

1 **Spatiotemporal mapping of gene expression landscapes and**
2 **developmental trajectories during zebrafish embryogenesis**

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30 **SUMMARY**

31 Vertebrate embryogenesis is a remarkably dynamic process during which numerous
32 cell types of different lineages generate, change, or disappear within a short period of
33 time. A major challenge in understanding this process is the lack of topographical
34 transcriptomic information that can help correlate microenvironmental cues within the
35 hierarchy of cell fate decisions. Here, we employed Stereo-seq, a high-definition
36 spatially resolved transcriptomic technology, to dissect the spatiotemporal dynamics of
37 gene expression and regulatory networks in the developing zebrafish embryos. We
38 profiled 91 embryo sections covering six critical time points during the first 24 hours of
39 development, obtaining a total of 139,391 spots at cellular size ($\sim 100 \mu\text{m}^2$) with spatial
40 coordinates. Meanwhile, we identified spatial modules and co-varying genes for
41 specific tissue organizations. By performing the integrative analysis of the Stereo-seq
42 and scRNA-seq data from each time point, we reconstructed the spatially resolved
43 developmental trajectories of cell fate transitions and molecular changes during
44 zebrafish embryogenesis. We further investigated the spatial distribution of ligand-
45 receptor pairs for major signaling pathways and identified novel interactions that
46 potentially crosstalk with the Notch signaling pathway during zebrafish development.
47 Our study constitutes a fundamental reference for further studies aiming to understand
48 vertebrate development.

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50 Key words: Stereo-seq; spatial transcriptomics; scRNA-seq; embryonic development;
51 zebrafish

52

53 INTRODUCTION

54 Vertebrate embryogenesis is an intricate and dynamic process with intense gene
55 expression changes and frequent cell state transitions within short time windows.
56 Extrinsic and intrinsic cues, including transcription factors (TFs), morphogens,
57 signaling pathways, and signals from the extracellular matrix (ECM), play pivotal roles
58 in determining different cell fates which present distinct morphologies, spatial positions
59 and functions (Bardot and Hadjantonakis, 2020; Marlow, 2020; Vining and Mooney,
60 2017). How these regulatory factors spatially interact and function together to induce
61 a complex vertebrate embryo in a precisely controlled manner is one of the
62 fundamental questions about embryogenesis that demands further investigation. In
63 particular, understanding how ligand-receptor pairs spatially interact to switch on/off
64 specific signaling pathways, such as Notch, Wnt, SHH and TGF- β during vertebrate
65 embryogenesis is crucial but poorly documented.

66 The zebrafish is a widely-used model organism for studying vertebrate embryonic
67 development thanks to its fast development, embryonic transparency, and accessibility
68 to both physical and genetic manipulation. The advances in sequencing technologies
69 have made possible the assembly of single-cell atlases of model organisms during
70 development, which enables genome-wide profiling of multimodal information
71 including gene expression, epigenetic state, and protein level in individual cells (Cao
72 et al., 2017; Han et al., 2020; Karaiskos et al., 2017; Stelzer et al., 2015; The Tabula
73 Muris Consortium et al., 2018; Trevino et al., 2020). Efforts have been made to map
74 the gene expression landscapes during zebrafish embryogenesis and developmental
75 trajectories have been constructed, which defined the transcriptomic states of cells as
76 they acquire their fates (Briggs et al., 2018; Farrell et al., 2018; Wagner et al., 2018).
77 However, how the time-course transcriptomic states correlate with each other in the
78 background of spatial localization in a complex developing zebrafish embryo remains
79 elusive and the spatial organization of different cell types in complex tissues remains
80 poorly understood due to the limitation of current spatial transcriptomic technologies
81 (Li et al., 2021; Liu et al., 2020; Rodrigues et al., 2020; Stickels et al., 2021; Vickovic
82 et al., 2019).

83 Based on the traditional *in situ* hybridization technology, a representative database
84 of gene expression in the developing zebrafish embryos and larvae (The Zebrafish
85 Information Network (ZFIN)) (Sprague, 2003) is available for scientists to inquire the
86 expression pattern of a specific gene, but the *in situ* images lack cell type information
87 and the global changes of transcripts cannot be explored. A spatial transcriptomic
88 technology named Tomo-seq has been applied in zebrafish embryos, but this
89 technology is based on bulk sequencing of cryosections and the spatial information on
90 the tissue sections is missing (Holler et al., 2021; Junker et al., 2014). Here, we
91 adopted the newly developed SpaTial Enhanced REsolution Omics-sequencing
92 (Stereo-seq) (Chen et al., 2021) to dissect the spatiotemporal gene expression
93 landscapes with high resolution and sensitivity during the first day of zebrafish embryo
94 development. Our study provides important data resources for the research of gene

95 expression, cellular organization and regulatory networks during zebrafish
96 embryogenesis.

97 **RESULTS**

98 **Generation of high-resolution spatiotemporal transcriptomic data by Stereo-seq** 99 **in zebrafish embryos**

100 Zebrafish embryos of different stages (3.3 hours post-fertilization (hpf), 5.25 hpf, 10
101 hpf, 12 hpf, 18 hpf, 24 hpf) were harvested for spatial transcriptomic analysis (Figure
102 1A, top). Embryos were dechorionated and embedded in optimal cutting temperature
103 (OCT) compound for the preparation of sagittal cryosections with a thickness of 15 μm
104 (close to cellular size). High resolution Stereo-seq chips (spot size 220 nm, center-to-
105 center size 715 nm, chip size 1 cm^2) were used for the capture of RNA transcripts
106 (Figure 1A, bottom). In order to avoid incomplete sampling of cell types and batch
107 effects, we obtained multiple sections of one embryo at each stage for Stereo-seq.
108 Taking 24 hpf as an example, 17 sections of the same embryo were attached on two
109 1 cm^2 chips and subjected to Stereo-seq (Figure 1B-1C). We applied a grid-based
110 strategy, which aggregates a number of DNA nanoball (DNB) spots, to cluster the
111 spatial signature into substructures (see Methods). As expected, by comparison of
112 spatial clustering with different grid (bin) size ranging from 143 μm (bin 200, 200 \times 200
113 DNB) to 10 μm (bin 15, 15 \times 15 DNB, equivalent to \sim 1 cell diameter), we found more
114 refined clustering with increased cluster number and accuracy was achieved by
115 employing a higher-resolution bin (Figure 1D). Meanwhile, the number of transcripts
116 and genes detected among different sections of the 24 hpf embryo were comparable
117 (Figure 1E-1F), and we recovered a median of 1250 unique molecular identifiers (UMIs)
118 and 394 genes with bin 15 (Figure S1A). The number of embryo sections and the
119 average numbers of UMIs and genes were overall sufficient for further analysis across
120 all the 6 developmental stages (Figure 1G and S1A).

121 Thus, our Stereo-seq with multiple-section strategy enables high-resolution and
122 comprehensive spatial transcriptomic analysis of small-sized samples such as
123 zebrafish embryos.

124 **Spatial clustering and molecular characterization of the zebrafish** 125 **embryogenesis spatiotemporal transcriptomic atlas**

126 Stereo-seq with multiple-section strategy at cellular size resolution (bin 15) generated
127 high-definition spatial transcriptomic data from developing zebrafish embryos, which
128 can hardly be profiled by other available technologies due to a much lower density of
129 signatures. This allowed us to create a high-quality Zebrafish Embryogenesis
130 Spatiotemporal Transcriptomic Atlas (ZESTA).

131 We pooled all the sections of each stage for unsupervised clustering (see
132 Methods), and revealed spatial heterogeneity of the embryos and spatial specification
133 dynamics of different regions during development (Figure 2A). As Stereo-seq captured

134 a high density of signals, we were able to identify more elaborate structures with
135 deeper clustering (Figure 2B and S1B-S1G). Some structures can be further divided
136 into detailed substructures. For example, the brain components from the Stereo-seq
137 data of the 24 hpf embryo could be further divided into subdivisions such as
138 'telencephalon' and 'dorsal diencephalon' (Figure 2C). Similarly, the eye can be divided
139 into 'optic lens' and 'pigment-epithelium' (Figure 2C). On the other hand, the spinal
140 cord, floor plate, and notochord are adjacent interacting structures and could be
141 separated precisely on the embryo sections by specific expression of genes including
142 *prdm8*, *ntn1b* and *ntd5*, respectively (Figure 2C). Gene expression kinetics across all
143 stages revealed restricted expression at specific time points and anatomic regions, as
144 exemplified by erythroid lineage cell (*hbbe1.1*), myotome (*tnnc2*), yolk syncytial layer
145 (YSL) (*apoa1a*) and hatching gland (*he1.1*) (Figure 2D). Genes of early embryogenesis,
146 i.e., *pou5f3* and *s100a1* showed specific expression at early stages, consistent with
147 known expression patterns identified by classic *in situ* hybridization experiments
148 (Figure 2D) (Diks et al., 2006; Onichtchouk and Driever, 2016).

149 Taken together, our Stereo-seq constituted a unique resource of ZESTA with
150 unprecedented resolution, and the publicly available interactive data portal can be
151 accessed at http://stereomap.cngb.org/zebrafish/data_index.

152 **Spatial gene modules uncover the interaction of different spatial regions**

153 The spatial patterns of gene expression depict different functional regions across
154 embryo sections. In order to identify co-varying genes showing similar spatial
155 distribution, we applied Hotspot (DeTomaso and Yosef, 2021) to identify gene modules
156 on our Stereo-seq dataset. We identified 12 spatial modules for the 24 hpf embryo
157 sections. Gene Ontology (GO) enrichment analysis on the spatially correlated genes
158 of each module confirmed the functions of these spatial modules (Figure 3A), which
159 also showed consistency with the spatial clusters (Figure 3B). Spatial visualization of
160 modules showed that the distribution of spatial modules reasonably matched their
161 region-specific biological functions (Figure 3A and 3C). For example, module 7 (M7,
162 posterior erythroid lineage cell) gathered genes involved in oxygen transport, and M8
163 (eye) was abundant in genes related to lens development. For the other developmental
164 stages, we identified 8 spatial modules for 10 hpf, 11 for 12 hpf and 16 for 18 hpf
165 (Figure S2A, S2D and S2G). Similarly, the GO function enrichment, spatial location
166 and cell types reasonably matched with each other for most spatial modules (Figure
167 S2B-C, S2E-F and S2H-I).

168 Interestingly, we found that a single gene module can correspond to multiple
169 subregions. For instance, M9 in 24 hpf embryo spread across various clusters
170 including neural crest, erythroid lineage cell, epidermis, and eye (Figure 3B and 3D).
171 GO enrichment of this module revealed functions related to the development of
172 pigment cells, mesenchymal cells, and sensory organ (Figure 3E). In order to explore
173 the connection among these three cell types/organs, STRING was employed to seek
174 potential gene interaction which was visualized with Cytoscape (Figure 3F). Intriguingly,
175 we found that the transcription factor gene *sox10* intensively interacts with genes from

176 these three cell types/organs, consistent with the reported function of *sox10* (Rocha et
177 al., 2020). As expected, projection of specific genes from pigment cell, sensory organ,
178 and mesenchymal cell on the same embryo section revealed spatial neighboring
179 distributions (Figure 3G).

