

1 Multi-Dimensional Immuno-Profiling of *Drosophila* Hemocytes by Single Cell  
2 Mass Cytometry

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26 **Abstract**

27 Single cell mass cytometry (SCMC) combines features of traditional flow cytometry  
28 (FACS) with mass spectrometry and allows the measurement of several parameters at  
29 the single cell level, thus permitting a complex analysis of biological regulatory  
30 mechanisms. We optimized this platform to analyze the cellular elements, the  
31 hemocytes, of the *Drosophila* innate immune system. We have metal-conjugated six  
32 antibodies against cell surface antigens (H2, H3, H18, L1, L4, P1), against two  
33 intracellular antigens (3A5, L2) and one anti-IgM for the detection of L6 surface  
34 antigen, as well as one anti-GFP for the detection of crystal cells in the immune  
35 induced samples. We investigated the antigen expression profile of single cells and  
36 hemocyte populations in naive, in immune induced states, in tumorous mutants  
37 (*hop<sup>Tum</sup>* bearing a driver mutation and *l(3)mbn<sup>1</sup>* carrying deficiency of a tumor  
38 suppressor) as well as in stem cell maintenance defective *hdc<sup>484</sup>* mutant larvae.  
39 Multidimensional analysis enabled the discrimination of the functionally different  
40 major hemocyte subsets, lamellocytes, plasmatocytes, crystal cell, and delineated the  
41 unique immunophenotype of the mutants. We have identified sub-populations of  
42 L2+/P1+ (*l(3)mbn<sup>1</sup>*), L2+/L4+/P1+ (*hop<sup>Tum</sup>*) transitional phenotype cells in the  
43 tumorous strains and a sub-population of L4+/P1+ cells upon immune induction. Our  
44 results demonstrated for the first time, that mass cytometry, a recent single cell  
45 technology combined with multidimensional bioinformatic analysis represents a  
46 versatile and powerful tool to deeply analyze at protein level the regulation of cell  
47 mediated immunity of *Drosophila*.

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49 **KEYWORDS:** Mass Cytometry; Innate Immunity; *Drosophila*; Single Cell Analysis,  
50 Hemocyte

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## 54 **Introduction**

55 In the animal kingdom, insects have multi-layered innate immune defence mechanisms  
56 against invading pathogens. Work on insects, including the fruit fly, *Drosophila*  
57 *melanogaster* which lacks an acquired immune response, plays an important role in our  
58 understanding of how innate immunity works [1, 2]. The conserved signaling pathways  
59 between insects and vertebrates, combined with the powerful genetic resources  
60 provided by *Drosophila*, make this organism an ideal system to model biological  
61 phenomena related to human biology and medicine. In *Drosophila*, microbial infection  
62 induces a powerful humoral immune response, the release of antimicrobial peptides,  
63 the regulation of which is now well understood [3]. Infection by parasites, development  
64 of tumours or wounding induce a cellular immune response by blood cells, the  
65 hemocytes, which are capable of sophisticated functions, as recognition, encapsulation  
66 and killing of parasites and phagocytosis of microorganisms [4–6]. These functions are  
67 exerted by specialized blood cells the phagocytic plasmatocytes, the encapsulating  
68 lamellocytes and the melanizing crystal cells. For the identification and  
69 characterization of the mechanisms of cell mediated immunity through which the  
70 immune cells and tissues can be specifically studied and manipulated, quantitative  
71 methods are required. For the definition of the functional hemocyte subsets transgenic  
72 reporter constructs and monoclonal antibodies have been developed. These systems  
73 generally use fluorescent molecules in the form of *in vivo* markers and antibodies, the  
74 use of which significantly contributed to our understanding of innate immunity [7–9].  
75 Recently, single cell mass cytometry was developed to monitor the expression of  
76 marker molecules in haematological and other pathological conditions [10,11]. The  
77 antibodies against cell type specific antigens are applicable to monitor blood cell  
78 differentiation during ontogenesis or following immune induction. However,  
79 traditional antibody staining against only one or two of the cell type specific antigens  
80 is not sufficient to describe individual hemocyte populations with complex antigen  
81 expression patterns. Therefore, we adopted and optimized single cell mass cytometry

82 for *Drosophila* by multiplex analysis of antibodies to transmembrane proteins and  
83 intracellular antigens of IgG and IgM type, routinely used for detecting and  
84 discriminating hemocyte subsets of *Drosophila melanogaster* [7, 12–16].

85 The circulating hemocytes of the *Drosophila* larva are classified into three categories,  
86 of which only two cell types are present in naive condition. These are the small round  
87 phagocytic plasmatocytes, which account for 95% of the circulating hemocytes, and  
88 the melanizing crystal cells, which are similar in size to plasmatocytes, but contain  
89 prophenoloxidase crystals in their cytoplasm. The third cell type, the large flattened  
90 lamellocytes differentiate only in tumorous larvae and in case of immune induction,  
91 such as wounding or parasitic wasp infestation [17]. Lamellocytes, together with  
92 plasmatocytes are capable of forming a multilayer capsule around the wasp egg,  
93 thereby killing the invader [18–20]. Plasmatocytes, crystal cells and lamellocytes can  
94 be distinguished with cell type specific monoclonal antibodies, and *in vivo* transgenic  
95 reporters [7–9, 12–15]. All plasmatocytes express the P1 antigen (coded by the  
96 *nimC1* gene) [21], while lamellocytes show a characteristic expression of L1 (the  
97 product of the *atilla* gene), L2, L4, and L6 [14]. Following immune induction, a  
98 portion of plasmatocytes transdifferentiate into lamellocytes to fight the parasitic  
99 wasp egg [22–25]. This transdifferentiation is accompanied by a stepwise alteration  
100 of lamellocyte specific antigen expression.

101 Understanding cancer, a devastating disease of multicellular organisms is a challenge  
102 for scientists. The conserved signal transduction pathways in *Drosophila* with  
103 mammals and the easy genetic manipulation made the fruit fly a frequently used  
104 model organism to study cancer [26]. Therefore, we investigated two different  
105 tumorous *Drosophila* strains, one bearing a driver mutation (*hop<sup>Tum</sup>*) and one carrying  
106 deficiency of a tumor suppressor (*l(3)mbn<sup>1</sup>*). Constitutive activation of the *Drosophila*  
107 Janus kinase namely, the Hopscotch (Hop) causes melanotic tumors, lymph gland  
108 hypertrophy in the larvae and malignant neoplasia of *hop<sup>Tum</sup>* *Drosophila* blood cells  
109 [27]. The homozygously mutated state of the tumor suppressor gene, called *lethal* (3)

110 *malignant blood neoplasm* causes malignant transformation, enhanced hemocyte  
111 proliferation and lamellocyte differentiation of *l(3)mbn<sup>1</sup>* *Drosophila* blood cells [28].  
112 We also investigated the immunophenotype of the mutation of the *hdc* gene (*hdc<sup>A84</sup>*),  
113 which encodes for the *Drosophila* homolog (Headcase) of the human tumor  
114 suppressor HECA and plays a role in hematopoietic stem cell maintenance [29, 30].  
115 Wild type *Oregon-R* (*Ore-R*) and *white* mutant *w<sup>1118</sup>* were used as reference strains  
116 because *w<sup>1118</sup>* was considered previously as wild type and used for the generation of  
117 mutants [31]. Immune activation was monitored successfully by infestation with the  
118 *Leptopilina boulardi* parasitoid wasp of *Drosophila* larvae in the *lozenge>GFP* strain  
119 (*lz-Gal4, UAS-GFP; +; +*), in which crystal cells were monitored by metal tag  
120 labeled anti- GFP antibody [32, 33].  
121 We are the first to demonstrate that single cell mass cytometry is a powerful tool for  
122 the characterization of hemocytes in different mutants of *Drosophila* strains at protein  
123 level. Bioinformatic analysis revealed the characteristic protein expression pattern of  
124 hemocyte subsets at single cell resolution from the studied different genetic variants.  
125 These together suggest that single cell mass cytometry is a valuable tool for  
126 characterizing immune phenotypes in any model organism, in which antibodies  
127 against immune components are available.

