprietary buffers for hybridization and dehybridization, and the use of a wide spectrum of
300 fluorophore-labeled complementary oligonucleotides provides a truly 'open source' system with
301 many advantages. In particular, the imaging method described here applies the same antibodies
302 used for scRNA-Seq, permitting direct comparison between imaging and CITE-Seq datasets while
303 providing a much-needed spatial context for the cell populations identified. Using this approach,
304 we were able to achieve high quality tissue staining with 5 unique fluorophores (Fig. S7C). This
305 method can be directly integrated into our IBEX protocol, alongside fluorophore-conjugated
306 antibodies when CITE-Seq antibodies to desired targets do not exist, as LiBH₄ bleaching leaves
307 oligonucleotide binding intact (Fig. 6C-D). Importantly, the quality of staining achieved with
308 oligonucleotide-conjugated antibodies, even after multiple cycles of LiBH₄ bleaching, is comparable
309 to conventional IF as quantitative differences, e.g., higher expression of MHCII on DCs versus B
310 cells, can still be observed (Fig. 6D, Fig. S7D, Movie S9). In summary, this protocol improves upon
311 existing high dimensional DNA-based imaging techniques by offering full flexibility in antibody-
312 fluorophore pairing, integrating commercially produced CITE-Seq reagents, reducing antibody
313 labeling to one step, and extending the number of fluorophores per cycle.

314

315 **Discussion**

316 Multi-plex imaging of tissues is increasingly important for studies of tumor-immune
317 interactions, for discovery efforts such as the Human Cell Atlas, for better understanding
318 pathological events in infected or physically damaged tissues, and for placing data from isolated
319 cells in the context of *in situ* tissue organization. IBEX is a broadly applicable technique that utilizes
320 conventional microscopes and commercially available antibodies to obtain these essential high
321 dimensional imaging data. IBEX improves upon existing iterative methods by addressing many of
322 the limitations inherent to these techniques. First, we have significantly reduced the fluorophore
323 inactivation step and antibody labeling time from >16 hours to <1 hour using a rapid chemical

324 bleaching agent and antibody labeling employing a commercial, non-heating microwave. Second,
325 our selection of the bleaching agent LiBH_4 provides an efficient means to bleach over 15 unique
326 fluorophores while preserving select fluorophores to serve as repeated markers for registration.
327 Importantly, LiBH_4 treatment does not cause tissue or epitope loss as evidenced by our ability to
328 obtain highly multi-plexed data over several cycles in a wide range of tissues with a very large
329 number of antibodies. Third, and integral to the preservation of tissue integrity through multiple fluid
330 handling cycles, was the use of the tissue adhesive chrome gelatin alum. Importantly, this adhesive
331 adheres delicate tissues to the slide or coverslip surface while maintaining key anatomical features.
332 Finally, the SimpleITK workflow described here represents a significant advancement for the
333 registration of images obtained via cyclic IF methods. In addition to offering flexibility in terms of
334 the repeated markers (membrane, nuclear, structural) used, it provides alignment of markers
335 present on the same cell but not utilized as the fiducial. This is a critical standard for all high
336 dimensional imaging methods because multiple markers are often required to phenotype a
337 particular cell type and staining for the relevant epitopes may occur in different imaging cycles.

338 In addition to developing an efficient method for highly multi-plexed imaging, the IBEX
339 workflow, unlike commercial all-in-one systems (9-11), offers flexibility in terms of cellular markers,
340 antibody-fluorophore combinations, and microscope configurations employed. Because the
341 chemistry of bleaching depends on the fluorophore and not the antibody to which it is conjugated,
342 once the bleaching conditions are defined, staining panels can be designed using specific
343 combinations of fluorophores without regard for the target epitopes of the antibodies employed,
344 providing the user with extreme versatility in experimental design. To this end, we report the
345 validation of more than 200 commercially available antibodies conjugated to fluorophores with
346 excitation and emission spectra ranging from 405 nm to 750 nm. Furthermore, we demonstrate that
347 commercially available oligonucleotide-conjugated antibodies can be seamlessly integrated into
348 our IBEX workflow, representing the first application of TotalSeqA™ antibodies for *in situ* IHC.
349 Given that the barcode sequences for TotalSeqA™ antibodies are disclosed, and a wide range of
350 fluorophore-conjugated oligos is readily available, fluorophore and antibody pairing can be fully
351 customized to match microscope configuration, epitope abundance, and unique tissue
352 characteristics. Taken together, the oligonucleotide-staining method described here provides a
353 completely 'open' method to achieve highly multi-plexed IF imaging using the same antibodies
354 employed for flow cytometry and/or CITE-Seq, enabling effective cross-referencing of datasets
355 derived from these complementary technologies.

356 As a proof-of-concept, we have used the IBEX workflow to examine such issues as the
357 visualization of difficult to extract myeloid populations in various tissues, changes in immune cell
358 composition following immune perturbation, and detection of low abundance epitopes. For the first
359 application, we were able to visualize tissue-resident macrophages that are difficult to characterize
360 using other methods such as flow cytometry because of their limited recovery upon enzymatic

361 tissue digestion (12). Using the panels of antibodies outlined here, we were able to deeply
362 phenotype medullary (CD169⁺F4/80⁺CD11b⁺Lyve-1^{+/-}) and subcapsular sinus (CD169⁺F4/80⁻
363 CD11b⁺) macrophages in the LNs (41) as well as alveolar (SiglecF⁺CD11b⁻CD11c⁺) and interstitial
364 (CD11b⁺CD11c⁺MHCII⁺) macrophages of the lung (42). Additionally, we show that the IBEX
365 method can be scaled to capture ultra-high content imaging in human tissues. The ability to survey
366 large areas of human tissue is critically important as all possible information needs to be extracted
367 to provide maximally useful clinical and research data.