180 The notochord provides directional signals to the surrounding tissues during
181 development through secreting sonic hedgehog (SHH), a key morphogen regulating
182 organogenesis (Corallo et al., 2015; Male et al., 2020). To investigate the interaction
183 of notochord with the neighboring regions, we constructed the interaction network of
184 genes in M6 (notochord (Figure 3A and 3H, right)) and the neighboring modules, M1
185 and M5 which correspond to the musculature system and the spinal cord respectively
186 (Figure 3A and 3H, left). Within the potential interacting genes revealed by STRING,
187 we found that *myod1* and *hhatlb* in M1 showed interaction with *shha* and *shhb* in M6.
188 Previous studies have found that *shh* plays a role in muscle development and adult
189 myogenesis by regulating the activity of *myod1* (Voronova et al., 2013), while to the
190 best of our knowledge, *hhatlb* has been rarely reported to interact with *shh*. On the
191 other hand, *fgf13* and two other neural stem cells maker genes (*gfap* and *pou4f2*) in
192 M5 show interaction with *shha/b*. The interaction of these genes was further confirmed
193 by their tightly adjacent characters of spatial distribution (Figure 3J).

194 In summary, spatial modules depict the functional regions or subregions in an
195 organism, provide a powerful tool to explore the genetic interaction between different
196 cell types, and serve as a reliable resource to discover novel function as well as
197 unknown interactions of genes.

198 **Construction of spatially resolved developmental trajectories by integrated** 199 **analysis of scRNA-seq and Stereo-seq data**

200 Both our Stereo-seq data and previously published scRNA-seq data indicate that
201 developmental time is a strong source of variation mainly due to active transition
202 between different cell states during embryogenesis. In order to construct the
203 spatiotemporal developmental trajectories of embryonic cells, we further performed
204 droplet based scRNA-seq (Liu et al., 2019) (see Methods) with zebrafish embryos at
205 the same developmental time points as in the Stereo-seq profiling (Figure 4A). We
206 obtained 86,307 cells from embryos of the 6 developmental stages and identified 71
207 cell types, which are mostly consistent with previous studies (Farrell et al., 2018;
208 Wagner et al., 2018) (Figure 4A and S3A-S3H).

209 At 3.3 hpf, the emergence of two cell types was observed, for which we used
210 Monocle 2 to undertake pseudo-chronological analysis to determine their differential
211 identities. Based on the pseudo-chronological relationship, we determined the two cell
212 types as early blastodisc and late blastodisc respectively (Figure S4B). To construct
213 the developmental trajectory, we integrated scRNA-seq data of adjacent time points
214 using Harmony (Korsunsky et al., 2019) and applied KNN mapping to predict the
215 developmental fate of each cell of the earlier time point (see Methods). We then
216 projected the developing trajectory using the Sankey diagram (Figure 4B).

217 Although single-cell studies have mapped the cell types and their molecular
218 features during vertebrate embryogenesis, the dynamics of the spatial organization of

219 cells is still poorly understood (Farrell et al., 2018; Wagner et al., 2018). To introduce
220 spatial information to the cell fate lineages, we conducted an integrative analysis with
221 the combination of the scRNA-seq and Stereo-seq data by applying SPOTlight
222 (Elosua-Bayes et al., 2021) to calculate the cell composition of each bin in the Stereo-
223 seq data, which allowed projection of the developmental trajectories to the embryo
224 sections (Figure 4C and S4A-S4B). These results revealed the spatial dynamics of
225 different developmental trajectories. Taking the two developmental branches of the
226 presumptive ectoderm as an example, the central nervous system branch and the
227 pigment cell branch both developed from the presumptive ectoderm which appeared
228 at 5.25 hpf with a discrete distribution pattern, and the two branches started to be
229 distinguished at 10 hpf. The central nervous system branch showed an anteriorly
230 concentrated pattern starting at 10 hpf and enriched in the brain region with some
231 leakage at the spinal cord. However, the pigment cell branch remained discrete on the
232 section at 10 hpf while appeared to gather at the anterior part of the embryo section at
233 12 hpf when the neural crest cell fate was evident and subsequently differentiated into
234 pigment cells (Figure 4C and S4A).

235 A prominent phenomenon of the developmental trajectory is that some cell types
236 bifurcate into multiple branches at certain time points (Figure 4D and S4C). We noticed
237 that the distributions of some developmental directions seem to be already spatially
238 separated and distinguishable in the progenitor cell type (Figure 4E, top and S4D, top).
239 We integrated the single-cell transcriptomic data and applied SCENIC (Aibar et al.,
240 2017) algorithm to predict TFs with high activity at each developmental directions (see
241 Methods) (Figure 4E, middle and S4D, middle). For example, different TFs were
242 identified to regulate the segregated developmental directions of the 12 hpf lateral plate
243 mesoderm, which gives rise to the cardiovascular system, erythroid lineage cell and
244 endothelial cell. The *nkx2.5* gene is predicted as crucial TFs for the development of
245 the cardiovascular system, while *cdx4* for erythroid lineage cell (Figure 4E, left).
246 Similarly, different TFs were identified for the separate developmental lineages of the
247 18 hpf neural crest (Figure 4E, right). We visualized the regulatory activities of these
248 key TFs by displaying the target gene expression on embryo sections, for which the
249 distribution of target genes is largely consistent with that of the corresponding cell type
250 (Figure 4E, bottom and S4D, bottom).

251 As described above, our spatiotemporal developmental trajectories revealed the
252 dynamic cell fate transitions with anatomical annotation during zebrafish early
253 embryogenesis, and can be used to investigate potential regulatory TFs for a specific
254 developmental direction.

255 **Dynamic spatiotemporal distribution of ligand-receptor pairs during zebrafish** 256 **embryogenesis.**

257 The extrinsic cues, including the interaction between ligands and receptors, are crucial
258 for the regulation of cell fate during embryogenesis. We, therefore, calculated the
259 relative distance of different ligand-receptor pairs at all six developmental stages with
260 the distance of adjacent bins (Figure 5A and S5A-S5B). Considering the dropout in the
261 transcriptomic data, we define that the ligand and receptor have strong potential to

262 interact when their average relative distance was less than 5 bins at least at one time
263 point. Combining the relative distance and expression strength, we illustrated the
264 dynamics of the ligand-receptor interactions during zebrafish embryonic development
265 (Figure 5B). We observed an increasing number of receptor-ligand pairs that come to
266 interact as the embryo develops. Moreover, the expressions of most active ligand-
267 receptor pairs were upregulated and the distances became smaller.

268 The Notch signaling pathway is conserved in most vertebrates and plays an
269 essential role in cell fate determination through lateral inhibition (Andersson et al.,
270 2011). We graphed the distance heat map of the Notch ligand-receptor pairs at different
271 developmental stages and found that multiple pairs showed time-dependent increase
272 in proximity (Figure S5C). Then we explored the dynamic spatial distribution of the
273 Notch components at all six time points, which revealed that the Notch receptors and
274 ligands (*delta*, *jagged*) were sparsely expressed at 3.3 hpf and 5.25 hpf, and the
275 expression was increased at 10 hpf without any obvious pattern. Higher Notch ligand-
276 receptor proximity was observed at the somite forming site on 12 hpf sections. The
277 expression of both ligands and receptors showed wider distribution but was enriched
278 in the brain and the somite at 18 hpf, and was mainly concentrated in the nervous
279 system at 24 hpf (Figure 5C, top). To illustrate the spatial ligand-receptor interactions
280 of Notch signaling, we selected the receptor-expressing bins with a relative distance
281 less than 2 bins to the ligands, plotted them together with the nearest ligand-expressing
282 bins on the embryo sections (Figure 5C, middle), and illustrated the corresponding cell
283 clusters (Figure S5D). We further analyzed Notch activities by mapping the expression
284 of downstream target genes (Figure 5C, bottom), which is consistent with the spatial
285 expression dynamics of Notch ligand-receptor pairs. Our results revealed a highly
286 dynamic spatial distribution of Notch components expression and Notch activity during
287 zebrafish embryogenesis.

288 We further explored gene modules that are spatially correlated with Notch
289 signaling pathway using Hotspot analysis on the Stereo-seq data of 12 hpf, 18 hpf, and
290 24 hpf zebrafish embryos (Figure 5D-5F) (see Methods). At 12 hpf, one Notch
291 correlated spatial module is identified with distribution in the somite-forming region and
292 contains genes such as *myf5*, *unc45b* and *hsp90aa1.1* which are functionally
293 associated with muscle development (Figure 5D). Two Notch correlated spatial
294 modules showed up at 18 hpf (Figure 5E). M1 gathers in the somitogenesis region and
295 is abundant in genes that function in organ development, collagen fibril organization
296 and anterior-posterior pattern specification such as *shha*, *cdx4* and *col2a1a*. M2 is
297 concentrated in the central nervous system comprising genes that are important in
298 neurogenesis and brain development, *i.e.* *gfap* and *neurod4*, which regulate
299 gliogenesis and neuronal differentiation respectively (Fukuoka et al., 2021). At 24 hpf,
300 the Notch correlated spatial modules are also enriched in the nervous system (Figure
301 5F). M1 contains genes such as *pcna*, *mcm5* and *nova2*, which are related to cell cycle,
302 RNA splicing and DNA metabolism, indicating that the dominant role of this module is
303 to regulate the development of neural progenitor cells (Ino and Chiba, 2000; Yano et
304 al., 2010). In contrast, genes involved in M2 such as *dpysl3*, *cxcr4b*, mainly function in
305 axonogenesis and neuronal projection development. Consistent with spatial

306 expression dynamics of Notch ligand-receptor pairs, correlated spatial modules
307 showed that the Notch correlated genes were weak and enriched at the somitogenesis
308 region at 12 hpf, then distributed widely but were enriched in the brain and at the
309 somitogenesis region at 18 hpf, and were concentrated in the nervous system at 24
310 hpf (Figure 5D-5F). In order to identify candidate genes that directly or indirectly
311 interact with the Notch pathway at each developmental stage, we further characterized
312 the co-varying tendency of module genes relative to the Notch pathway at 12 hpf, 18
313 hpf and 24 hpf (Figure 5G). Therefore, the spatial gene modules showed co-varying
314 expression patterns and functional correlation with Notch signaling pathway at specific
315 time points, which indicates that the genes involved can very likely interact with Notch
316 signaling, either directly or indirectly, to play an important role in the development of
317 defined cell types.

318 We thereby demonstrated a spatiotemporal expression atlas of various ligand-
319 receptor pairs and provided a useful database for the investigation of the dynamics of
320 different signaling pathways as exemplified by the comprehensive analysis on Notch
321 pathway.

322 **DISCUSSION**

323 The mapping of a spatial transcriptomic landscape at cellular resolution is essential for
324 understanding vertebrate embryogenesis. Due to the limitation of current spatial
325 transcriptomic technologies, there is a lack of high-resolution spatial transcriptomic
326 resources for the zebrafish embryos, a popular model organism for developmental
327 biology. The newly developed Stereo-seq enables the depiction of the spatial
328 transcriptomic landscape at cellular resolution with high sensitivity during zebrafish
329 embryogenesis (Chen et al., 2021). In addition, the flexible size of the Stereo-seq chip
330 allows us to simultaneously attach multiple sections on one chip, which can greatly
331 eliminate incomplete sampling of cell types and batch effects introduced by separate
332 experimental runs. The present study, for the first time, employed the Stereo-seq, and
333 drafted the dynamic spatiotemporal landscape of gene expression as well as spatial
334 regulatory factors at single-cell size resolution during the first day of zebrafish
335 embryonic development. Our high-resolution Stereo-seq data successfully
336 distinguished detailed anatomical structures such as the hypoblast, EVL, hatching
337 gland, and spinal cord at different developmental stages and identified organ
338 subdivisions such as the telencephalon, midbrain-hindbrain boundary, and dorsal
339 diencephalon of the brain. Besides, certain structures and gene expression patterns
340 can be further zoomed in by extracting the tissue-specific data from our online resource
341 to perform personalized analyses (http://stereomap.cngb.org/zebrafish/data_index).