## 128 **Results and Discussion**

### 129 **Single cell mass cytometry revealed the transitional phenotypes of hemocytes in** 130 **the tumorous *hop<sup>Tum</sup>* and *l(3)mbn<sup>1</sup>* strains.**

131 We have built the metal tag labelled panel of discriminative antibodies recognizing  
132 *Drosophila melanogaster* hemocytes and hemocyte subsets for mass cytometry. We  
133 have conjugated six antibodies against cell surface antigens (H2, H3, H18, L1, L4, P1),  
134 against two intracellular antigens (3A5, L2) and one anti-IgM for the detection of L6  
135 surface antigen. List of the antibodies can be found in Table 1. The H18 and 3A5  
136 antibodies reported herein first were characterized and validated before the study by

137 indirect immunofluorescence and Western-blot analysis (Figure S1 and S2). The  
138 analysis revealed that 3A5 molecule is expressed in plasmatocytes and lamellocytes in  
139 *l(3)mbn<sup>1</sup>*, but not expressed in lamellocytes of immune (*L.b.*) induced larvae (Figure  
140 S1), while H18 molecule as a pan-hemocyte marker is expressed in all tested samples  
141 (Figure S2). To test and optimize the reactions of the antibodies, a comparative  
142 analysis was carried out by correlating the fluorescence activated cell sorting (FACS)  
143 (Figure S3A) and the mass cytometry histograms (Figure S3B). The comparison  
144 showed similar reactivity patterns. Hemese (H2) pan-hemocyte marker positive single  
145 live cells were gated for mass cytometry analysis (Figure S4). All metal-tag labelled  
146 antibodies were titrated for mass cytometry as shown in Figure S5. Next, we  
147 compared the expansion of the hemocyte populations in the mutants in relation to the  
148 two wild type *Ore-R* and *w<sup>1118</sup>*. The proportion of hemocytes expressing the  
149 investigated markers were similar in wild type (wt) *Ore-R* and *w<sup>1118</sup>*. However, we  
150 detected a significant proliferation of hemocytes expressing the L1, L2, and L4  
151 markers in *l(3)mbn<sup>1</sup>* and *hop<sup>Tum</sup>* mutant larvae, reflecting an extensive differentiation  
152 of lamellocytes, a phenotypic characteristic to the blood cell malignancy. A slight  
153 elevation in the proportion of L6 expressing hemocytes was also detected (Figure S6  
154 and **Figure 1A**). The explanation for this moderate change may be the fact that L6 is  
155 only expressed by a subset of lamellocytes in tumorous larvae [14]. All lamellocyte  
156 markers showed a higher expression level in the tumorous *hop<sup>Tum</sup>* mutant compared to  
157 the control (Figure S7 and **Figure 1B**). In the *hdc<sup>Δ84</sup>* mutant larvae, we detected a  
158 moderate elevation in the expression level of L2, and a decrease in the expression  
159 level of P1 (Figure 1B), however, the number of hemocytes expressing lamellocyte  
160 markers did not show a significant increase compared to the controls (Figure 1A).  
161 This is in line with the finding that in the *hdc<sup>Δ84</sup>* mutant larvae, lamellocytes  
162 differentiate in low numbers, while the number of plasmatocytes are reduced [30].  
163 This reduction of plasmatocyte number is also observable in Figure 1A.

164 Multidimensional analysis by the algorithm of t-distributed stochastic neighbor  
165 embedding (tSNE) and the visualization of stochastic neighbor embedding (viSNE)  
166 was carried out within the H2 (Hemese) positive live singlets based on H3, H18, L1,  
167 L2, L4, L6, P1, and 3A5 marker expression in order to map high parametric single  
168 cell data on biaxial plots [34]. The viSNE patterns of hemocyte marker expression  
169 correlated to the data shown in Figure 1 (**Figure 2**). The viSNE bioinformatic analysis  
170 revealed the characteristic protein expression pattern of hemocyte subsets at single  
171 cell resolution from the studied genetic variants. We observed a dramatic difference in  
172 the viSNE patterns between hemocytes isolated from the tumorous *l(3)mbn<sup>1</sup>* and  
173 *hop<sup>Tum</sup>* larvae as compared to either control *Ore-R* or *w<sup>1118</sup>* larvae (Figure 2). Control  
174 *Ore-R* or *w<sup>1118</sup>* hemocytes were not discriminated on the viSNE plots showing their  
175 minimal genetic distance but tumorous *l(3)mbn<sup>1</sup>* and *hop<sup>Tum</sup>* larvae delineated viSNE  
176 maps with the expansion of lamellocytes (Figure 2). In the *hdc<sup>Δ84</sup>* larvae, we detected  
177 a subset of hemocytes that express the 3A5 marker at a high level. This subset was  
178 detected neither in the control, nor in the tumorous larvae, and may represent a cell  
179 type that differentiate as a precursor for lamellocytes as a consequence of the defect in  
180 the maintenance of the hematopoietic niche [30].

181 The Uniform Manifold Approximation and Projection (UMAP) analysis was  
182 performed by the hemocyte subset specific, discriminating markers: L1, L2, L4, L6  
183 for lamellocytes and P1 for plasmacytes on the 5 studied genetic variants of  
184 *Drosophila melanogaster*. The UMAP analysis resulted in the same conclusion as  
185 tSNE, namely, that lamellocyte expansion occurs in in tumorous strains *l(3)mbn<sup>1</sup>* and  
186 *hop<sup>Tum</sup>* (Figure S8). Both the viSNE and UMAP analysis demonstrate transitional  
187 phenotypes of certain lamellocytes and plasmacytes by the transitional coloration of  
188 marker expression (partially overlapping L2+ or L4+ with some P1+ cells) at protein  
189 level in *l(3)mbn<sup>1</sup>* and *hop<sup>Tum</sup>*. Merging viSNE graphs outlined characteristic maps of  
190 each strain based on high parametric mass cytometry data (**Figure 3A-C**). The *Ore-R*  
191 and *w<sup>1118</sup>* controls showed overlapping patterns on the viSNE diagram (Figure 3A-C),

192 with a somewhat lower expression of all markers observed in case of the  $w^{1118}$ , which  
193 may be due to uncontrollable genetic background variations. The dots representing to  
194  $hdc^{\Delta 84}$  hemocytes, mutant of the  $hdc$  regulator of hematopoietic stem cell maintenance  
195 [30], were detected as a zone in between the control and the tumorous patterns (Figure  
196 3C). The most likely explanation to this phenomenon is that  $hdc^{\Delta 84}$  homozygous  
197 larvae produce lamellocytes, but in a much lower proportion than tumorous larvae, the  
198  $l(3)mbn^1$  and  $hop^{Tum}$  [30]. Tumorous hemocytes  $l(3)mbn^1$  and  $hop^{Tum}$  were closely  
199 mapped and partially overlapping, giving a population clearly separated from the  
200 cloud of the controls, due to the lamellocyte-expansive malignant phenotype (Figure  
201 3A-C).

### 202 **Single cell mass cytometry revealed the transitional phenotypes of hemocytes** 203 **upon immune induction**

204 In order to monitor the changes in the composition of hemocyte subsets following  
205 immune induction, we used  $lz>GFP$  larvae and complemented the experiment with  
206 anti-GFP labeling, which enables the detection of crystal cells [32, 33]. The tSNE  
207 analysis of H3, H18, L1, L2, L4, L6, P1, 3A5 markers and anti-GFP (marking crystal  
208 cells in this particular system) was carried out within the population of pan-hemocyte  
209 H2 (Hemese) positive live singlets (**Figure 4A**). We observed a new subset of  
210 hemocytes appearing 72 h after infestation of the  $lz>GFP$  larvae with the parasitic  
211 wasp (Figure S9. and Figure 4A). This subset of cells accounts for the lamellocytes  
212 that differentiate as a result of the immune induction, since these cells fall into the  
213 high expression part of the viSNE for the L1, L2, L4, and L6 lamellocyte markers  
214 (Figure S9. and Figure 4A). This finding is in correlation with the increase of the  
215 number of hemocytes expressing the L1 (35.1% vs. 1.81%), L2 (32% vs. 1.6%), L4  
216 (34.36% vs. 1.39%) and L6 (13.82 vs. 0.935%) markers (**Figure 4B**), and the elevated  
217 expression levels of the lamellocyte markers detected in immune induced larvae  
218 compared to the naive control (**Figure 4C**). Interestingly, a new subset of crystal cells  
219 (anti-GFP<sup>+</sup> cells) also appeared in immune induced ( $lz>GFP$  *i.i.*) larvae compared to