368 Beyond simple visualization of diverse cell types, we have shown compatibility between
369 IBEX-generated data and downstream single-cell, spatially-resolved analysis using the open
370 source method histoCAT. From the 10 cycle IBEX experiments described above, the histoCAT
371 workflow identified 29 phenotype clusters characterized by the expression of several different
372 markers present in distinct imaging cycles. Importantly, this approach identified well described
373 changes following immunization such as an increase in Tfh cells and GC B cells (43). Finally,
374 incorporation of multi-plex Opal IHC into our IBEX workflow facilitated the detection of low
375 abundance epitopes present in conventionally fixed tissues while aiding in the detection of epitopes
376 lost under extreme fixation conditions. The latter represents a significant achievement because few
377 studies have visualized the immunopathology induced by select agents and, to date, the largest
378 number of parameters examined in a single section has been limited to 3 (44). The ability to use
379 IBEX in its native format and with the Opal-plex variation is especially valuable in the context of the
380 current COVID-19 pandemic. Preliminary data show that these methods work well with highly fixed
381 post-mortem samples from such patients.

382 In summary, IBEX constitutes a versatile technique for obtaining high content imaging data
383 using conventional microscopes and commercially available antibodies. In addition to providing a
384 valuable resource for studying tissue-based immunity in animal models of disease, ongoing studies
385 have shown the value of the IBEX method to provide a spatially-defined assessment of complex
386 cell phenotypes from diverse organs including lung, kidney, heart, and lymphoid tissues from
387 surgical specimens as well as post-mortem samples from human COVID-19 patients. We believe
388 that the open nature of the reagents that can be utilized, and the variety of instruments suitable for
389 implementation of IBEX, make it an attractive method for many laboratories seeking to obtain a
390 deeper understanding of cell composition and spatial organization in tissues of interest.

391

392 **Materials and Methods**

393 Detailed descriptions of animals, immunization and tissue preparations, reagents, imaging
394 protocols with Opal fluorophores and oligonucleotide-conjugated antibodies, microscopy
395 configurations, and image acquisition and analysis details are reported in the SI Materials and
396 Methods.

397

398 **Mouse and human tissues**

399 Murine organs and human LNs (1 cm³ or smaller in size) were fixed with BD
400 CytoFix/CytoPerm (BD Biosciences) diluted in PBS (1:4) for 2 days. Following fixation, all tissues
401 were washed briefly (5 minutes per wash) in PBS and incubated in 30% sucrose for 2 days before
402 embedding in OCT compound (Tissue-Tek). All mice were maintained in specific pathogen-free
403 conditions at an Association for Assessment and Accreditation of Laboratory Animal Care-
404 accredited animal facility at the National Institute of Allergy and Infectious Diseases (NIAID). All
405 procedures were approved by the NIAID Animal Care and Use Committee (NIH). De-identified
406 human LN samples were obtained from patients undergoing elective risk-reducing gastrectomies
407 or colon resections for colon adenocarcinoma at the National Cancer Institute (NCI) based on an
408 Institutional Review Board (IRB) approved tissue collection protocol (#13C-0076).

409 **IBEX using inverted confocal microscope**

410 20-30 µm sections were cut on a CM1950 cryostat (Leica) and adhered to 2 well
411 Chambered Coverglasses (Lab-tek) coated with 15 µl of chrome alum gelatin (Newcomer Supply)
412 per well. Frozen sections were permeabilized, blocked, and stained in PBS containing 0.3% Triton
413 X-100 (Sigma-Aldrich), 1% bovine serum albumin (Sigma-Aldrich), and 1% mouse or human Fc
414 block (BD Biosciences). For conventional IF, sections were first blocked 1-2 hours at room
415 temperature and then stained for 12 hours at 4 °C in a humidity chamber. For microwave-assisted
416 IF, we utilized the PELCO BioWave Pro 36500-230 microwave equipped with a PELCO
417 SteadyTemp Pro 50062 Thermoelectric Recirculating Chiller (Ted Pella). A 2-1-2-1-2-1-2-1-2
418 program was used for immunolabeling where '2' denotes 2 minutes at 100 watts and '1' denotes 1
419 minute at 0 watts. The above program was executed once for blocking and secondary antibody
420 labeling and twice for primary antibody labeling. A complete list of antibodies and tissue-specific
421 panels can be found in Tables S1-S5. Cell nuclei were visualized with JOJO-1 (Thermo Fisher
422 Scientific) or Hoechst (Biotium) and sections were mounted using Fluoromount G (Southern
423 Biotech). Mounting media was thoroughly removed by washing with PBS after image acquisition
424 and before chemical bleaching of fluorophores. Samples were treated with 1 mg/mL of LiBH₄
425 (STREM Chemicals) prepared in diH₂O for 15 minutes to bleach all fluorophores except JOJO-1,
426 Hoechst, eF615, and Alexa Fluor 594. To bleach antibodies conjugated to Brilliant Violet 421
427 (BV421) and Brilliant Violet 510 (BV510) dyes, tissue sections were illuminated using the metal
428 halide lamp with the DAPI filter of the Leica TCS SP8 X inverted confocal microscope. The
429 efficiency of fluorophore bleaching was assessed in real time by viewing the LiBH₄-incubated
430 samples on the microscope. Following efficient bleaching, the LiBH₄ solution was removed and
431 samples were washed in 3 exchanges of PBS, restained with the next panel, and mounted with
432 Fluoromount G. Fluorophore inactivation with H₂O₂ was conducted as described previously (5) with