342 Taking advantage of our Stereo-seq data, we identified spatial modules at different
343 developmental stages and integrated the spatial modules with spatial regions to
344 investigate key co-varying genes. Nevertheless, how the spatially correlated gene sets
345 interact and mutually regulate each other both inside and outside the modules to form
346 a regulatory network remains elusive, and unraveling it would improve our
347 understanding of the molecular mechanisms of vertebrate embryogenesis. For

348 example, the notochord provides directional signals to the surrounding tissue during
349 embryogenesis, in which SHH plays a critical role. Consistent with the known
350 knowledge, our analysis of the spatial modules showed that the notochord interacts
351 with the adjacent spinal cord and muscle through *shh*. In addition to *shh*, we found that
352 *cav3* in M6 could interact with the genes related to muscle contraction in M1. The Cavin
353 protein (including Cav1/Cav3) has been reported as essential for the development of
354 notochord and muscle (Lim et al., 2017). We, therefore, discovered the potential
355 interacting genes of *cav3* through analyzing the interaction of spatial module genes,
356 which will provide the rationale for further mechanistic investigations on the function of
357 *cav3* during organogenesis (Figure 3I).

358 To accurately map the cell type determination and developmental trajectory
359 reconstruction, we have constructed the spatiotemporal developmental trajectories of
360 the zebrafish embryo by combining the scRNA-seq and Stereo-seq data to delineate
361 cell state transitions with spatial coordinates. The developmental trajectories showed
362 clear and specific spatiotemporal characteristics, which are consistent with previously
363 known facts about zebrafish embryonic development. Besides illuminating the spatial
364 separation of closely correlated cell types, our spatiotemporal developmental
365 trajectories resolved some previously unanswered questions. For instance, it has been
366 unclear when the lateral plate mesoderm starts to differentiate into different tissues.
367 Our results disclosed that the fate of this structure is already decided to differentiate in
368 three different directions, namely, the cardiovascular system, erythroid lineage cell,
369 and endothelial cell at 12 hpf (Figure 4E and S4A).

370 TFs, together with their downstream gene networks, are one of the key factors that
371 drive cell fate transition during embryogenesis. We investigated candidate TFs that
372 potentially dominate the cell types at different time points in our spatiotemporal
373 developmental trajectories. Our data identified known TFs, whose spatiotemporal
374 distribution was consistent with their reported functions. The TF gene *cdx4* has been
375 shown to be involved in the development of hematopoietic stem cells (Davidson and
376 Zon, 2006) and our analysis showed that *cdx4* regulates the cell fate transition from
377 lateral plate mesoderm to erythroid lineage cell (Figure 4E, left). We also discovered a
378 potential new role of certain TFs such as *foxn4* in the differentiation of the presumptive
379 ectoderm from the blastodisc, while *foxn4* was previously known to function in the
380 regulation of neural stem cell division (Misra et al., 2014) (Figure S4D).

381 Based on our high-resolution Stereo-seq data, we were able to precisely calculate
382 the spatial distance between different ligands and receptors embryo-wide in zebrafish.
383 Besides the Notch components, we uncovered interesting dynamics of many other
384 ligand-receptor pairs. The ligand *uba52* has a strong interaction with multiple receptors
385 at different developmental stages, e.g., interacting with the BMP receptor *bmpr1ba*
386 from as early as 3.3 hpf while starting to interact with *lrp2a* at 10 hpf. The bmp family
387 receptor (*bmpr1ba*) shows spatial proximity with the ligand as early as 3.3 hpf in our
388 analysis, while the receptor gene *lrp2a* and the corresponding ligand genes are co-
389 expressed at 10 hpf when the somite and the nervous system start to develop. The
390 integrin gene *itgb1b.2*, which encodes the molecules that function to attach the cells to
391 the ECM and transduce signals from the ECM to the cells, starts to get close to the

392 ligands at 18-24 hpf. The highly dynamic spatiotemporal changes of ligand-receptor
393 expression suggested that different ligand-receptor pairs are precisely programmed to
394 coordinate the cell fate determination and organogenesis during zebrafish embryonic
395 development (Figure 5B).

396 In conclusion, we demonstrated a spatiotemporal landscape of the transcriptional
397 dynamics in the developing zebrafish embryo and provided a useful resource for
398 studying the cellular and molecular mechanisms of germ layer specification,
399 organogenesis and cell fate determination. Future perspectives can be the
400 construction of the spatiotemporal zebrafish developmental atlas at a longer time
401 window with shorter intervals, 3D reconstruction based on multiple sectioning strategy,
402 and further exploration of the regulatory networks by integrating more spatial omics
403 data such as the spatial genomics and chromatin accessibility.

404 **METHODS**

405 **Tissue collection and sample mounting for Stereo-seq**

406 All relevant procedures involving animal experiments presented in this paper were
407 approved by Animal Care and Use Committee of Huazhong Agriculture University
408 (HZAUMO-2015-016). Zebrafish embryos from AB wild-type crosses were collected at
409 3.3 hours, 5.25 hours, 10 hours, 12 hours, 18 hours, and 24 hours after fertilization.
410 After being dechorionated manually with forceps, embryos were anesthetized by
411 soaking in tricaine solution (Bomeibio, 886-86-2). Embryos were then placed in
412 cryomold with OCT (Sakura, 4583), and extra egg water was removed using a glass
413 pipette. The embryos were oriented with a blunt metal needle to the right position. After
414 the OCT freezes solidly on the flat surface of a block of dry ice, the embryos were
415 sectioned on a cryostat machine or stored at -80°C .

416 **Stereo-seq library preparation and sequencing**

417 Stereo-seq library preparation and sequencing were adapted according to the
418 standard protocol V1.1 with minor modification (Chen et al., 2021). Tissue sections
419 were adhered to the Stereo-seq chip, and incubated in -20°C methanol for 30 min
420 fixation, followed by nucleic acid dye staining (Thermo fisher, Q10212) and imaging
421 (Ti-7 Nikon Eclipse microscope). For permeabilization, tissue sections before 18 hpf
422 were permeabilized at 37°C for 3 minutes, while tissue sections of 18 hpf and 24 hpf
423 were permeabilized at 37°C for 5 minutes. The cDNA was purified using AMPure XP
424 beads (Vazyme, N411-03). The indexed single-cell RNA-seq libraries were constructed
425 according to the manufacturer's protocol. The sequencing libraries were quantified by
426 Qubit™ ssDNA Assay Kit (Thermo Fisher Scientific, Q10212). DNA nanoballs (DNBs)
427 were loaded into the patterned Nano arrays and sequenced on MGI DNBSEQ-Tx
428 sequencer (50 bp for read 1, 100 bp for read 2).

429 **Zebrafish embryo collection and single cell isolation for scRNA-seq**

430 The protocol for zebrafish embryos dissociation and single cell suspension isolation
431 was adapted according to published protocol with a few modifications (Manoli and
432 Driever, 2012). Briefly, 250–1000 embryos were collected and transferred into a
433 Petridish containing Pronase E protease solution (Sigma-Aldrich, P5147-1G). When
434 20–30% embryos were hatched, 1 mL 56°C pre-heated Hi-Fetal Bovine Serum
435 (Biological Industries, 04-001-1ACS) was added. Then embryos were washed once
436 with 0.5X Danieau's solution containing 10% Hi-FBS, and thrice with 0.5X Danieau's
437 solution. Then deyolking buffer was added and pipetted up and down until only the
438 bodies of the embryos were visible. After dissociation with 1× trypsin-EDTA solution
439 (Biosharp, BL512A), incubation was stopped by adding Hi-FBS to a final concentration
440 of 5%. Finally, single cells were resuspended in 0.04% BSA (Sigma, A8022-50G) in
441 PBS (Meilunbio, MA0015).

442 **Single cell RNA-seq library construction and sequencing**

443 The DNBelab C Series Single-Cell Library Prep Set (MGI, 1000021082) was utilized
444 according to the manufacture's protocol (Liu et al., 2019). In brief, single cell
445 suspensions were loaded into the chip for droplet generation. Then the droplets were
446 gently removed from the collection and incubated at room temperature for 20 minutes
447 to capture mRNA release from cells. And then emulsion breakage and beads collection
448 were performed. After reverse transcription, and cDNA amplification, purified PCR
449 products were used for DNB generation and finally sequenced on an MGI DNBSEQ-
450 Tx using the following read length: 41bp for read1, 100bp for read2, and 10bp for
451 sample index.

452 **Binning data of spatial Stereo-seq data**

453 Raw data were processed in the same procedure as previous work (Chen et al., 2021).
454 Transcripts captured by 15*15 DNBs were merged as one bin 15. We treated the bin
455 15 as the fundamental analysis unit. Bin IDs were synthesized by their spatial
456 coordination (spatial_x and spatial_y) at the capture chip. In specific, the DNB at the
457 left bottom of bin 15 was selected to represent the location of bin 15. Sample contours
458 were manually drawn to exclude bins that were not from the tissue samples. For
459 comparison with other resolutions, we also binned DNBs in other bin sizes, *i.e.*, bin 50,
460 100, 150, 200, and performed unsupervised clustering. The thresholds of gene number
461 used for filtering low-quality bins were: 150 for bin 15, 1500 for bin 50, 2500 for bin 100,
462 4000 for bin 150 and 5000 for bin 200.

463 For a demonstration of the spatial location of sparsely expressed genes in Figure
464 3G, we plotted every captured mRNA molecule in embryo in the resolution of bin 1
465 using function 'ggplot' in R package ggplot2 (Villanueva and Chen, 2019). And the
466 location of molecules captured by the same DNB was jittered to avoid overlapping by

467 using the function 'geom_jitter'.
468

469 **Unsupervised clustering of Stereo-seq data**

470 Data normalization, scaling, and bins clustering were processed using the R package
471 Seurat (v3.5.1) (<https://github.com/satijalab/seurat>). Sections from the same embryo
472 were pooled together as a data set for analysis. Cell identities of clusters were
473 annotated using marker genes. Gene number captured was used for quality control,
474 with 100 genes for 5.25 hpf and 10 hpf, 150 genes for embryos at other stages, and
475 bins with a lower number of genes captured were filtered out. Bins located in the yolk
476 with low gene numbers were also filtered out in this procedure. To get more detailed
477 cell types, bins belong to a specific cell type, or groups of relevant cell types were
478 further clustered and annotated.

479 **Single-cell RNA-seq data processing: cell clustering and identification of cell 480 types**

481 Raw sequencing reads of each sample were processed using
482 DNBelab_C_Series_HT_scRNA-analysis-software ([https://github.com/MGI-tech-
483 bioinformatics/DNBelab_C_Series_HT_scRNA-analysis-software](https://github.com/MGI-tech-bioinformatics/DNBelab_C_Series_HT_scRNA-analysis-software)) which include
484 alignment, primary filtering, and gene expression generation of each cell. We merged
485 all data sets for each time point using the merge function and used Seurat (v4.0.3) to
486 filter the low-quality cells by gene number of each cell and reads mapped to
487 mitochondrial genes. The data were then log normalized using 'NormalizeData'
488 function with the scaled factor set to the default value of 10,000. We scale the data
489 using 'ScaleData' function and identified the 2,000 high variable features using
490 'FindVariableFeatures' function. All these variable genes were used to do principal-
491 component analysis (PCA). The cells were clustered using 'FindNeighbors' function
492 using the first 20 principal components, followed by 'FindClusters' function. All cell
493 clusters were identified by using known cell type-specific markers for each cluster.