220 the control ( $lz > GFP$ ) (Figure 4A). The viSNE pattern of the 3A5 marker also changed  
221 significantly after the immune induction, which may be due to the newly  
222 differentiating hemocytes, similarly to that observed in the  $hdc^{\Delta 84}$  larvae (Figure 4A).  
223 Taken together, we report herein the first panel of metal-conjugated anti-*Drosophila*  
224 antibodies to present the applicability of mass cytometry for that canonical model  
225 organism of genetics. Recent studies identified novel subpopulations of *Drosophila*  
226 hemocytes based on single cell RNA data [35–38]. These findings largely contributed  
227 to the definition of hemocyte clusters and to the characterization of intermediate cells  
228 in the transition from plasmatocyte to lamellocyte. In these experiments, clusters were  
229 defined by the gene expression patterns of individual hemocytes. The application of  
230 CyTOF (cytometry by time-of-flight) can complement these comprehensive  
231 transcriptomic studies and verify the existence of transitional phenotypes at protein  
232 level. The comparative analysis of *Ore-R* and *white*<sup>1118</sup> with  $l(3)mbn^1$ ,  $Hop^{Tum}$ ,  $hdc^{\Delta 83}$   
233 revealed transitional phenotypes at protein level and the differences among reference  
234 stains: *Ore-R* and *white*<sup>1118</sup>. Both the viSNE and UMAP analysis demonstrated  
235 transitional phenotype of certain sub-populations of lamellocytes and plasmatocytes  
236 by the transitional coloration of common marker expression (partially overlapping  
237 L2+ or L4+ with P1+ cells) at protein level in  $l(3)mbn^1$ ,  $hop^{Tum}$ . This has been verified  
238 by a functional assay of immune induction (Figure 4). Our study demonstrates  
239 transitional phenotypes (Figure 2, Figure 4, Figure S8) from single cell data at protein  
240 level which places the innate immunity of *Drosophila* in a new biological insight.  
241 Additionally, we report herein two novel hemocyte markers, H18 located on the cell  
242 surface and 3A5 with intracellular localization. The simultaneous detection of several  
243 antigens provided by CyTOF could not be achieved earlier by traditional microscopy.  
244 The main advantage of CyTOF is the multidimensionality coupled with complex  
245 computational tools, therefore we propose the extension of the basic panel used in our  
246 study with antibodies recognizing signaling molecules (e.g. MAP kinases), enzymes  
247 (to follow metabolic pathways), cellular structural proteins (e.g. cytoskeletal, cargo

248 proteins) up to 42 markers in one single tube. Another advantage of the presented  
249 method is that CyTOF enables investigations at protein level (data of transcriptomics  
250 should be also verified at protein level) with single cell resolution. However, we may  
251 consider the limitations of the CyTOF which are a.) the availability of antibodies  
252 against the protein of interest (which is also a limitation for other antibody-based  
253 detection approaches). Moreover, anti-tag antibodies are available when the protein of  
254 interest is labelled with a fusion tag, or the cell of interest is labelled with the  
255 expression of a marker protein (we report herein the use of anti-GFP). Another  
256 limitations are b) the availability of the CyTOF technology (it is increasing and most  
257 of the research centres are supposed to own the technology, as there were 94  
258 instruments already installed in Europe in 2020 January), c) the relative high cost of  
259 the CyTOF technology (although the cost should be taken into account by the number  
260 of investigated markers at protein level and the number of single cells).

261 We believe that our method serves as a rapid and cost-effective tool to monitor the  
262 alteration of hemocyte composition influenced by various agents or mutations. In  
263 those cases, it is less expensive and easier to perform than single-cell transcriptome  
264 analysis. Additionally, the CyTOF can complement transcriptomic studies verifying  
265 up to 42 simultaneous markers at protein level with single cell resolution.

## 266 **Conclusion**

267 The SCMC combines the features of traditional cytometry with mass spectrometry  
268 and enables the detection of several parameters at single cell resolution, thus  
269 permitting a complex analysis of biological regulatory mechanisms. We optimized  
270 this platform to analyze the cellular elements, the hemocytes of the *Drosophila* innate  
271 immune system. The SCMC analysis with 9 antibodies to all hemocytes and  
272 hemocyte subsets showed a good accordance of fluorescence flow cytometry results,  
273 in terms of positivity on hemocytes of the tumor suppressor mutant *l(3)mbn<sup>1</sup>*. Further,  
274 we investigated the antigen expression profile of single cells and hemocyte  
275 populations in *Ore-R* and *w<sup>1118</sup>* controls, and tumorous (*l(3)mbn<sup>1</sup>*, *hop<sup>Tum</sup>*) strains, as

276 well as in a stem cell maintenance defective mutant (*hdc*<sup>Δ84</sup>). The immunophenotype  
277 of immune activation upon infestation with a parasitoid wasp, the differentiation of  
278 lamellocytes was detected by 10 antibodies in the *lz>GFP*.  
279 Multidimensional analysis (viSNE) enabled the discrimination of the major  
280 hemocytes: lamellocytes, plasmatocytes, crystal cells and delineated the unique single  
281 cell immunophenotype of the mutant strains under investigation. Single cell mass  
282 cytometry identified sub-populations of L2+/P1+ (*l(3)mbn*<sup>1</sup>), L2+/L4+/P1+ (*hop*<sup>Tum</sup>)  
283 transitional phenotype cells in the tumorous strains and a sub-population of L4+/P1+  
284 cells upon immune induction. We demonstrated that mass cytometry, a recent single  
285 cell technology coupled with multidimensional bioinformatic analysis at protein level  
286 represents a powerful tool to deeply analyze *Drosophila*, a key multicellular model  
287 organism of genetic studies with a wide inventory of available mutants.

## 288 **Materials and methods**

### 289 ***Drosophila* stocks**

290 The following *Drosophila* lines were used in the study: *w*<sup>1118</sup> (BSC#9505), *ORE-R*  
291 (wild type), *w*; *hdc*<sup>Δ84</sup>/*TM3*, *Kr>GFP* [30], *lz-Gal4*, *UAS-GFP*; +; + (a gift from  
292 Bruno Lemaitre, Lausanne, Switzerland) [32], *l(3)mbn*<sup>1</sup>/*TM6 Tb* [28], a homozygous  
293 *hop*<sup>Tum</sup> (BSC#8492) line generated by dr. Gábor Csordás (BRC, Szeged, Hungary).  
294 The flies were grown on a standard cornmeal-yeast substrate at 25 °C.

### 295 **Production of the H18 and 3A5 antibodies**

296 Monoclonal antibodies against *Drosophila* hemocytes were raised as described  
297 previously [14]. Briefly, BALB/c mice were immunized by i.p. injection of 10<sup>6</sup>  
298 hemocytes from late third instar larvae of the *lethal(3)malignant blood neoplasm*  
299 [*l(3)mbn*<sup>1</sup>] mutant larvae in *Drosophila* Ringer's solution (Sigma-Aldrich, St. Louis,  
300 MI, USA). Booster injections were given 4, 8, and 13 weeks later. Three days after  
301 the last immunization, spleen cells were collected and fused with SP2/O myeloma  
302 cells by using polyethylene glycol (PEG1450, P5402 Sigma-Aldrich). Hybridomas  
303 were selected in HAT medium (HAT = hypoxanthine-aminopterin-thymidine

304 Supplement, 21060017 Thermo Fischer Scientific Waltham, MA, USA) and  
305 maintained as described by Kohler and Milstein [14, 39]. Hybridoma culture  
306 supernatants were screened by indirect immunofluorescence on acetone fixed,  
307 permeabilized and on live hemocytes. The selected hybridomas were subcloned three  
308 times by limiting dilution.

### 309 **Isolation of hemocytes**

310 Hemocytes were isolated from late third stage larvae by dissecting the larvae in  
311 *Drosophila* Schneider's solution (21720001 Thermo Fisher Scientific, Waltham, MA,  
312 USA)) supplemented with 5% fetal bovine serum albumin (FBS, F7524-500ML  
313 Sigma-Aldrich) plus 0.003% 1-phenyl-2-thiourea (P7629 Sigma-Aldrich).

### 314 **Immune induction**

315 *lz-Gal4; UAS-GFP* flies (*lz>GFP*) laid eggs for three days in bottles containing  
316 standard *Drosophila* medium. After 72 hours, larvae were infected with *Leptopilina*  
317 *boulardi* wasps for 6 hours. Larvae with visible melanotic nodules were selected 72  
318 hours after infestation for isolation of hemocytes. Age and size-matched larvae were  
319 used as control.