433 tissue sections being treated for 1 hour at room temperature with 4.5% H₂O₂ (Sigma) prepared in
434 an alkaline solution. Tissue sections were imaged as described in the SI Materials and Methods.

435

436 **Image alignment and registration**

437 The alignment of all IBEX panels to a common coordinate system was performed
438 using SimpleITK (23, 24). To facilitate registration, we utilized a common channel present in all
439 panels. As the images may differ by a significant translational motion, we use a Fourier domain-
440 based initialization approach (45) that accommodates for this motion. In addition, we
441 utilized SimpleITK's multi-scale registration framework with four levels, reducing the resolution by
442 a factor of two per level. Directly using the original voxel sizes, on the order of 10⁻³
443 mm, in the gradient descent optimizer computations leads to numerical instability. We therefore
444 normalized the voxel dimensions during optimization, while preserving anisotropy. The final,
445 optimal transformations are then used to resample all channels from each panel to the common
446 coordinate system. The software repository for SITK_IBEX can be found on [github.com/niaid/sitk-
447 ibex](https://github.com/niaid/sitk-ibex).

448

449 **Extensions of IBEX protocol**

450 Integrating IBEX with Opal dyes or oligonucleotide-conjugated antibodies was performed
451 as detailed in the SI Materials and Methods. For multi-plex Opal IHC the following steps—primary
452 antibody incubation, incubation with HRP-conjugated secondary, labeling with Opal dye, and
453 antibody stripping—were repeated to achieve 6-plex imaging using the Opal 520, 540, 570, 620,
454 650, and 690 fluorophores. After representative images were captured, coverslips were removed
455 and tissue sections were treated with 1 mg/mL of LiBH₄ prepared in diH₂O for 30 minutes to bleach
456 the Opal 570, 650, and 690 dyes. Cycles of multi-plex Opal IHC and IBEX were repeated to achieve
457 the desired number of markers. For integration of oligonucleotide-conjugated antibodies into the
458 IBEX workflow, TotalSeq-A™ antibodies were co-incubated with fluorophore-conjugated antibodies
459 which were imaged first before LiBH₄ bleaching. Oligonucleotides are preserved and
460 complementary fluorophore-conjugated oligonucleotides are used to reveal immunostaining, then
461 either dehybridized or bleached again *in situ* across multiple cycles.

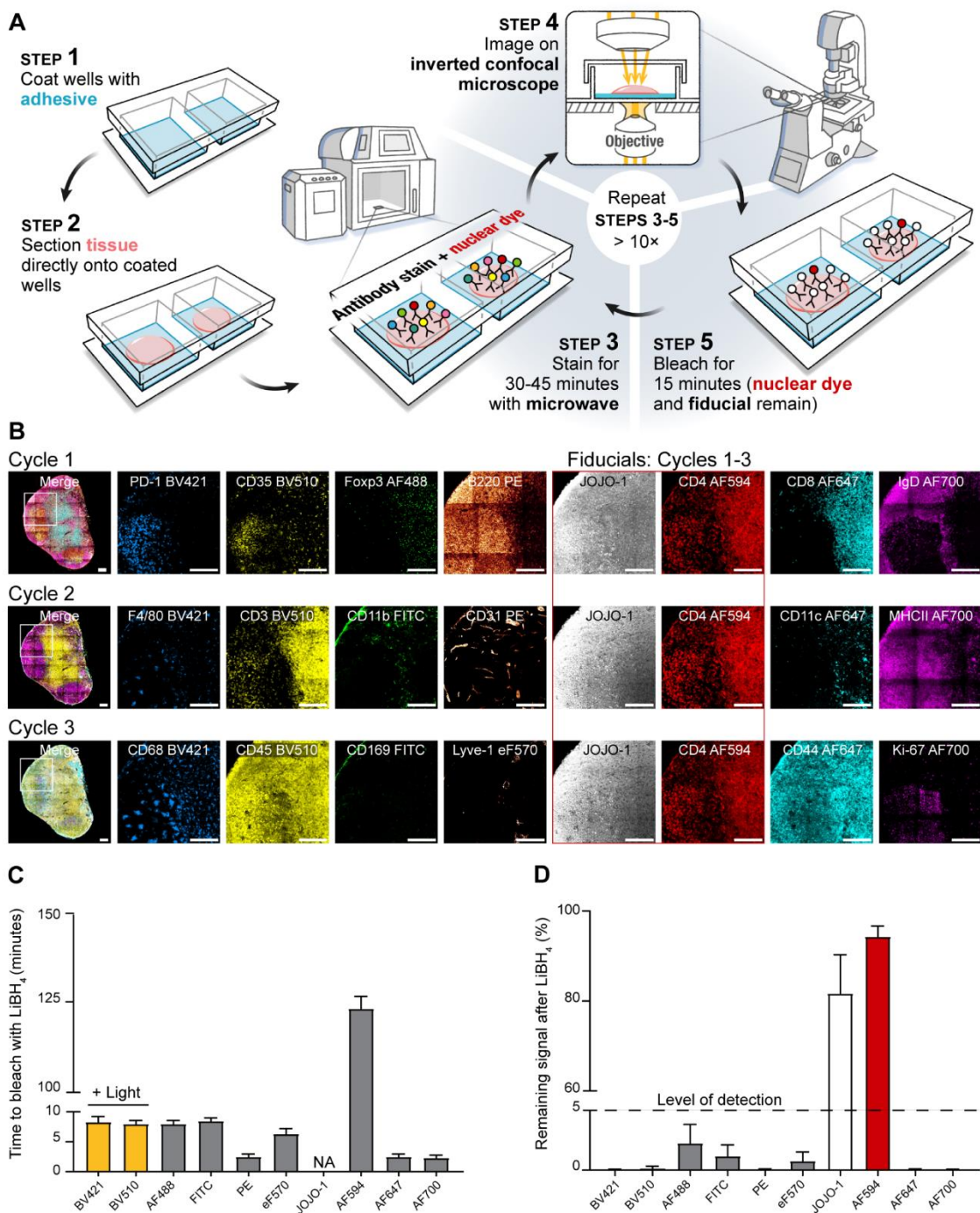
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Figure 1. IBEX: A high dimensional, iterative imaging technique.

