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### ► To cite this version:

Céline Trébeau, Jacques Boutet de Monvel, Gizem Altay, Jean-Yves Tinevez, Raphael Etournay.  
Extracting multiple surfaces from 3D microscopy images in complex biological tissues with the Zellige  
software tool. 2022. pasteur-03983119v1

**HAL Id: pasteur-03983119**

**<https://pasteur.hal.science/pasteur-03983119v1>**

Preprint submitted on 9 Jun 2022 (v1), last revised 10 Feb 2023 (v2)

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# 1 Extracting multiple surfaces from 3D microscopy images in 2 complex biological tissues with the Zellige software tool.

3

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9

## 10 **Abstract.**

11 Efficient tools allowing the extraction of 2D surfaces from 3D-microscopy data are essential for studies  
12 aiming to decipher the complex cellular choreography through which epithelium morphogenesis takes  
13 place during development. Most existing methods allow for the extraction of a single and smooth manifold  
14 of sufficiently high signal intensity and contrast, and usually fail when the surface of interest has a rough  
15 topography or when its localization is hampered by other surrounding structures of higher contrast.  
16 Multiple surface segmentation entails laborious manual annotations of the various surfaces separately. As  
17 automating this task is critical in studies involving tissue-tissue or tissue-matrix interaction, we developed  
18 the Zellige software, which allows the extraction of a non-prescribed number of surfaces of varying  
19 inclination, contrast, and texture from a 3D image. The tool requires the adjustment of a small set of control  
20 parameters, for which we provide an intuitive interface implemented as a Fiji plugin. As a proof of principle  
21 of the versatility of Zellige, we demonstrate its performance and robustness on synthetic images and on  
22 four different types of biological samples, covering a wide range of biological contexts.

23

## 24 Introduction.

25 The interplay between gene regulatory networks and physical forces in driving collective cell behaviors is  
26 key to tissue morphogenesis during development and to tissue homeostasis throughout life. Recent  
27 quantitative studies of epithelial morphogenesis have begun to unravel the basic cellular and physical  
28 principles of tissue development, by providing the tools to integrate multiple scales of tissue dynamics [1–  
29 4]. These tools are instrumental to quantify how cell shape changes, cell divisions, cell rearrangements and  
30 cell extrusions contribute to tissue remodeling, and to establish data-driven computational models of tissue  
31 morphogenesis.

32 Quantitative analysis of an epithelium starts with the extraction of its apical surface from 3D-  
33 microscopy images (z-stacks of xy-optical sections) encompassing the volume immediately surrounding the  
34 epithelium. However, this is a difficult task because this surface is usually not flat (it is best modelled as a  
35 curved surface, or 2D submanifold embedded in 3D space), and is often surrounded by other biological  
36 structures such as cell layers, acellular membranes, extracellular matrix, and vesicles that hamper its  
37 visualization and reconstruction. To make the surface extraction tractable, these studies rely on specific  
38 preparations of the specimen, allowing to expose the entire epithelial surface labeled with junctional  
39 fluorescent markers to reveal the network formed by epithelial cell-cell contacts. Once the epithelial surface  
40 has been extracted, automated cell segmentation and cell contour tracking tools can be used to follow the  
41 dynamics of every cell within the epithelium.

42 Another challenging experimental limitation of these studies is that some of the structures  
43 surrounding the epithelium exert external physical constraints that are known to critically affect epithelial  
44 morphogenesis by directing cellular dynamics and signaling pathways [1, 5–7]. To understand the physical  
45 forces controlling tissue morphogenesis, it is thus essential to also characterize how the dynamics of these  
46 extra-epithelial surfaces relate to that of the epithelium (see [8] for review). This calls for the development  
47 of dedicated tools allowing the automated extraction of information from several surfaces of interest in a  
48 given sample, since the sheer volume of the data precludes any attempt at a manual analysis.

49 Several surface extraction tools have been developed, some of which are available as open access  
50 software, such as PreMosa [9], FastSME [10], and LocalZProjector [11]. These tools focus on the extraction  
51 of a single, near-horizontal epithelial layer, which is assumed to (i) be sufficiently smooth, (ii) show enough  
52 contrast against surrounding background signals, and (iii) cover the entire image field-of-view. Specifically,  
53 it is assumed that the fluorescent marker used to label the epithelial cell network should provide the highest  
54 contrast in the image and allow to select it out from autofluorescent extracellular structures such as the  
55 cuticle in flies, or other acellular membranes in mammalian epithelia. The surface is then localized using  
56 heuristic algorithms based on the detection of the pixels of maximum contrast and/or brightness. However,  
57 applying these tools on more complex biological images with several epithelia of weaker contrast often  
58 leads to incorrect localization of the surface of interest, and its blending with the nearby unwanted  
59 biological structures.

60 MinCostZ on the other hand, is the only available open-source tool that allows the extraction of up  
61 to two surfaces from a 3D stack, and imposes explicit continuity constraints on the reconstructed surfaces.  
62 MinCostZ surface extraction relies on a previously developed formulation of the problem as a graph-cut  
63 optimization [12]. It is implemented as an ImageJ plugin [13], taking as control parameters, the number of  
64 surfaces to extract, the maximum slope and the range of distances allowed between the surfaces, as well  
65 as some user-defined cost function that should reflect the characteristics of the surfaces in term of signal  
66 intensity, contrast and texture. Despite its interest, this approach remains computationally costly and  
67 difficult to apply in practice due to the non-trivial choice of the cost function and the need to know  
68 beforehand the relative positions of the surfaces to be extracted.