### 320 **Immunofluorescent staining**

321 Immunofluorescent staining was performed as described previously [23]. Briefly,  
322 hemocytes were attached to multispot slides (SM-011, Hendley-Essex, Loughton,  
323 UK) at 21 °C for 45 min. Fixation was performed with acetone for 6 min, rehydrated  
324 and subsequently blocked for 20 min in PBS supplemented with 0.1% BSA (PBS =  
325 phosphate buffered saline, P4417 Sigma-Aldrich; BSA = bovine serum albumin,  
326 A2058 Sigma-Aldrich), incubated with the indicated antibodies for 1 h at 21 °C,  
327 washed three times with PBS and incubated with CF-568 conjugated anti-mouse IgG  
328 (H+L), F(ab')<sub>2</sub> fragment (1:1000, SAB4600082 Sigma-Aldrich) for 45 min. Nuclei  
329 were labeled with DAPI (D9542 Sigma-Aldrich). The microscopic analysis was  
330 carried out using a Zeiss Axioskope 2MOT epifluorescent microscope and Axiovision  
331 2.4 software (Zeiss, Oberkochen, Germany).

332 **Western blotting**

333 Western blotting was performed in order to test the specificity of the anti-3A5 and  
334 anti-H18 antibodies as described previously [12]. Briefly, proteins were differentiated  
335 by SDS-PAGE. Following the electrophoresis, the proteins were blotted onto  
336 nitrocellulose membrane (Hybond-C, 10564755 Amersham Pharmacia,  
337 Buckinghamshire, UK) in the transfer buffer (25 mM Tris pH 8.3, 192 mM glycine,  
338 20% (V/V) methanol). The nonspecific binding was blocked with PBS supplemented  
339 with 0.1% Tween 20 (PBST, P1379 Sigma-Aldrich) and 5% non-fat dry milk at 21 °C  
340 for 1 h. The blotted proteins were reacted to the indicated antibody (anti-3A5 in  
341 Figure S1, and anti-H18 in Figure S2) with rotation at 21 °C for 3 h. Washing was  
342 performed with PBST three times for 10 min and then incubated with  
343 HRPO-conjugated anti-mouse antibody (62-6520 Thermo Fisher Scientific). After  
344 three washes with PBST for 10 min, the proteins were detected by the ECL-Plus  
345 system (32132 Thermo Fisher Scientific) following the manufacturer's  
346 recommendations.

347 **Flow cytometry**

348 Flow cytometry was executed as published previously [12]. Briefly, 20 µl of 10<sup>7</sup>/ml  
349 hemocyte suspension was plated in insect Schneider's medium (supplemented with  
350 10% FCS) into each well of a 96-well U-bottom microtiter plate (3635 Corning Life  
351 Sciences, Tewksbury, MA, USA). Samples for intracellular staining were treated by  
352 2% paraformaldehyde (158127 Sigma-Aldrich). Hybridoma supernatants (50 µl) were  
353 measured to each well, and reacted at 4 °C for 45 min. The negative control  
354 monoclonal antibody was a mouse IgG1 (clone T2/48, anti-human anti-CD45) [40].  
355 After the incubation, cells were washed three times with ice-cold Schneider's  
356 medium. The secondary antibody, Alexa Fluor 488-labeled anti-mouse IgG  
357 (AP124JA4 Sigma-Aldrich) was added (1:1000). After 45 min incubation at 4 °C, the  
358 cells were washed (three times) with ice-cold Schneider's medium and acquired on  
359 FACSCalibur (Beckton Dickinson, Franklin Lakes, NJ, USA).

360 **Mass cytometry**

361 Mass cytometry was performed as we published earlier with some modifications [10,  
362 41]. The affinity purified monoclonal antibodies were provided by Istvan Ando's  
363 group (BRC, Szeged, Hungary) (Table 1) or purchased: anti-IgM, (406527 Biolegend,  
364 San Diego, CA, USA [42]), anti-GFP (A11122 Thermo Fisher Scientific [43]),  
365 anti-CD45 (3089003B Fluidigm, South San Francisco, CA, USA [44]) and conjugated  
366 in house according to the instructions of the manufacturer (Maxpar antibody labeling  
367 kit, Fluidigm). Optimal antibody concentrations were titrated prior use (Figure S5).  
368 The following antibody concentrations were used: H2: 5 µg/ml, H3: 5 µg/ml, H18: 5  
369 µg/ml, L1: 1 µg/ml, L2: 7.5 µg/ml, L4: 7.5 µg/ml, L6: 10 µg/ml, anti-IgM: 10 µg/ml,  
370 P1: 7.5 µg/ml, 3A5: 5 µg/ml, anti-GFP: 10 µg/ml. The negative control monoclonal  
371 antibody was a mouse IgG1 (clone H130, anti-human 89Y labeled anti-CD45) in  
372 1:100 dilution. The isotypes of anti-*Drosophila* antibodies were determined by the  
373 IsoStrip™ Antibody Isotyping Kit (11493027001 Roche, Basel, Switzerland)  
374 according to the instructions of the manufacturer.

375 Single cell suspensions were centrifugated at 1100 g at 6 °C for 4 min and incubated  
376 with viability marker (5 µM cisplatin, 195 Pt, 201064 Fluidigm) on ice in 40 µl PBS  
377 for 3 min. Cells were washed twice with 200 µl Maxpar Cell Staining Buffer (MCSB,  
378 201068 Fluidigm) and centrifugated at 1100 g at 6°C for 4 min. Cells were  
379 resuspended in 50 µl MCSB and 50 µl surface antibody cocktail (2 ×) was added,  
380 incubated on ice for 30 min. Cells were washed with 200 µl MCSB and stained with  
381 anti-IgM antibody (volumes were the same as in the surface staining), incubated on  
382 ice for 30 min. Cells were washed with 200 µl MCSB and suspended in 100 µl 1 ×  
383 Maxpar Fix I buffer (201065 Fluidigm), incubated on ice for 20 min. Cells were  
384 washed twice with 200 µl PermS buffer (201066 Fluidigm) then stained with the  
385 intracellular antibody cocktail (L2, 3A5 and anti-GFP in *Lz>GFP* samples), left on  
386 ice for 30 min. Cells were washed once with MCSB then fixed with 200 µl 1.6%  
387 formaldehyde solution (freshly diluted from 16% Pierce formaldehyde in PBS, 28906

388 Thermo Fisher Scientific), incubated on ice for 10 minutes then centrifugated at 1300  
389 g at 6°C for 4 min. After fixation, cells were resuspended in 300 µl Maxpar Fix and  
390 Perm buffer (201067 Fluidigm) containing 125 nM Cell-ID DNA intercalator  
391 (191/193 Iridium, 201192A Fluidigm) and incubated at 4 °C overnight. Before the  
392 acquisition samples were washed in MCSB twice and in PBS once (without Mg<sup>2+</sup> and  
393 Ca<sup>2+</sup>, 10010015 Thermo Fisher Scientific) by centrifugation at 1300 g at 6°C for 4  
394 min. Cells were counted using Bürker chamber. For the measurement on Helios, the  
395 concentration of cells was set to  $0.5 \times 10^6$ /ml in cell acquisition solution (CAS,  
396 201240 Fluidigm) supplemented with 10% EQ Calibration Beads (201078 Fluidigm).  
397 Cells were filtered (30 µm, 04-0042-2316 Celltrics, Sysmex Partec, Görlitz,  
398 Germany) prior to acquisition. Samples were run on CyTOF (cytometry by  
399 time-of-flight) Helios (Fluidigm). Bead based normalization of CyTOF cytofddata was  
400 performed. After randomization, normalization and FCS file generation the files were  
401 further analyzed in Cytobank (Beckman Coulter, Brea, CA, USA). Analysis of the  
402 cells was carried out on live singlets within the pan-hemocyte marker, H2 positive  
403 population. The viSNE (visualization of stochastic neighbour embedding) analysis  
404 was carried out on  $3 \times 10^4$  cisplatin negative (live) singlets with the following  
405 settings: iterations = 1000, perplexity = 30, theta = 0.5).