579 (A) Schematic depicting IBEX protocol using an inverted confocal microscope. (B) Mice were
580 immunized s.c. with 25 μ l of SRBCs on day 0 and 7. On day 14, pLN tissue sections were labeled
581 with 3 separate imaging panels. JOJO-1 and CD4 AF594 were present throughout cycles 1-3 and
582 served as fiducials. Left-most panel is a composite of all channels except for JOJO-1. Scale bar
583 represents 150 μ m. Light refers to bleaching with LiBH₄ while sample was illuminated with a metal
584 halide lamp and DAPI filter (C) Time required to bleach respective fluorophores using LiBH₄. NA
585 indicates no appreciable loss of signal over multiple hours of LiBH₄ exposure. (D) Percentage of

586 fluorophore signal remaining after 15 minutes of LiBH₄ treatment. Data are pooled from 2 similar
587 experiments.

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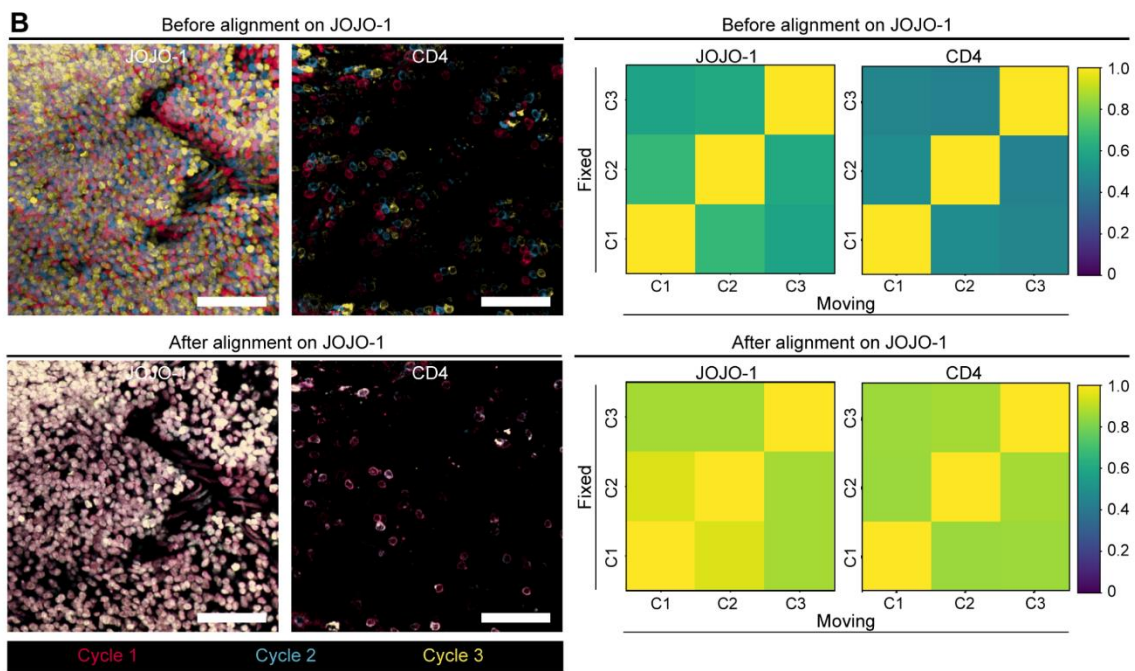
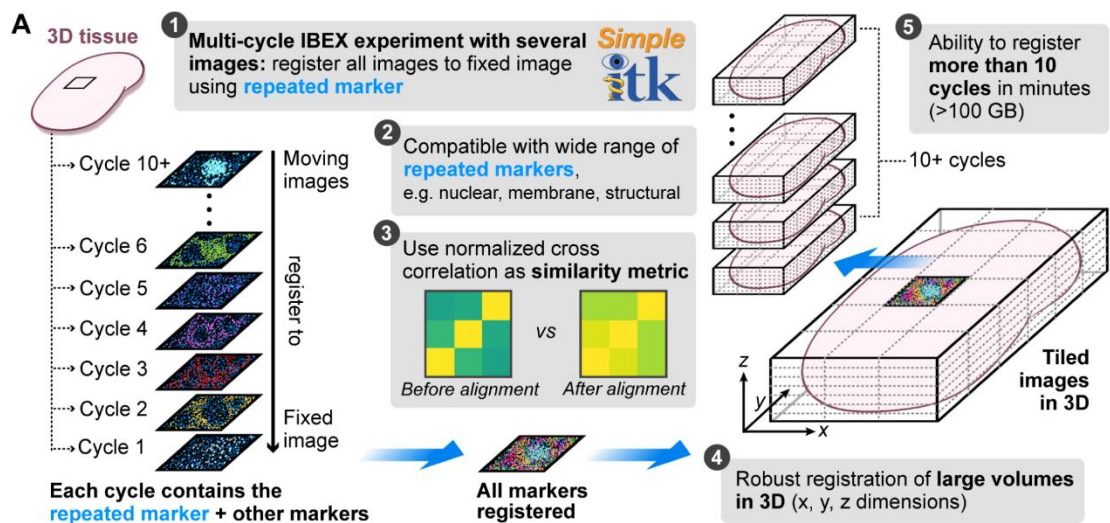
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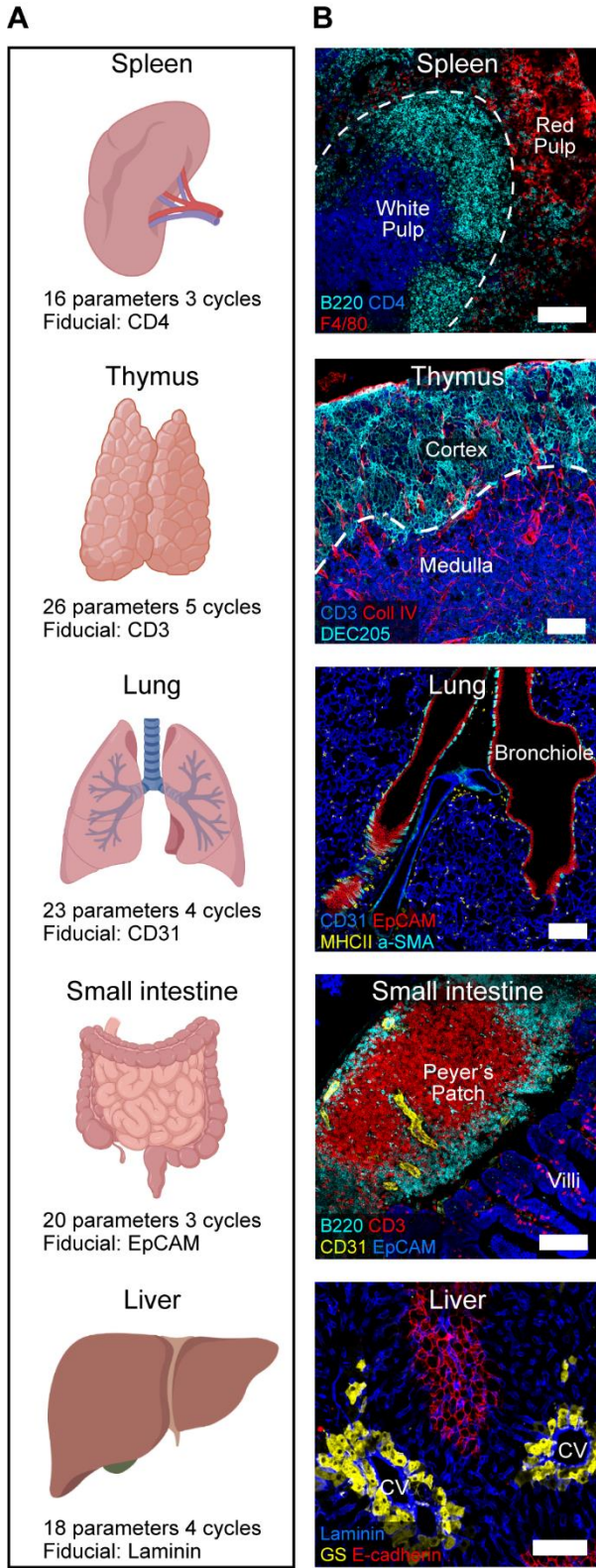
617 **Figure 2. Image alignment with SimpleITK image registration pipeline.**

618 (A) Workflow for SimpleITK image registration pipeline. (B) Confocal images showing JOJO-1 and
 619 CD4 from 3 consecutive IBEX cycles before and after alignment using the nuclear marker JOJO-1
 620 as a fiducial across all 3 cycles. CD4 was also repeated and shows cell-cell registration after JOJO-
 621 1 alignment. Cycle (C), scale bar is 50 μ m. Cross correlation similarity matrices before and
 622 after alignment with JOJO-1 for JOJO-1 and CD4 channels. All experiments are representative of at
 623 least 2 similar experiments.