494 **Marker genes of clusters and GO and KEGG gene enrichment analysis**

495 Marker genes of different clusters were found by 'FindMarkers' of Seurat R package,
496 with parameter (min.pct = 0.25, logfc.threshold = 0.25). Gene set enrichment analysis,
497 were processed with function 'enrichGO' using parameters (OrgDb=org.Dr.eleg.db,
498 pAdjustMethod="BH", pvalueCutoff = 0.01, qvalueCutoff = 0.05) and function
499 'enrichKEGG' with parameter (organism='dre', pvalueCutoff=0.05) of R package
500 clusterProfiler
501 (<https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>).

502 Identification of spatially auto-correlated gene modules

503 Modules of spatially correlated genes were identified using Hotspot (DeTomaso and
504 Yosef, 2021). The expression matrixes of genes with minimum UMIs number (50 for
505 10, 12, and 18 hpf; 300 for 24 hpf) of all embryo sections from 4 development stages
506 (10, 12, 18 and 24 hpf) were used as input. The data were first normalized by the total
507 UMIs number of these genes of each bin, then K-Nearest Neighbors (KNN) graph of
508 genes was created using the 'create_knn_graph' function with the parameters:
509 n_neighbors = 8, then genes with significant spatial autocorrelation (FDR < 0.05) were
510 kept for further analysis. The modules were identified using the 'create_modules' with
511 the parameters: min_gene_threshold (20 genes for 10 and 12 hpf, 30 genes for 18 hpf,
512 and 50 genes for 24 hpf) and fdr_threshold = 0.05. For the cell type enrichment
513 analysis for each module, we calculated the cell type composition of bins with high
514 module score (with the criteria that module scores bigger than 3, the method of
515 calculation of the module score see the Hotspot reference (DeTomaso and Yosef,
516 2021)). As a comparison, we also calculated the cell type composition of all sections
517 of the same developmental stage (Figure 3B, S2B, S2E and S2H).

518 To find genes that spatially correlated with Notch pathway, we further conducted
519 module analysis in selected sections at 12, 18 and 24 hpf (section #8, #8, and #4, with
520 minimum UMIs number for gene selection as 50, 30, 50 respectively.). We first
521 calculated gene set expression score (Notch-score) of Notch pathway genes (genes
522 with very low expression were removed from the Notch pathway gene list) using
523 function 'AddModuleScore' of R package Seurat. Then we added the Notch-score into
524 the gene expression matrix and calculated the spatial correlations as above. Genes
525 with Z-scores for the significance of the correlation with Notch-score bigger than 5 were
526 chosen for further analysis. Modules of these Notch pathway correlated genes were
527 identified using 'create_modules', with parameter min_gene_threshold, 5, 10, 20 for
528 12, 18, 24 hpf respectively. Notch genes used for analysis included: *dlc*, *dla*, *dld*, *dlb*,
529 *dll4*, *jag1a*, *jag1b*, *jag2b*, *notch1a*, *notch1b*, *notch2*, *notch3*, *her6*, *her9*, *her12*, *her15.1*,
530 *her15.2*, *her4.2*, *her2*, *her4.4*, *her3*, *her13*, *her8.2*, *her8a*, *her4.1*, *her4.3*, *her4.5*, *her7*,
531 *hey1*, *hey2*.

532 Protein-protein interaction (PPI) network analysis of spatial modules

533 We applied the search tool for the retrieval of interacting genes (STRING)
534 (<https://string-db.org>) database to seek potential interactions among genes in
535 one/multiple modules. Active interaction sources including co-expression as well as
536 species limited to "*Danio rerio*", and an interaction score >0.4 were applied to construct
537 the PPI networks. The PPI networks were visualized by Cytoscape software 3.8.2. In
538 these networks, the nodes correspond to the proteins and the node size is proportional
539 to the relative connectivity in each network. The dark lines represent positive
540 associations and the thickness represents the strength of the interactions. We chose
541 "co-expression" as edge weights to represent the strength of the interactions.

542 **Construction of single-cell developmental trajectory**

543 We integrated scRNA-seq data from adjacent time points using Harmony ($\lambda =$
544 0.1 , $\epsilon_{\text{harmony}} = -\text{Inf}$) (Korsunsky et al., 2019). Then we calculated the 10
545 nearest neighbors in the latter time point for each spot of the earlier time point using
546 K-Nearest Neighbors algorithm. We took the most frequent clusters of the 10 nearest
547 spots as the target states that the earlier spots would develop into. If two or more target
548 clusters had the same frequencies in the nearest neighbors, the cluster with the
549 shortest weighted distance was used. We repeated this procedure on each pair of
550 adjacent time points from 3.3 hpf to 24 hpf. While constructing the developmental
551 trajectory between each two adjacent time points, we reserved only the connections
552 with a proportion of source cells more than 30% of its type.

553 **Deconvolution of cell types**

554 We divided single-cell clusters into subtypes according to the single-cell developmental
555 trajectory. Then we calculated the cell composition of each spatial data bin using
556 SPOTlight (Elosua-Bayes et al., 2021) with scRNA-seq data as a reference.

557 **TF regulation activity prediction**

558 We downloaded the motif database of zebrafish from the CIS-BP Database
559 (<http://cisbp.ccb.utoronto.ca/bulk.php>) and constructed the cisTarget databases for
560 zebrafish according to the SCENIC tutorial instructions (Aibar et al., 2017)
561 (https://github.com/aertslab/create_cisTarget_databases). Then we calculated the
562 single-cell TF regulation activity using pySCENIC ($\text{rank_threshold} = 10000$,
563 $\text{auc_threshold} = 0.1$, $\text{nes_threshold} = 2.5$). We used the weighted average expression
564 of predicted target genes to illustrate the regulation activity of each transcription factor
565 in the spatial transcriptomic data.

566 **Ligand-receptor analysis**

567 Ligand-receptor pairs were extracted from the LRBase.Dre.eg.db database
568 (<http://bioconductor.org/packages/release/data/annotation/html/LRBase.Dre.eg.db.html>).
569 For each bin expressing a receptor, we calculated its Euclidian distances to all the
570 bins expressing the corresponding ligand and take the nearest distance as the distance
571 of this ligand-receptor pair in this bin. Then the distances were scaled regarding the
572 distance of adjacent cells as 1. The average distance of each ligand-receptor pair in
573 each time point was taken as the ligand-receptor distance for that time point. Ligand-
574 receptor pairs with an average distance less than 5 in one or more time points were
575 kept for analysis. The distance score was calculated as:

576 $\text{Distance_score} = \log_2(1 + 1/\text{distance})$

577 We calculated the expression score of each ligand-receptor pair as:

578 $\text{Expression_score} = \log_2(1 + (E_r \times E_i))$
579 Where E_r and E_i represent the average expression of the receptor and the ligand
580 respectively.
581 For receptor-ligand interaction of notch signal, we normalized the interaction
582 frequency by dividing the bin number of each cell type.

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587 **AUTHOR CONTRIBUTIONS**

588 X.X., Z.D. and L.L. designed and supervised the work. C.L., X.X., Z.D. and L.L.
589 designed the experiment. C.L., X.L., S.W., K.Z., X.S., C.P., H.W. and W.B. performed
590 the library preparation and sequencing. R.L., Young.L, Q.L. Y.M. and X.Y. performed
591 the bioinformatics analysis. K.M. and J.H. provided technical support. A.C. and
592 Yuxiang.L. gave the relevant advice. M.A.E., M.A.B., S.K.S., S.L. and X.X. participated
593 in the manuscript editing and discussion. C.L., Z.D. and L.L. wrote the manuscript. All
594 authors edited and approved the manuscript.

595 **DECLARATION OF INTERESTS**

596 The chip, procedure and applications of Stereo-seq are covered in pending patents.
597 Employees of NGI have stock holding in BGI.

598 **DATA AND CODE AVAILABILITY**

599 All raw data have been deposited to CNGB Nucleotide Sequence Archive (accession
600 code: CNP0002220).

601

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726

727 **FIGURE LEGENDS**

728 **Figure 1. High resolution Stereo-seq on multiple sections of the developing**
729 **zebrafish embryos.**

730 (A) Experimental outline (top): diagram of the zebrafish embryos at different
731 development stages which were subjected to Stereo-seq. Scale bar: 0.5 mm. Stereo-
732 seq process diagram (bottom): the enlarged image shows the size of each spot and
733 the distance between 2 adjacent spots.

734 (B) Nucleic acid dye staining of the 24 hpf zebrafish embryo sections attached on two
735 1-square-centimeter Stereo-seq chips. Scale bar: 0.5 mm.

736 (C) Spatial visualization of the distribution of captured transcripts (unique molecular
737 identifiers (UMIs)) on all 24 hpf zebrafish embryo sections. Scale bar: 0.5 mm.

738 (D) Unsupervised clustering of the 24 hpf zebrafish embryo section analyzed by

739 Stereo-seq at different bin sizes. Scale bar: 0.5 mm.
740 (E and F) Violin plot of the number of captured transcripts (E) and genes (F) of each
741 24hpf zebrafish embryo sections.
742 (G) Table summarizing the number of sections used in Stereo-seq at each embryonic
743 development stage.

744

745 **Figure 2. A spatial transcriptomic atlas at cellular size resolution of the**
746 **developing zebrafish embryo.**

747 (A) Unsupervised clustering of the zebrafish embryo section across sequential
748 developmental stages analyzed by Stereo-seq at bin 15. Cells are colored by different
749 regions.

750 (B) Unsupervised clustering of the 24hpf zebrafish embryo sections at bin 15. Cells are
751 colored by spatial identities inferred from expressed marker genes. Scale bars 0.5 mm.

752 (C) Spatial visualization of indicated areas of the 24 hpf embryo on the left: detailed
753 anatomical structures identified in the brain and the eye, and a combined structure
754 including the spinal cord, the floor plate, and the notochord. The expression patterns
755 of marker genes for each anatomical structure are shown on the right. Scale bars: 0.5
756 mm and 0.05 mm.

757 (D) Spatial visualization of the expression of indicated genes for erythroid lineage cell,
758 myotome, YSL, hatching gland and early development stages. Scale bar: 0.5 mm.

759

760 **Figure 3. Spatial modules identified by Hotspot uncover the interaction among**
761 **spatial regions in 24 hpf zebrafish embryo.**

762 (A) Heatmap shows the genes with significant spatial autocorrelation (5,089 genes,
763 FDR<0.05) grouped into 12 gene modules based on pairwise spatial correlations of
764 gene expression in multiple sections of the 24 hpf zebrafish embryo. Selected genes
765 and GO term related to representative modules are highlight on the right side.

766 (B) Heatmap shows the Pearson correlation of the module score for each spatial
767 autocorrelation module and the expression sets of specific genes for each spatial
768 cluster of the 24 hpf embryo from Stereo-seq dataset in Figure 2B.

769 (C) Spatial visualization of representative modules on 24 hpf embryo sections. Scale
770 bars 0.5 mm.