#### 406 **Authors' contributions**

407 JAB carried out the mass cytometric experiments, analysis and visualization  
408 VH participated in *Drosophila* work, drafted the manuscript and supervised the  
409 analysis  
410 EK produced and affinity purified the antibodies, carried out flow cytometric  
411 experiments, prepared graphs and supervised the analysis, and revised the manuscript  
412 LGP supervised the study and revised the manuscript  
413 IA provided the antibodies, supervised the study, and revised the manuscript  
414 GJS designed and supervised the study, designed the experiments and analysis,  
415 prepared the figures, drafted the manuscript.

416 The authors read and approved the final version of the manuscript.

### 417 **Competing interests**

418 The authors have declared no competing interests.

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559 decreased proportion of non-classical monocytes and increased proportion of Th17  
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561

## 562 **Figure legends**

563 **Figure 1 Single cell mass cytometry revealed the expansion of hemocytes in**  
564 ***hop<sup>Tum</sup>* and *l(3)mbn<sup>1</sup>***

565 (A) The percentage of H3, H18, L1, L2, L4, L6, P1, and 3A5 cells were plotted on  
566 radar plots for *Drosophila* mutants on *Ore-R* or *w<sup>1118</sup>* background. (B) Comparative  
567 heatmap of mass cytometry data (arcsinh-transformed median intensity values)  
568 regarding marker density at single cell resolution show increased expression of H18,  
569 L1, L2, L4 markers in the mutant *hop<sup>Tum</sup>* and *l(3)mbn<sup>1</sup>* in relation to control, the wild  
570 type *Ore-R*. Analysis was performed within the H2 (Hemese) positive live singlets.

571

572 **Figure 2 Multidimensional comparative analysis by the tSNE algorithm**  
573 **dissects the cell relatedness of 5 different *Drosophila* strains, namely *Ore-R*, *w<sup>1118</sup>*,**  
574 ***l(3)mbn<sup>1</sup>*, *hop<sup>Tum</sup>* and *hdc<sup>484</sup>***

575 The wild type *Ore-R* and *white* mutant  $w^{1118}$  (genetic backgrounds) are overlapping  
576 while both tumorous strains  $l(3)mbn^1$  and  $hop^{Tum}$  represent H18, L1, L2, L4 expansion.  
577 The tSNE analysis of H3, H18, L1, L2, L4, L6, P1, and 3A5 markers was carried out  
578 within the population of pan-hemocyte H2 (Hemese) positive live singlets and  
579 visualised as viSNE plots. Subpopulations of cells with common marker expression  
580 patterns are grouped close in the multidimensional space, while cells with different  
581 marker expression are plotted separately. Coloration is proportional with the intensity  
582 of the expression of a given marker: the hotter the plot, the higher the level of  
583 expression (red plots). Red boxes mark transitional phenotypes expressing both  
584 lamellocyte (L2 or L4) and plasmatocyte (P1) markers.

585

586 **Figure 3 Merging viSNE graphs (based on H3, H18, L1, L2, L4, L6, P1, and**  
587 **3A5 marker expression within the pan-hemocyte H2 (Hemese) positive live**  
588 **singlets) outlines characteristic maps of each strain (green = *Ore-R*, blue =  $w^{1118}$ ,**  
589 **red =  $l(3)mbn^1$ , lilac =  $hop^{Tum}$ , yellow =  $hdc^{A84}$ ) based on high parametric mass**  
590 **cytometry data**

591 (A) The viSNE comparison of  $l(3)mbn^1$  and its wt counterpart, the *Ore-R*. (B) The  
592 viSNE comparison of  $w^{1118}$ ,  $hop^{Tum}$ , and  $hdc^{A84}$ . (C) The viSNE islands of the control  
593 cells (*Ore-R* and  $w^{1118}$ ) localize separately from the tumorous  $l(3)mbn^1$  and  $hop^{Tum}$   
594 hemocytes while  $hdc^{A84}$  represents a transition phenotype.

595

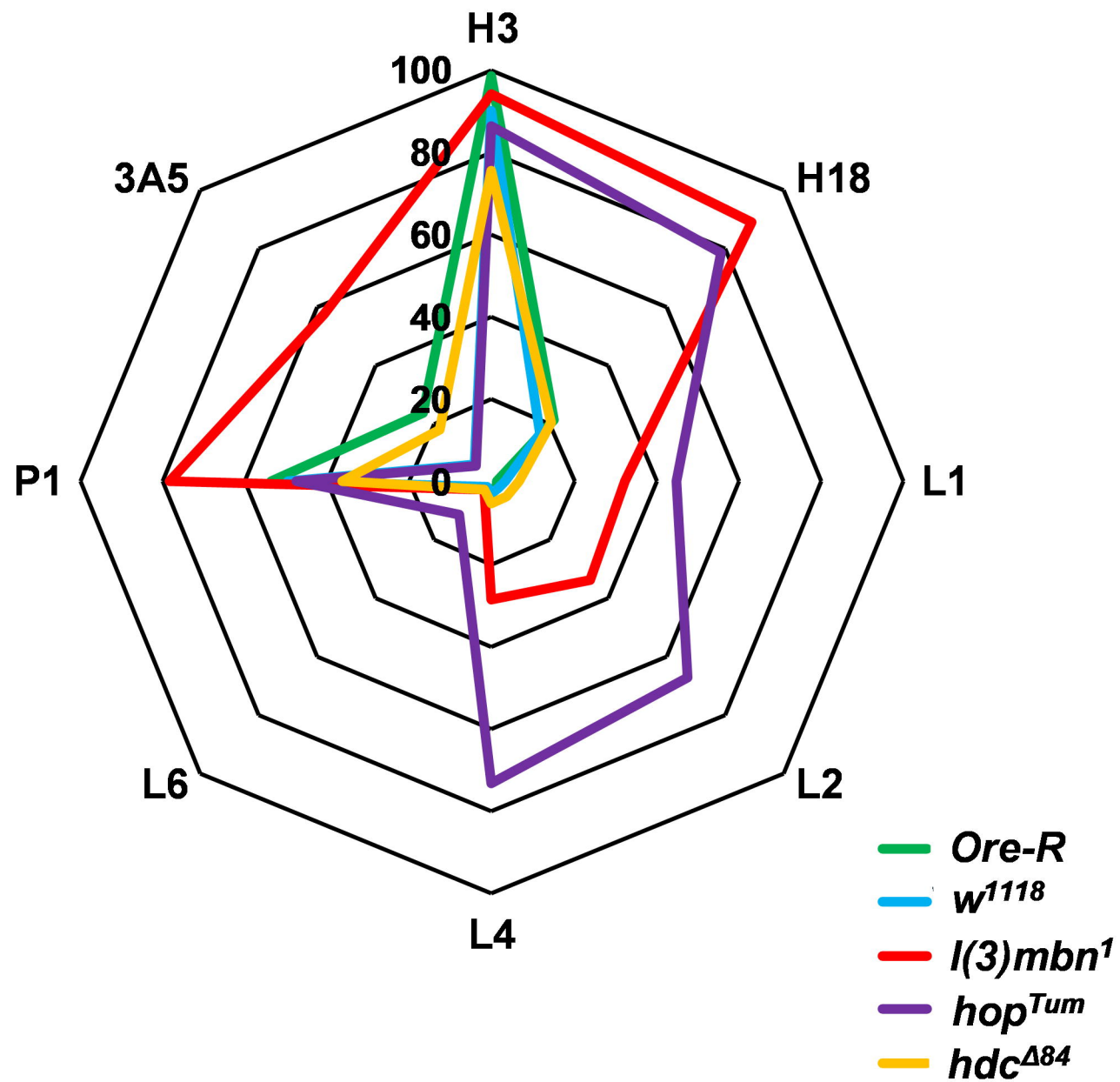
596 **Figure 4 Immune activation was monitored successfully by infestation with the**  
597 ***Leptopilina boulardi* parasitoid wasp of the *lozenge>GFP* strain**

598 (A) viSNE analysis of naive ( $lz>GFP$ ) and immune induced ( $lz>GFP$  *i.i.*) *Drosophila*  
599 larvae. The tSNE analysis of H3, H18, L1, L2, L4, L6, P1, 3A5 markers and anti-GFP  
600 (marking crystal cells in this particular system) was carried out within the population  
601 of pan-hemocyte H2 (Hemese) positive live singlets. Red boxes mark a  
602 subpopulation, the transitional phenotype of hemocytes expressing both lamellocyte  
603 (L4+) and plasmatocyte (P1) markers upon immune induction. (B) The percentage of  
604 H3, H18, L1, L2, L4, L6, P1, anti-GFP (crystal cells), and 3A5 positive cells. (C) The  
605 heatmap of the (arcsinh-transformed) median values shows the expression changes of  
606 the hemocyte marker expression upon immune induction. Analysis was performed  
607 within the pan-hemocyte marker H2 (Hemese) positive live singlets.