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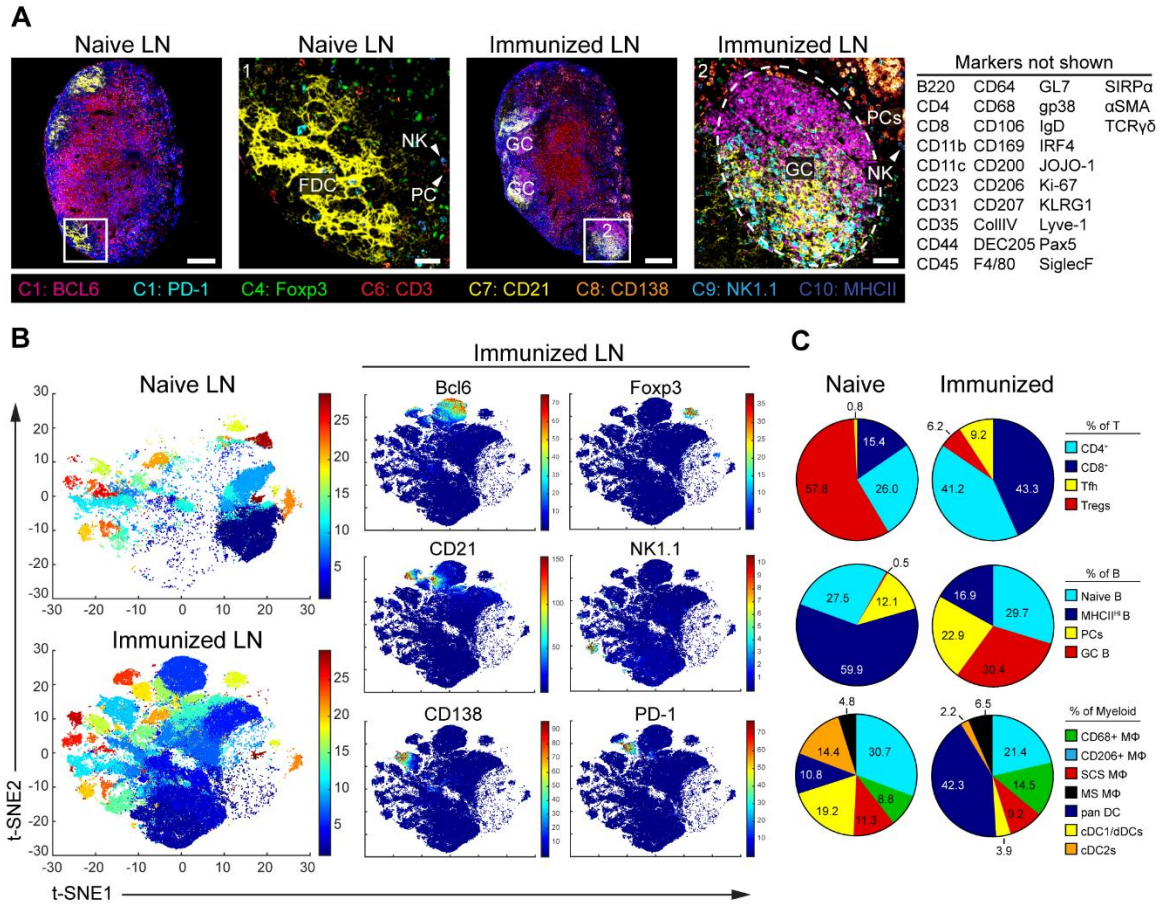


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628 **Figure 3. IBEX in multiple murine organs.**

629 (A) IBEX experimental parameters. (B) Confocal images from IBEX experiments in various mouse
630 organs. Liver: central vein (CV) and glutamine synthetase (GS). Scale bar is 100 μ m. See Movies
631 S1-S5 for additional details.

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684 **Figure 4. Visualization and quantification of LN populations using IBEX and histoCAT**
685 **following immune perturbation.**
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687 (A) Confocal images of pLNs from naïve and SRBC-immunized mice from 10 cycle (C) 41
688 parameter IBEX experiments, GC (germinal center), NK (natural killer), FDC (follicular dendritic
689 cell), PC (plasma cells). Scale bars from left to right: 100 μm, 25 μm, 100 μm, and 50 μm. (B)
690 t-SNE plots from naïve and immunized LNs identified by Phenograph clustering using segmented
691 cells in histoCAT (naïve n = 32,091; immune n = 80,355). Color reflects the cluster ID number (1-
692 29). Single plots show separation of representative markers into discrete clusters with color map
693 showing relative expression levels based on Z-score normalized marker intensity values. (C)
694 Phenograph clusters identified by histoCAT were phenotyped based on marker expression and
695 expressed as a proportion of lineage. Tfh (T follicular helper), MΦ (macrophage), SCS (subcapsular
696 sinus), MSM (Medullary sinus), DC (dendritic cell), dDC (dermal DC). Data are from one experiment
697 and representative of 2 similar experiments. See Fig. S6 and Movie S6.