771 (D) Spatial visualization of module 9 (M9).

772 (E) Bar graph shows significantly enriched selected gene ontology terms in M9.

773 (F) Protein-protein interaction (PPI) network of genes in M9. The network is visualized
774 by Cytoscape. Node size represents the relative connectivity, and the thickness of the
775 dark line represents the strength of interactions.

776 (G) Spatial expression pattern of genes in M9. These genes are related to the
777 development of pigment cell (blue), mesenchymal cell (purple), and sensory organ
778 (red).

779 (H) Spatial visualization of modules related to notochord and neighboring tissues on
780 24 hpf embryo sections including M1, M5 and M6.

781 (I) PPI network of genes in the 3 modules in H. The red lines represent interactions
782 with *shha/b*. (J) Spatial expression patterns of *shha* and the interactive genes.

783

784 **Figure 4. Construction of the spatially resolved developmental trajectories by**
785 **integrated analysis of the scRNA-seq and Stereo-seq data.**

786 (A) Schematic representation of the workflow for scRNA-seq of zebrafish embryos at
787 different developmental stages using the C4 system.

788 (B) Application of Sankey diagram to visualize zebrafish embryo developing trajectory
789 with scRNA-seq data. The abbreviations of the cell types in B are also marked in
790 parentheses after each cell type in the color legend.

791 (C) Application of SPOTlight to integrate Stereo-seq with scRNA-seq data to infer the
792 spatially resolved developmental trajectories. Two developmental branches which are
793 developed from the presumptive ectoderm, namely, pigment cell, and central nervous
794 system (left) are simultaneously displayed on six sequential spatial sections to show
795 the different spatial developmental trajectories (right).

796 (D) Two selected developmental branching points at 12 hpf and 18 hpf. The lateral
797 plate mesoderm (left) and neural crest (right), are shown in the Sankey diagram.

798 (E) Cell fate regulatory maps of different developmental destinations at 12 hpf and 18
799 hpf related to Figure 4D. SPOTlight was applied to visualize the spatial distribution of
800 cell subgroups with different differentiation (top); selected representative crucial TFs
801 for different developmental branches are shown in the chart (middle); the spatial
802 expression distributions of TFs target genes are visualized on embryonic sections
803 (bottom). The scale bars represent 0.5 mm.

804

805 **Figure 5. The relative spatial distance of different ligand-receptor pairs reveals**
806 **the arrangement and interaction of signaling pathways.**

807 (A) Model diagram of the analysis of ligand-receptor relative distance.

808 (B) A dotted heat map shows the expression score and relative distance score of
809 ligand-receptor pairs.

810 (C) Spatial expression pattern of *notch* family genes (blue), *delta* family genes (red),
811 and *jagged* family genes (green) on embryo sections at the 6 time points (top). Spatial
812 expression pattern of Notch receptors (red), ligands (blue) with a relative distance less
813 than 2, and the ligand-receptor pairs expressed in the same bin (purple) (middle).
814 Spatial expression pattern of Notch target genes (red) on the embryonic sections at
815 different time points (bottom). Scale bar: 0.5 mm.

816 (D-F) Gene modules identified using Hotspot that showed spatial correlation with
817 Notch pathway in 12 hpf (D), 18 hpf (E), and 24 hpf (F) embryos. The spatial expression
818 pattern of Notch pathway genes at each developmental stage is shown on the left, and
819 selected genes and GO terms related to spatially correlated modules are highlight on
820 the right. Scale bars: 0.5 mm.

821 (G) A heatmap shows the temporal expression change of genes selected from
822 modules in D-F.

823

824 **Figure S1. Spatially resolved transcriptomic atlas across 6 sequential**
825 **developmental stages.** Related to Figure 1 and 2.

826 (A) The summary of total bins, average captured number of UMIs and genes at bin 15

827 resolution for each embryonic development stage.
828 (B-G) Unsupervised clustering of 3 selected sections from 3.3 hpf (B), 5.25 hpf (C), 10
829 hpf (D), 12 hpf (E), 18 hpf (F) and 24 hpf (G) embryos by Stereo-seq (top) and the
830 images of nucleic acid dye staining of the same embryo sections (middle). Scale bars:
831 0.1 mm. Heatmap shows the expression pattern of marker genes of different clusters
832 at each developmental stage (bottom).

833

834 **Figure S2. Hotspot identified spatial modules at multiple zebrafish embryo**
835 **sections at different developmental stages.** Related to Figure 3.

836 (A, D, G) Heatmap shows the genes with significant spatial autocorrelation (449 genes
837 at 10 hpf, 791 genes at 12 hpf, 2320 genes at 18 hpf, FDR<0.05) grouped into 8 gene
838 modules at 10 hpf (A), 11 gene modules at 12 hpf (D) and 16 gene modules at 18 hpf
839 (G). Selected genes and representative GO terms related to the corresponding
840 modules are highlighted on the right side.

841 (B, E, H) Heatmap shows the Pearson correlation of the module score for each spatial
842 autocorrelation module and the expression sets of specific genes for each spatial
843 cluster of 10 hpf (B), 12 hpf (E) and 18 hpf (H) embryos.

844 (C, F, I) Spatial visualization of modules on 10 hpf (C), 12 hpf (F) and 18 hpf (I) embryo
845 sections. Scale bars: 0.5 mm.

846

847 **Figure S3. Quality control of the single-cell RNA-seq libraries.** Related to Figure
848 4.

849 (A) UMAP visualization of all single-cell RNA-seq data of the 6 developmental stages.
850 Visualization of each cell type colored by tissue/organ. The color legend of A is the
851 same as that in Figure 4B.

852 (B) Pseudotime analysis of the 3.3 hpf embryo is taken by Monocle 2, and cells are
853 colored by cell type.

854 (C-H) Violin plots show the number of genes (left), the number of UMIs (middle left),
855 and the percentage of mitochondrial genes (middle right) from multiple library
856 repetitions with 3.3 hpf (C), 5.25 hpf (D), 10 hpf (E), 12 hpf (F), 18 hpf (G), and 24 hpf
857 (H) zebrafish embryos. Heatmaps showing expression of marker genes of the
858 indicated cell type of each development stage (right).

859

860 **Figure S4. The selected spatially resolved developmental trajectories.** Related to
861 Figure 4.

862 (A) The representative developmental branches selected from Figure 4B are displayed
863 respectively on sequential spatial sections across all developmental time points to
864 show the spatial trajectory. The red arrows indicated the branches shown in Figure 4C
865 and S4B.

866 (B) Application of SPOTlight to integrate Stereo-seq with scRNA-seq data to infer the
867 spatially resolved developmental trajectories. Three developmental branches which
868 are developed from the dorsal margin and presumptive mesoderm, namely, erythroid
869 lineage cell, hatching gland and notochord are simultaneously displayed on spatial
870 sections.

871 (C) The selected developmental branching points at 3.3 hpf are shown in the Sankey
872 diagram.

873 (D) Cell fate regulatory maps of different developmental destinations at 3.3 hpf. Scale
874 bar: 0.5 mm. SPOTlight was applied to visualize the spatial location of cell subgroups
875 with different differentiation (top); crucial TFs for different developmental branches are
876 shown in the chart (middle); the spatial expression distributions of TFs target genes
877 are visualized on embryonic sections (bottom).

878

879 **Figure S5. Analysis of the relative spatial distance of ligand-receptor pairs**
880 **during zebrafish embryo development.** Related to Figure 5.

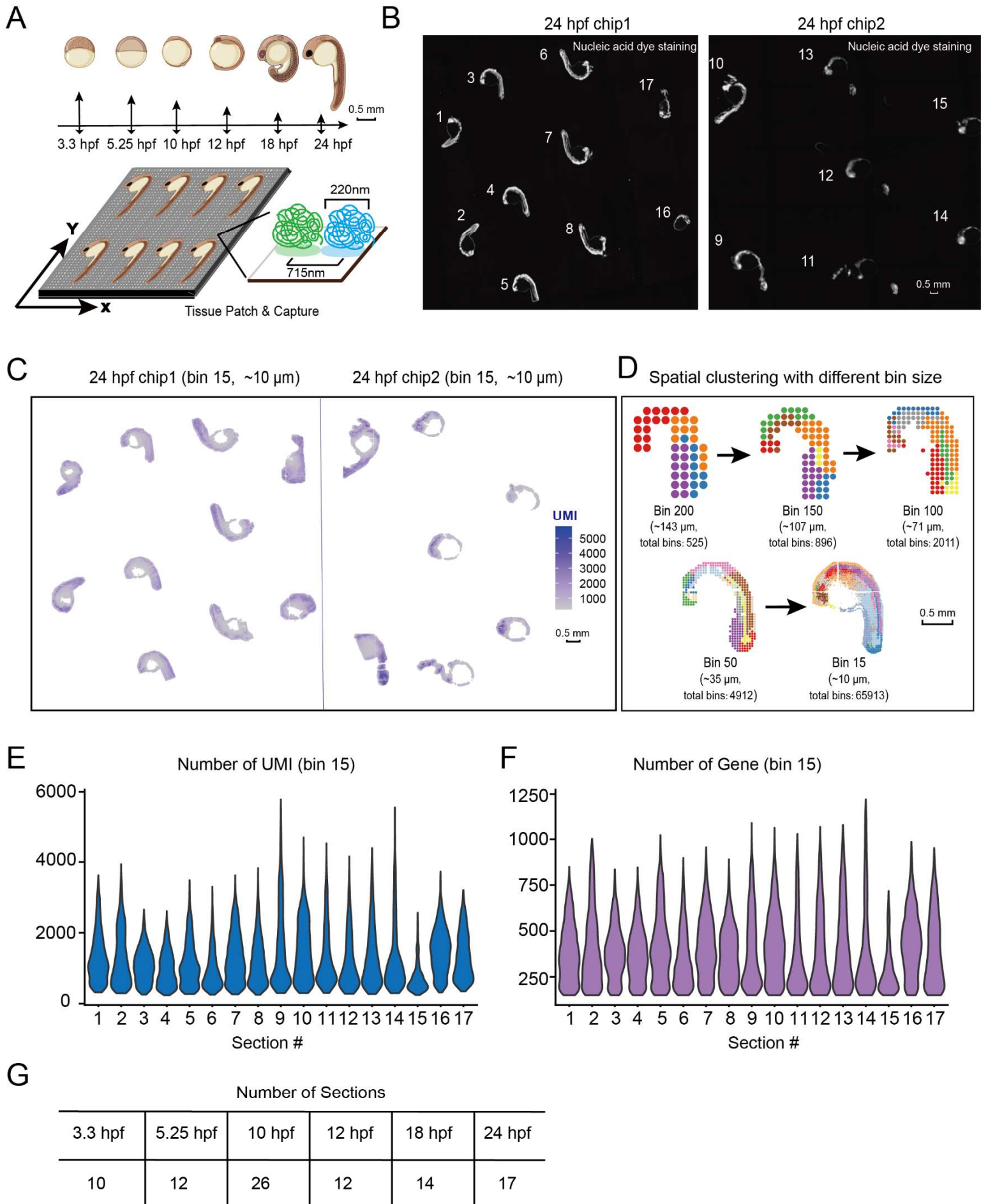
881 (A) Boxplot shows the relative distance values of all ligand-receptor pairs at different
882 time points.

883 (B) Boxplot shows the relative distance values of filtered ligand-receptor pairs at the
884 different time points. Filtered ligand-receptor pairs refer to those which have a relative
885 distance less than 5 for at least one time point.