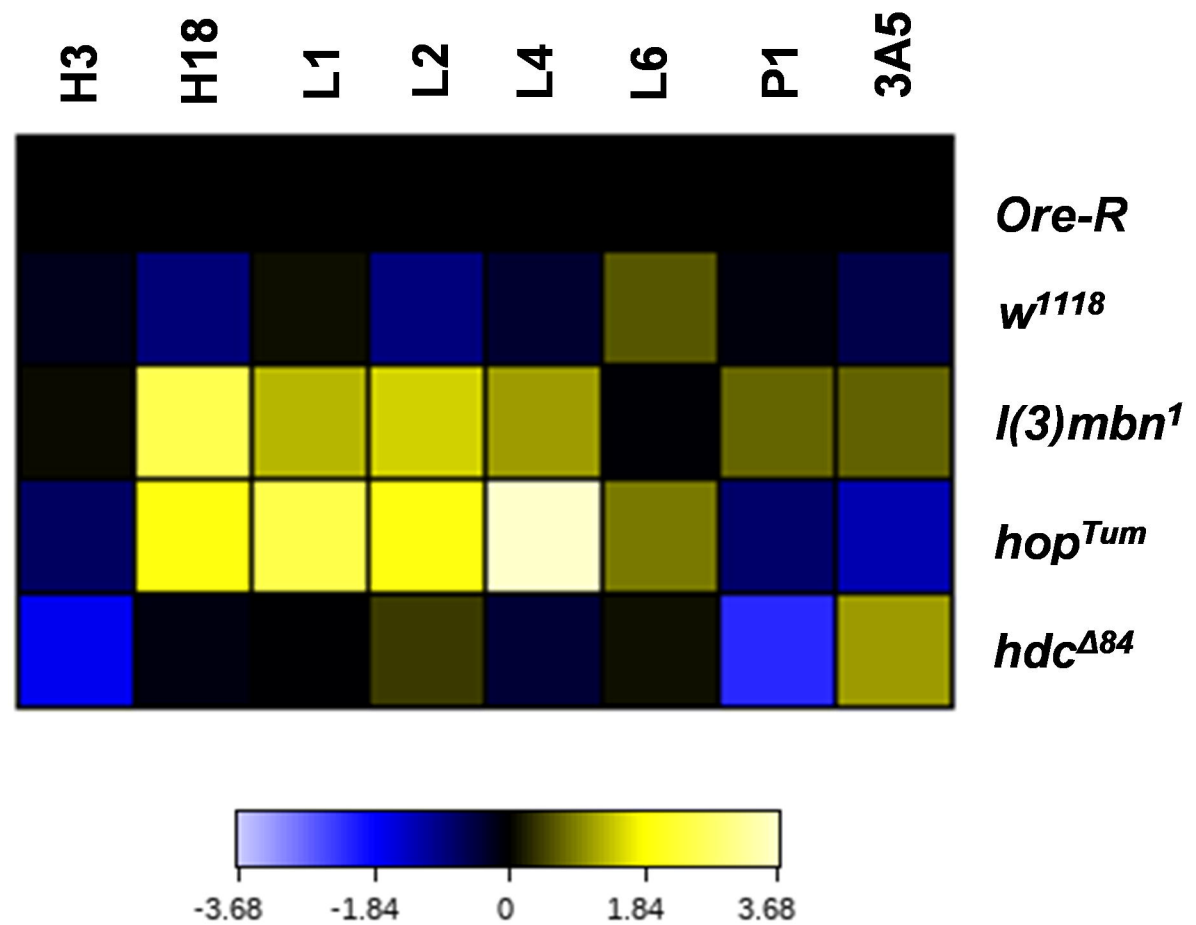
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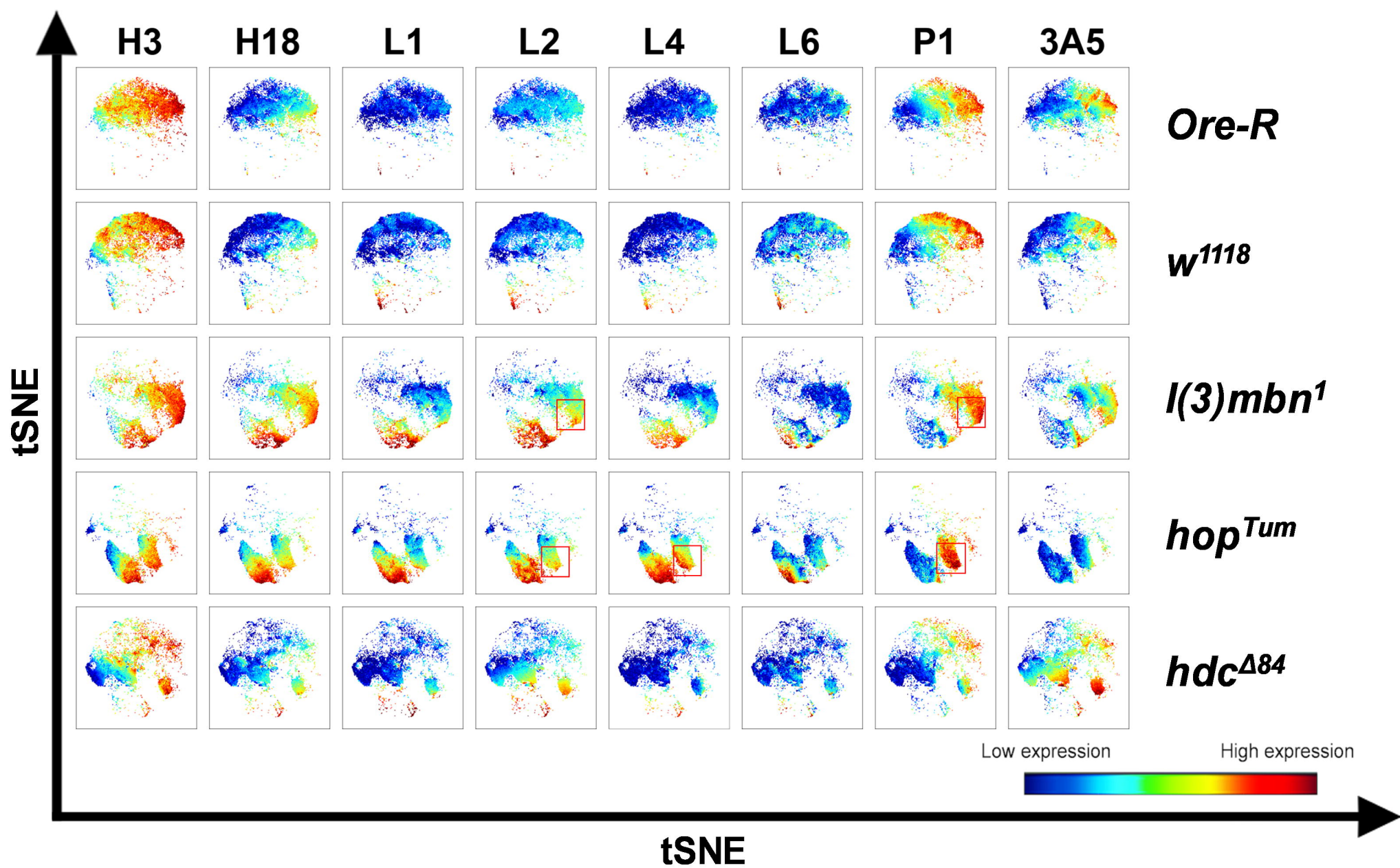
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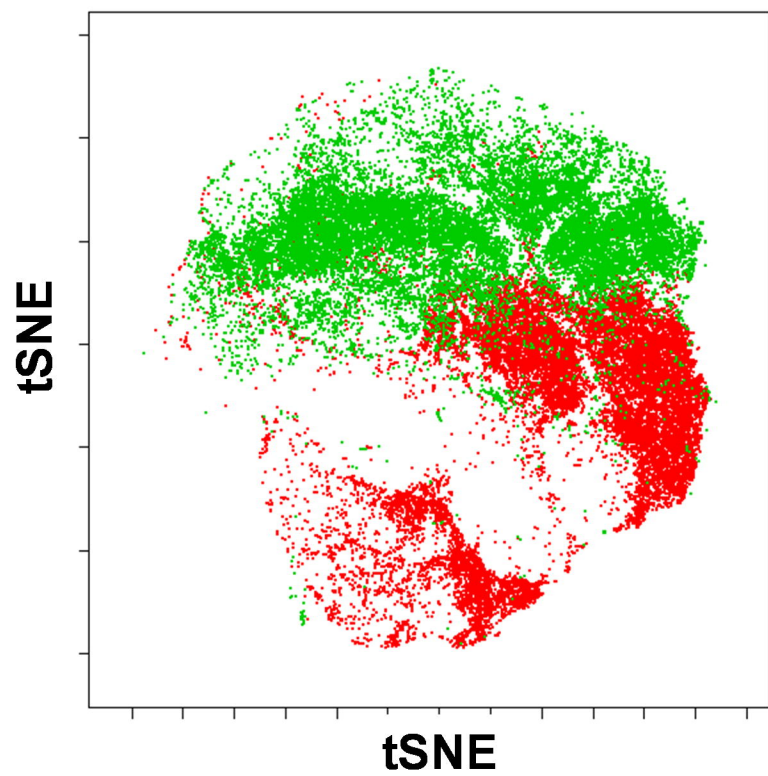