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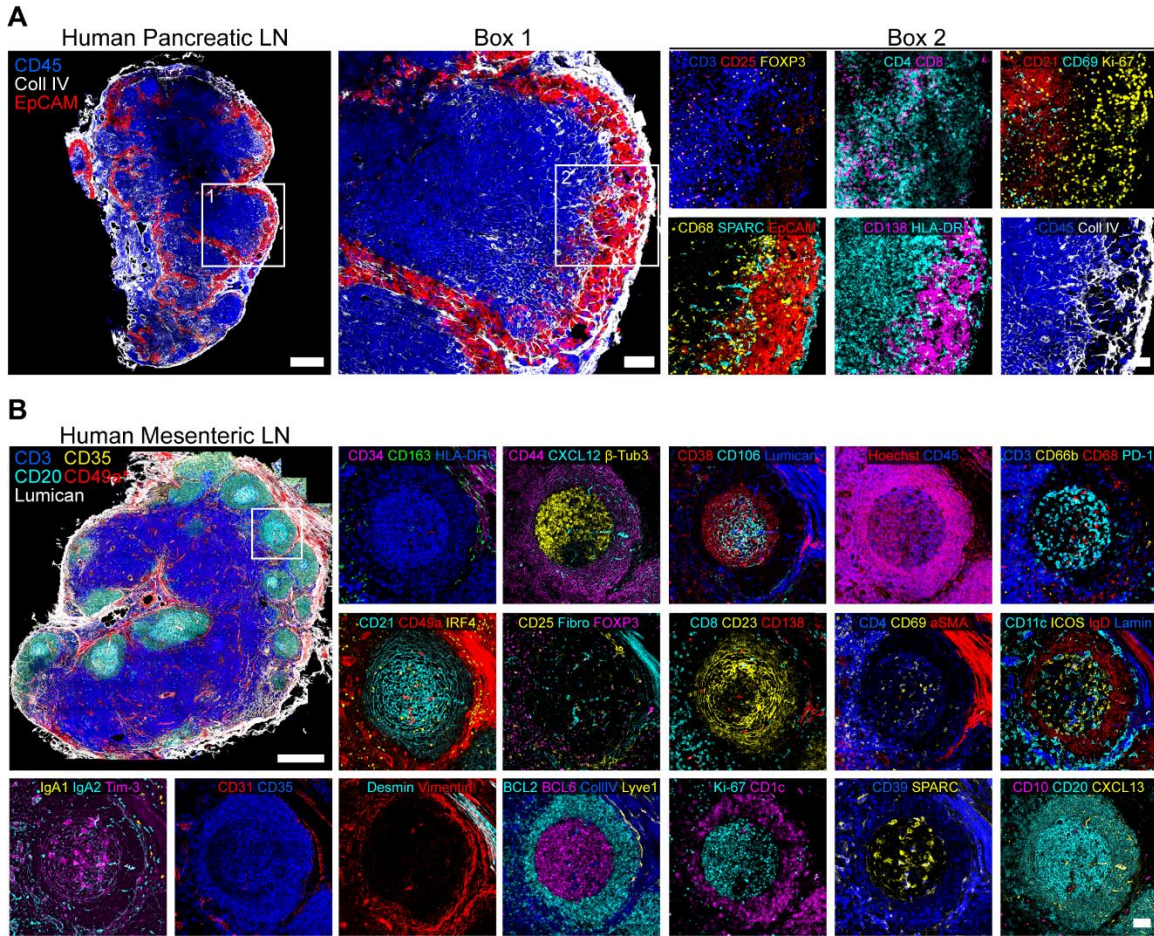
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