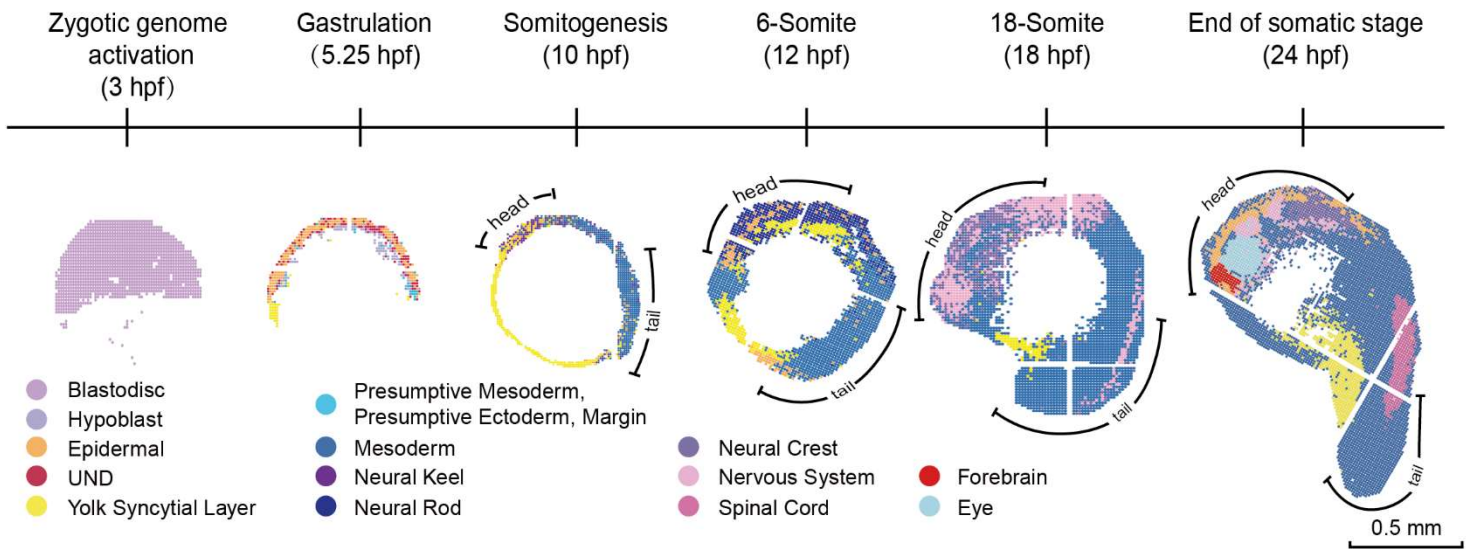
886 (C) Heat map shows the relative distance of Notch ligand-receptor pairs.

887 (D) Heat maps show the relative distance between Notch ligands and receptors in
888 representative cell types at 10 hpf, 12 hpf, 18 hpf and 24 hpf.

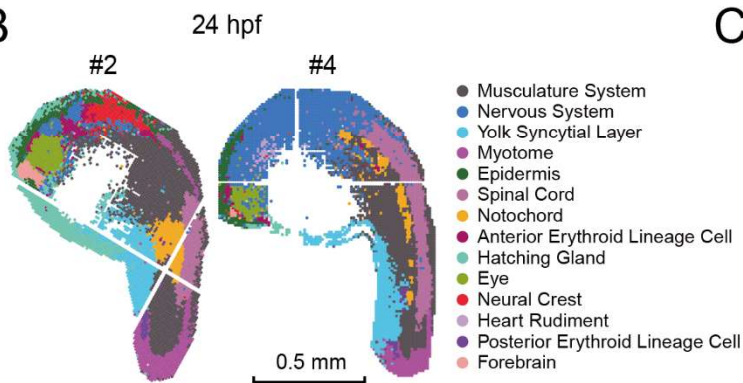
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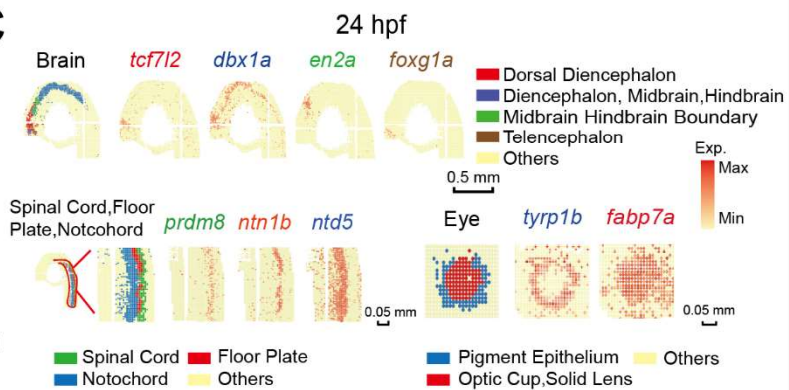
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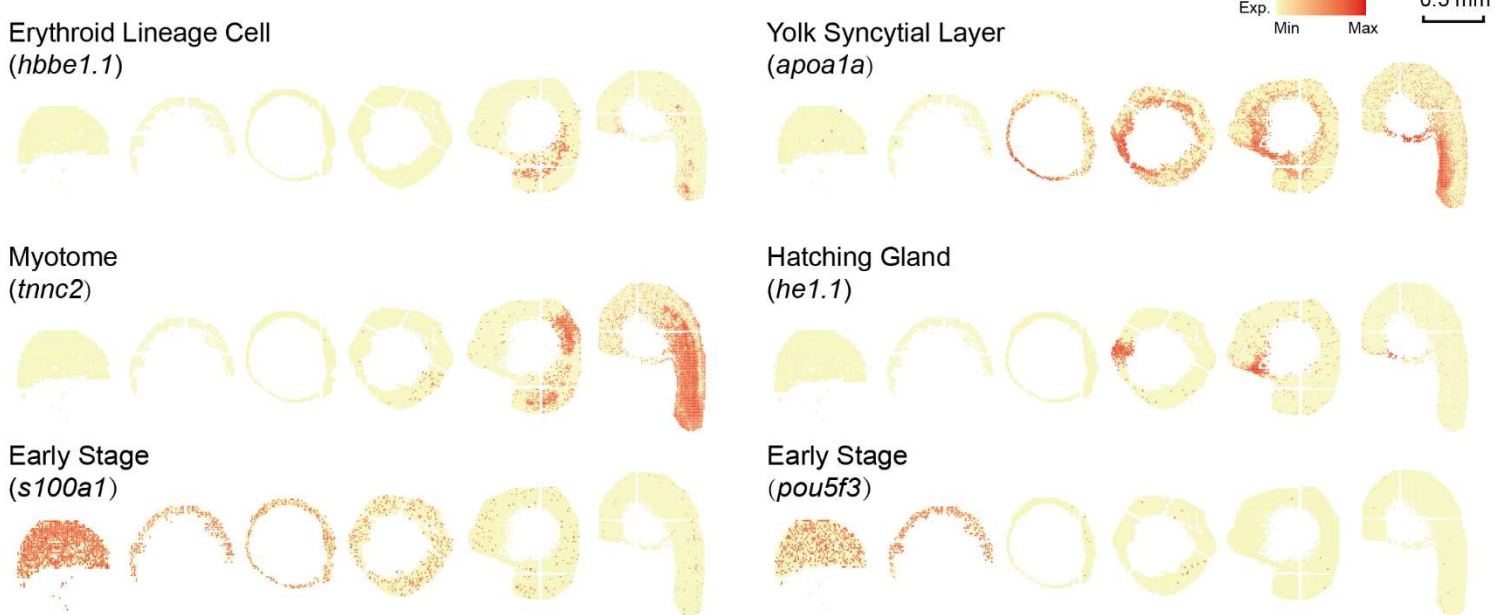
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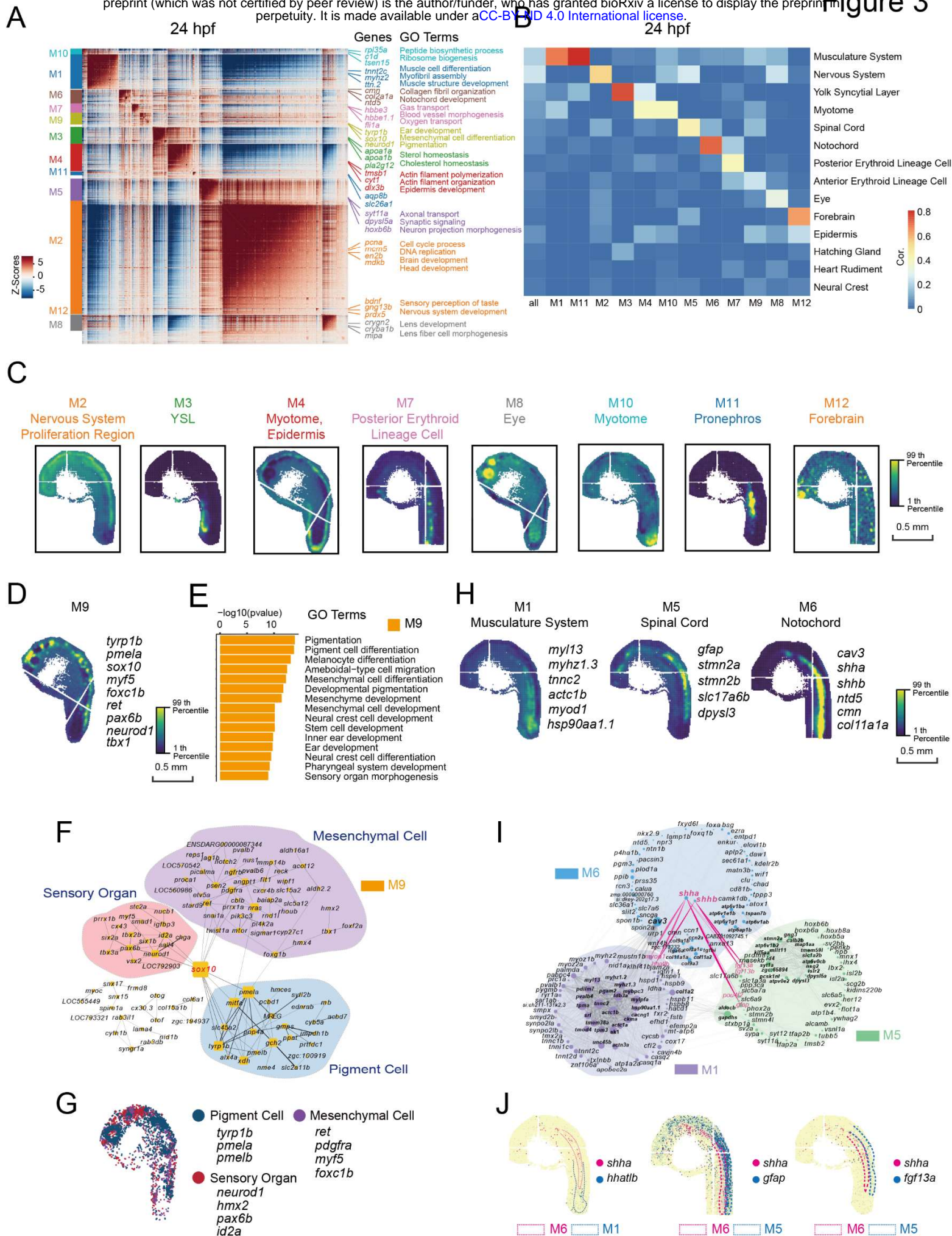