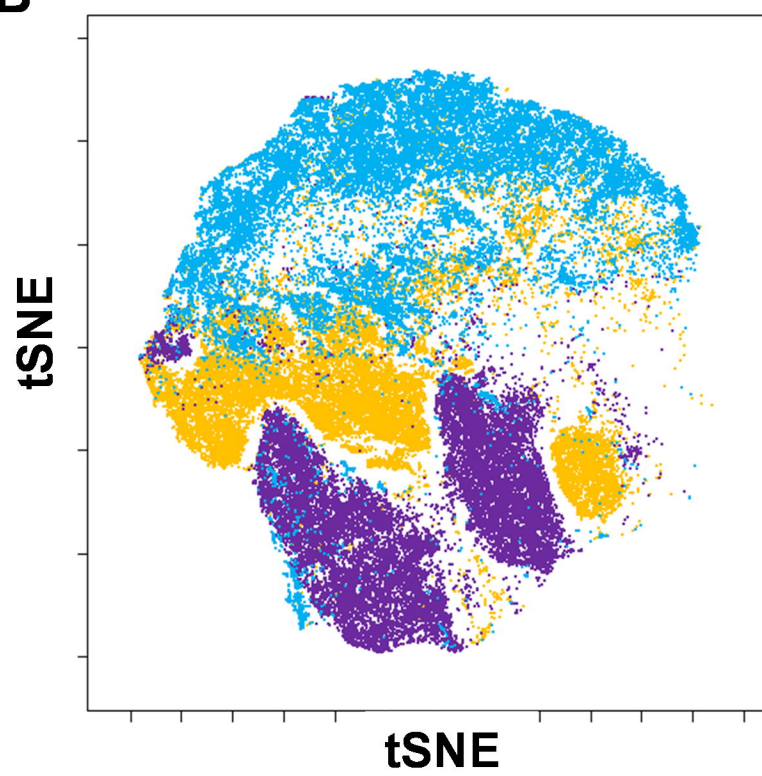
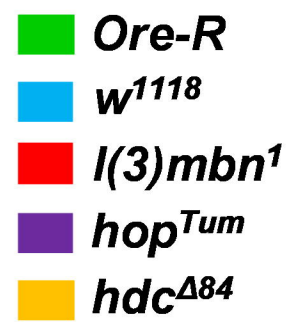
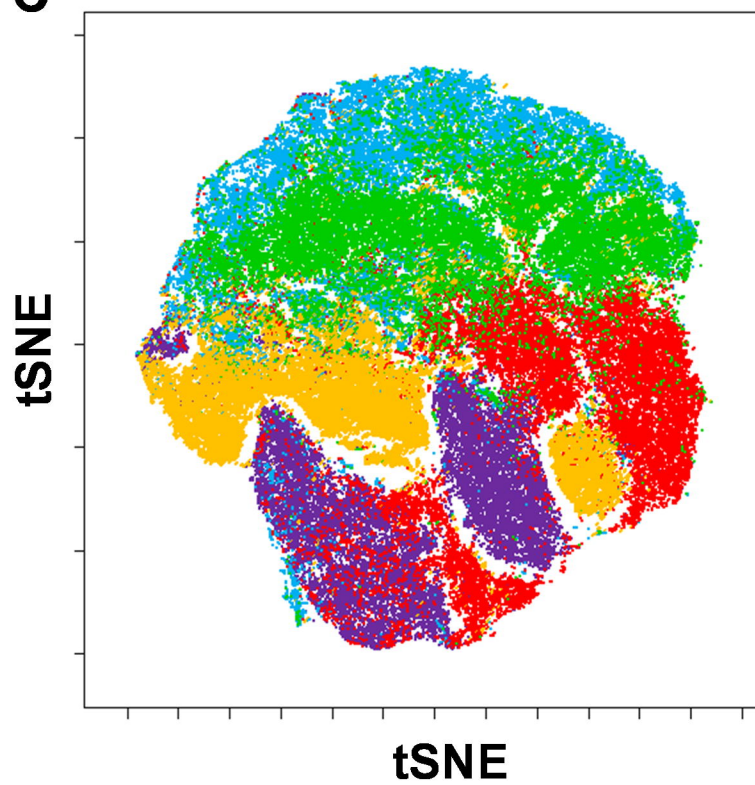
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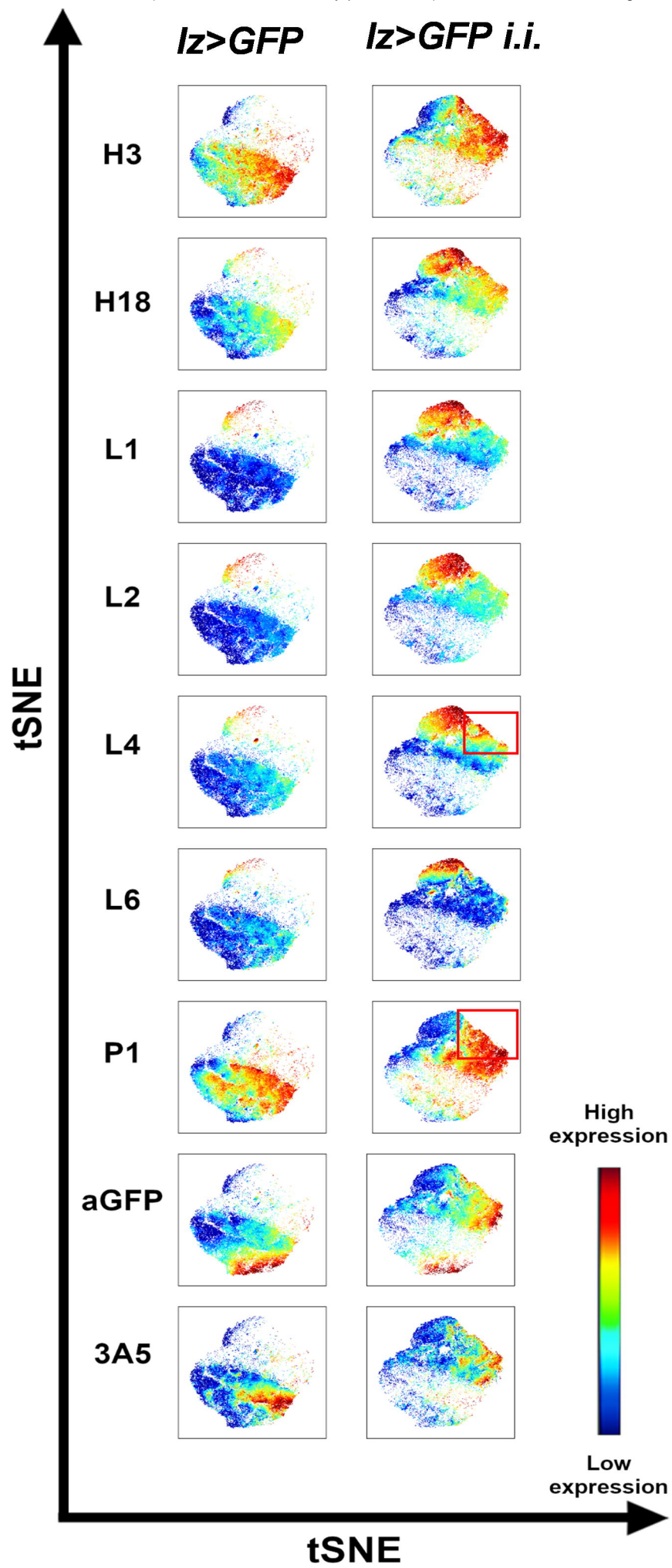
B