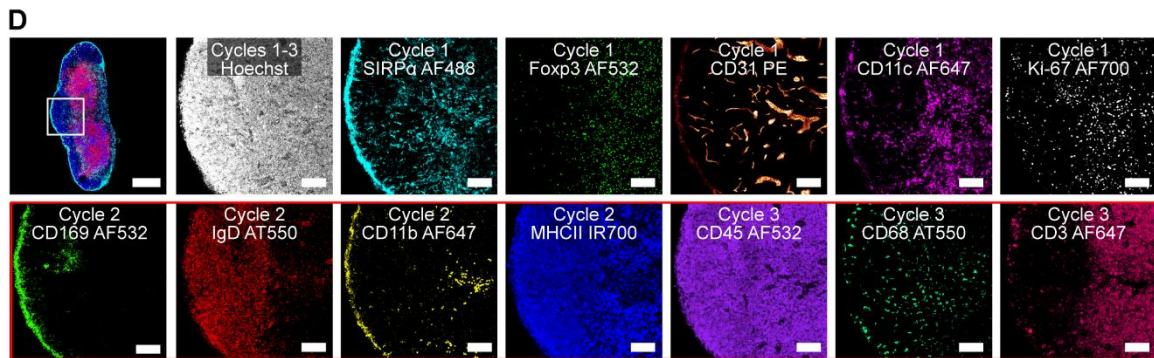
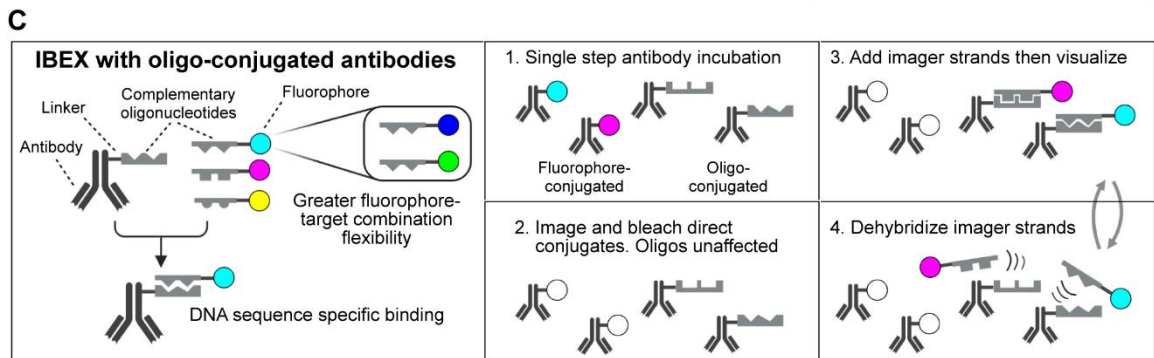
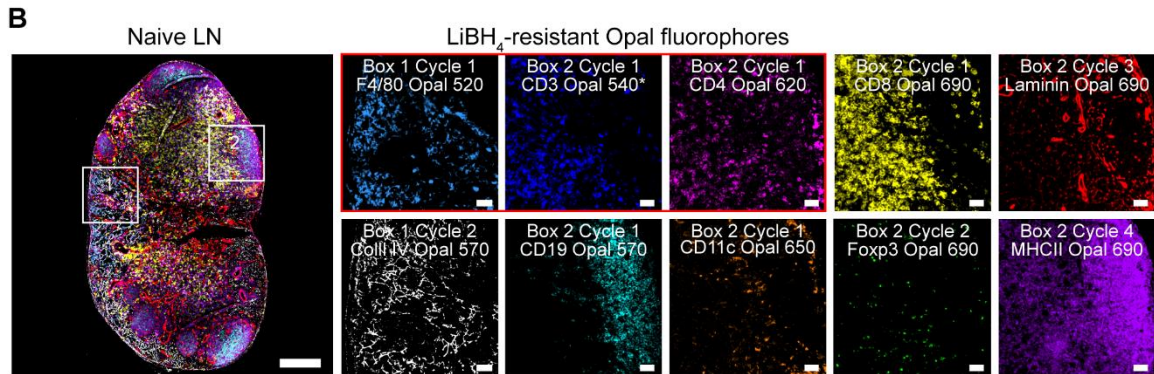
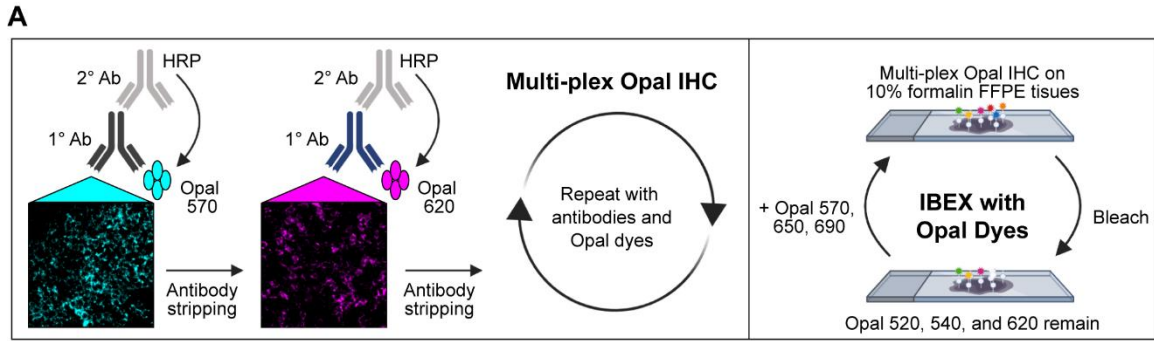