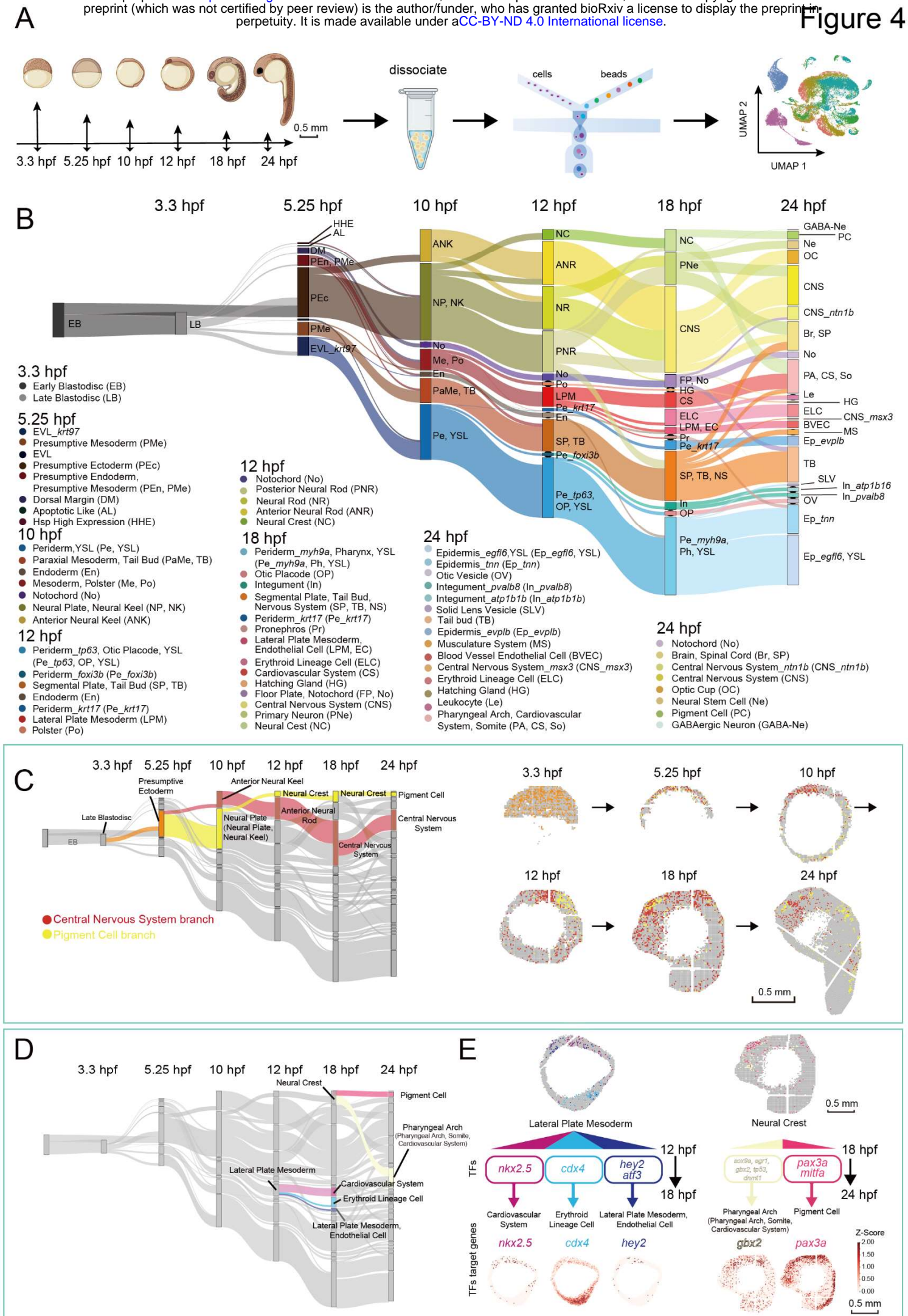
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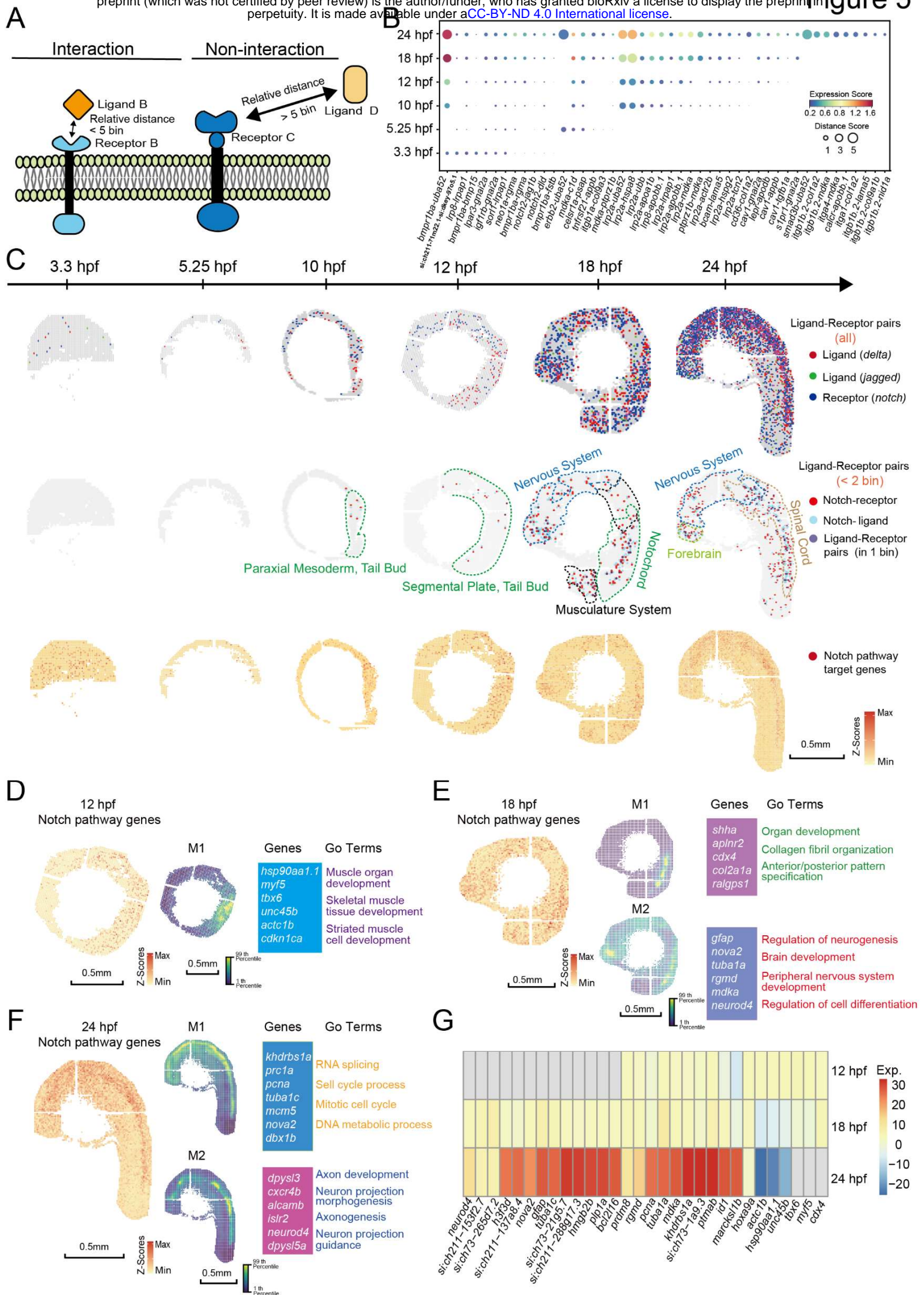


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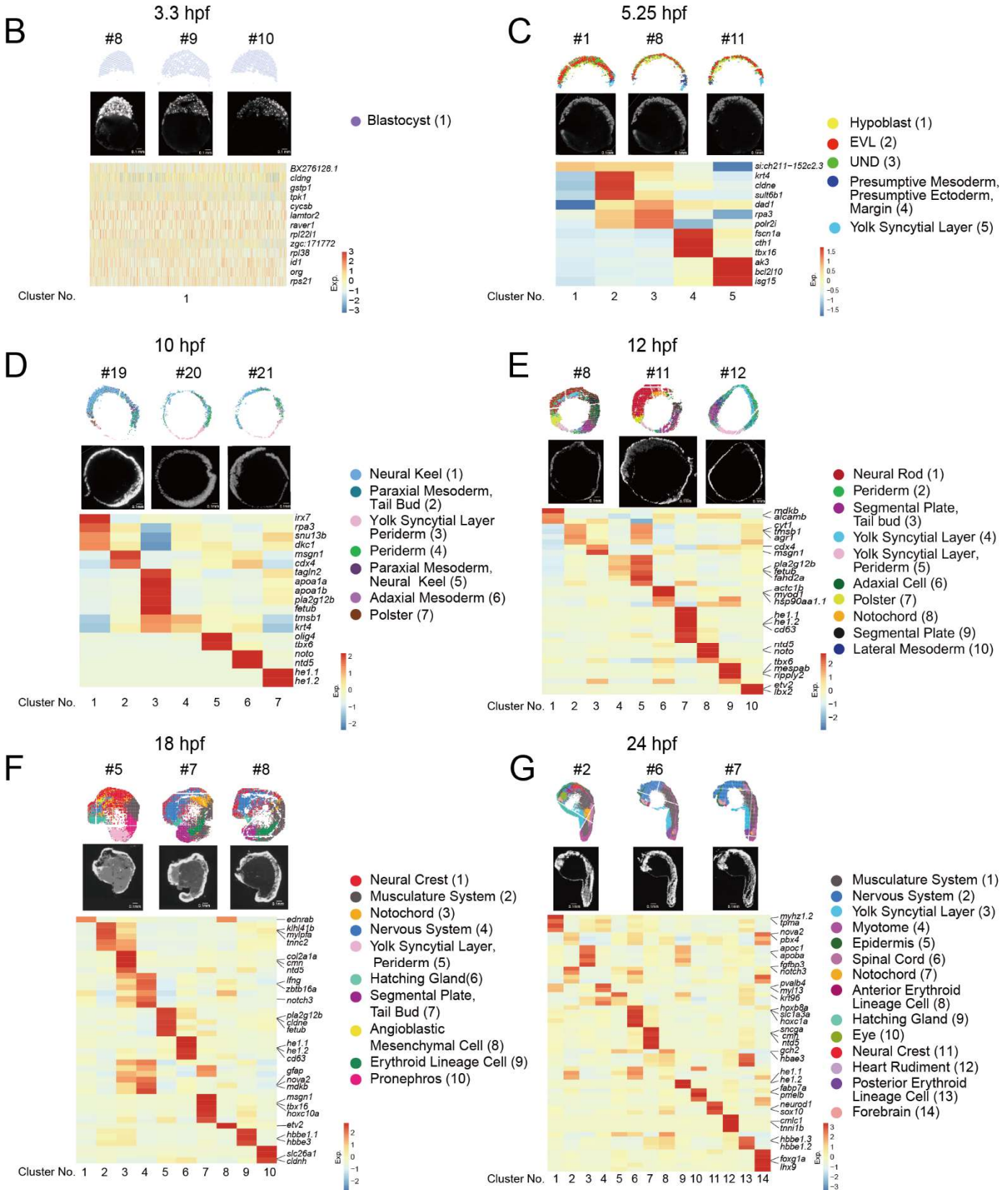