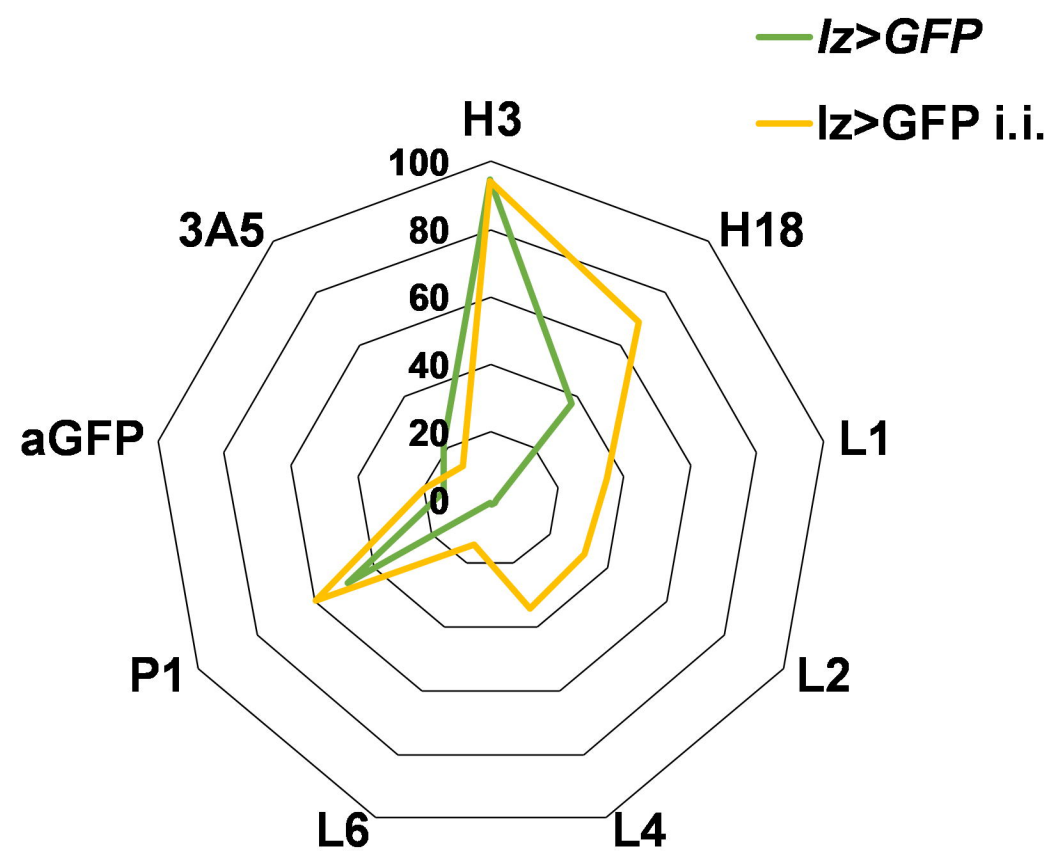


**A****B****C**

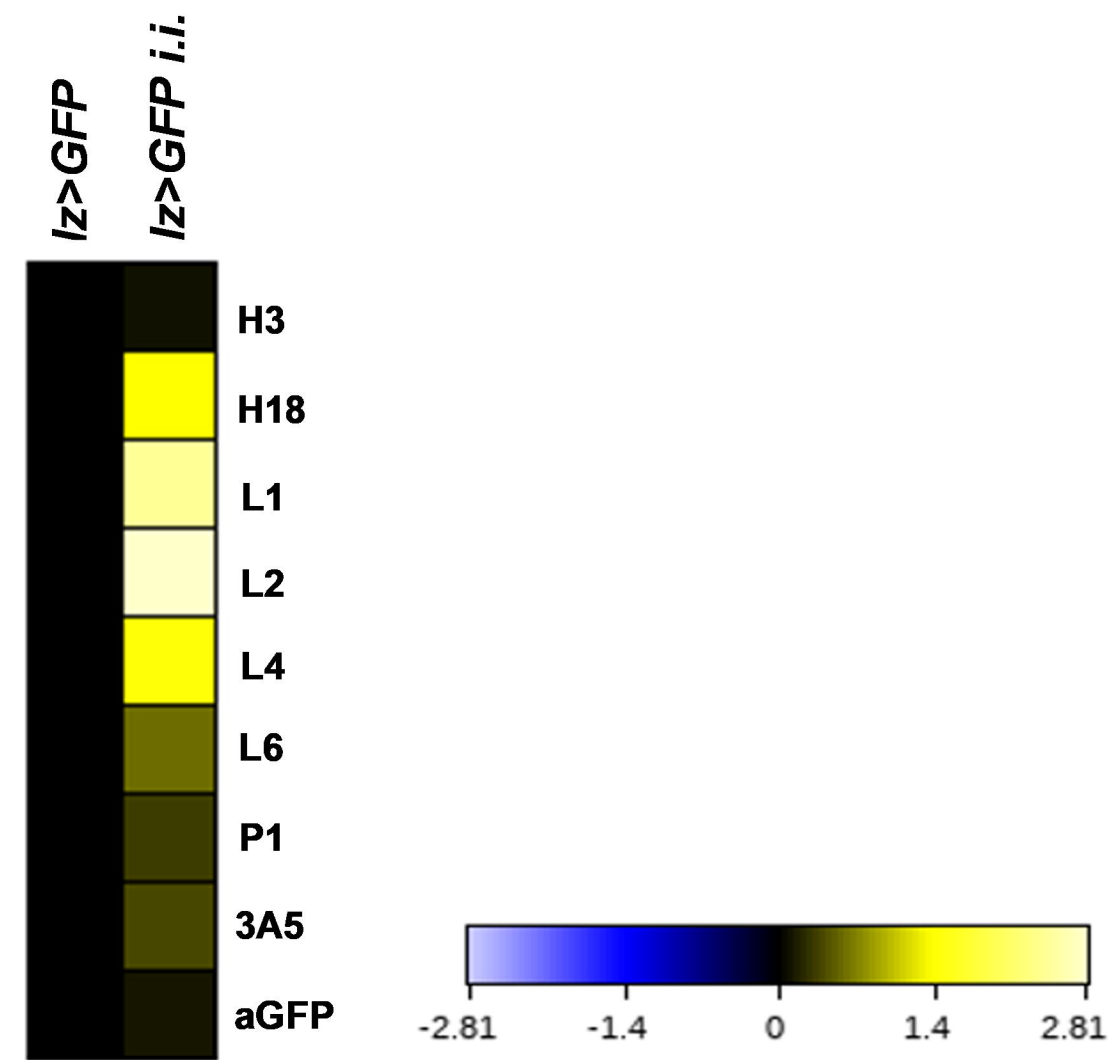
**A**



**B**



**C**





**Table 1 List of the antibodies used for mass cytometry**

<b>Marker</b>	<b>Clone</b>	<b>Isotype</b>	<b>Metal tag</b>	<b>References</b>
H2 (Hemese)	1.2	mouse IgG2a	147 Sm	12, 14
H3	4A12	mouse IgG1	155 Gd	14
H18 (Tetraspanin42Ed)	H18	mouse IgG1	164 Dy	-
L1 (Atilla)	H10	mouse IgG1	149 Sm	14, 15, 23
L2	31A4	mouse IgG2a	158 Gd	14, 23
L4 (Integrin beta-PS)	1F12	mouse IgG1	159 Tb	14, 23
L6 (IgM)	H3	mouse IgM	-	14, 23
anti-IgM	RMM-1	rat IgG2a	172 Yb	42
P1 (NimC1)	N47	mouse IgG1	154 Sm	13, 14, 21
3A5	3A5	mouse IgG2b	169 Tm	-
anti-GFP	-	rabbit polyclonal IgG	175 Lu	43
anti-CD45	HI30	mouse IgG1	89 Y	44