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Figure 5. IBEX scales to capture ultra-high content imaging in human tissues.

706 (A) Confocal images from a pancreatic LN with metastatic lesions from a patient with colorectal
 707 cancer (4 cycle 17 parameter IBEX experiment). Scale bar is 500 µm (left), 100 µm (Box 1), or 50
 708 µm (Box 2). (B) Representative confocal images from human mesenteric LN obtained by IBEX
 709 method (20 cycle 66 parameters). Scale bars (500 or 50 µm). Fibronectin (Fibro), Laminin (Laminin).
 710 See Movie S8 for additional details.

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Figure 6. Incorporation of Opal fluorophores and oligo-conjugated antibodies into IBEX workflow.

723 (A) Opal-plex imaging method consisting of several rounds of labeling with marker-specific primary
724 antibodies, an HRP-conjugated secondary antibody, Opal dyes, and antibody stripping for each
725 marker-Opal fluorophore pair followed by cycles of IBEX (imaging, removal of coverslip, and
726 bleaching). (B) Representative images from a 10 parameter 4 cycle Opal-plex experiment

727 performed on 5 μm FFPE tissue sections from heavily fixed mouse pLNs. CD3 Opal 540 was
728 present throughout cycles 1-4 and served as a fiducial (*). Scale bars (200 μm , left-most panel or
729 50 μm). (C) Schematic depicting principle behind tissue imaging with oligo-conjugated antibodies
730 and incorporation of these reagents into IBEX workflow. (D) Confocal images from a 13 parameter
731 3 cycle IBEX experiment performed on 20 μm tissue sections from an immunized inguinal mouse
732 LN. Cycle 1: Fluorophore-conjugated antibodies. Cycles 2-3: Oligo-conjugated antibodies, Atto550
733 (AT550). Scale bars (400 μm , top-left panel or 50 μm). Data are representative of 3 similar
734 experiments. See Fig. S7 and Movie S9.