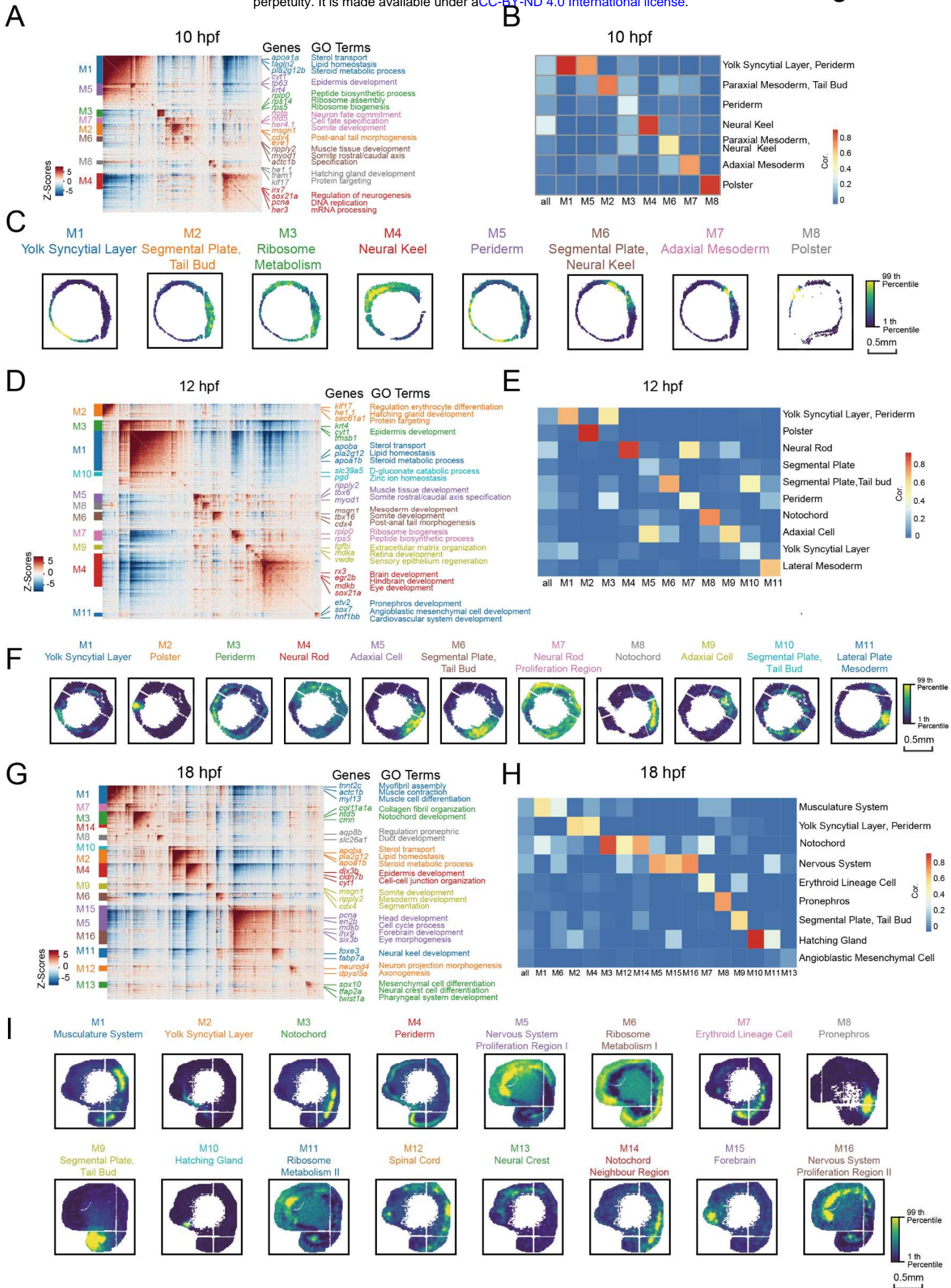




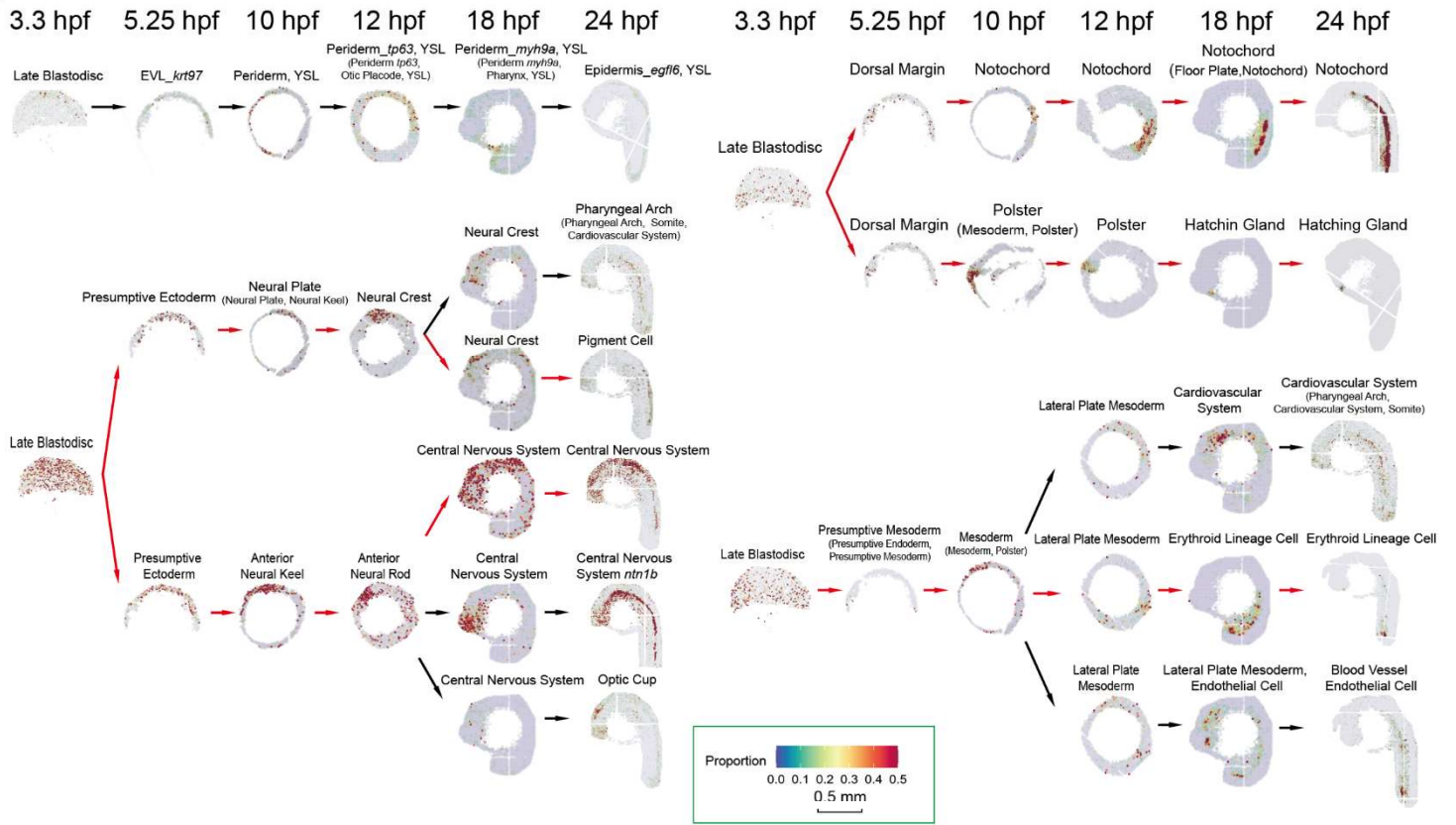
A

stages	3.3 hpf	5.25 hpf	10 hpf	12 hpf	18 hpf	24 hpf
Bin number (bin15, ~10 μm)	8529	5841	19520	16418	29712	65913
Average number of gene (bin 15)	257	154	153	169	321	394
Average number of UMI (bin 15)	405	284	294	302	798	1250

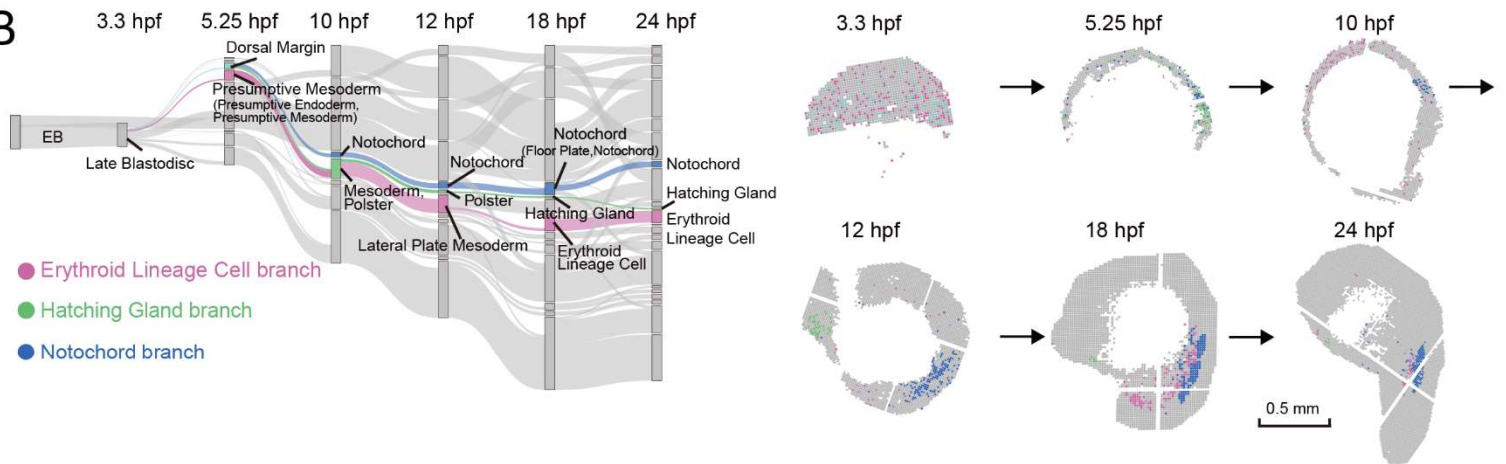




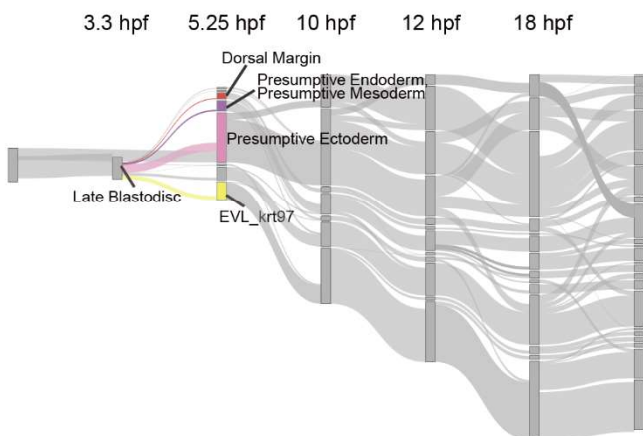
A



B



C



D