69           Alternatively, one can segment the surfaces of interest by using supervised machine learning tools  
70 such as the software solutions Weka [14] or Ilastik [15], as proposed in the ImSAnE surface reconstruction  
71 framework [16]. A deep learning approach, using a network of the U-net type to segment the pixels  
72 belonging to a single surface of interest, has also recently been reported [17]. While promising as they can  
73 provide state of the art segmentations of epithelial surfaces in difficult imaging conditions, machine learning  
74 approaches require the prior manual annotation of a sufficiently large set of surfaces to generate suitable  
75 training sets. This process can be very time consuming, often necessitating several rounds of trials and  
76 errors to obtain satisfactory results, without guarantees to be generic, *i.e.*, to generalize to a wide range of  
77 datasets. So far, no solution to the multiple surface extraction problem has been proposed, which is  
78 satisfactory both in terms of genericity and ease of use.

79           However, such a tool is highly desirable for modern biology studies. Indeed, tissue organization in  
80 the context of developmental biology emerges from the interaction of several neighboring structures [8]  
81 through the interplay of molecular signals [18, 19], as well as electrical [20, 21], hydraulic [22, 23] and  
82 mechanical contact interactions [24, 25]. The importance of such interaction is exemplified in embryonic  
83 explanted tissue cultures that develop abnormally when separated from their neighboring structures [26].  
84 Similarly, in the context of tissue engineering, stem-cell derived aggregates harbor various types of tissues  
85 surrounding the genuine organoid, and these tissues presumably influence organoid shape, fate and  
86 differentiation (see [27] for review). The ability to simultaneously study the dynamics of neighboring  
87 structures together with the structure of interest is therefore essential for an integrated understanding of  
88 tissue development, and for any attempt to harness tissue self-organization *in vitro*.

89           Here, we introduce Zellige, a tool based on a novel constructive approach that allows the automatic  
90 extraction of a non-prescribed number of surfaces from a 3D image. To do this, the user is only required to  
91 adjust a small set of intuitive control parameters, a task largely facilitated by a user-friendly interface  
92 implemented as a plugin for the open-source Fiji platform [28]. We tested the performance and robustness  
93 of Zellige for multiple surface extraction by applying it to synthetic images and 3D microscopy images from  
94 four different types of biological samples, containing multiple surfaces of interest of widely varying texture  
95 and contrast. These experiments demonstrate the ability of the approach to extract several (up to 4)  
96 surfaces of potentially very low contrast, selectively from other highly contrasted and complex structures,  
97 with a single set of reconstruction parameters. A sensitivity analysis also reveals a high robustness of Zellige  
98 against small variations of these parameters. This will make it a tool of choice in terms of versatility and  
99 ease of use for the investigation of biological surfaces.

## 100 Results & discussion.

### 101 Proof of concept of multiple surface extraction on a synthetic image.

102 The implementation of Zellige is summarized in **Figure 1** and in the *Methods* section (**Figure S1**). **Figure 2**  
103 shows the results produced by Zellige on a phantom 3D image [29] containing three distinct synthetic  
104 surfaces generated as described in Supplementary note 2. The three surfaces are extracted with little errors.  
105 We assessed the quality of the reconstruction by comparing each of the height-maps produced by Zellige  
106 to the corresponding ground truth (GT) height-map, which is exactly known in this case (**Figure 2A-C**, and  
107 Supplemental note 3). For the three surfaces, the reconstruction has subpixel accuracy over >99% of the  
108 GT pixels (**Figure 2D-E**), with a root mean square error (RMSE) of  $\leq 0.6$  in pixel units, showing that the  
109 surface localization is highly accurate. In addition, the coverage, which measures the proportion of the  
110 reconstructed surface relative to the GT, is near 100% for the three surfaces. To achieve these results, the  
111 control parameters of the two steps of the surface extraction were adjusted manually to some adequate  
112 reference values (see Supplementary Table S1) using the Zellige Fiji interface. Only the parameters  
113 controlling the pixel classification step (amplitude and Otsu threshold parameters  $T_A$  and  $T_{otsu}$ , minimal  
114 island size  $S_{min}$ , and smoothing parameters  $\sigma_{xy}$  and  $\sigma_z$ ) did actually require a modest adjustment. The  
115 parameters of the surface assembly step (parameters  $T_{OSE1}$ ,  $R_1$ ,  $C_1$  and  $T_{OSE2}$ ,  $R_2$ ,  $C_2$  of the 1<sup>st</sup> and 2<sup>nd</sup>  
116 construction rounds, respectively) were set to their default reference values (see **Figure S2** and  
117 Supplemental note 4) and did not need to be adjusted.

118 Thus, using a single set of control parameters, Zellige can extract multiple surfaces of various  
119 shapes and textures with virtually no error, without requiring the user to provide information about their  
120 number or relative position, nor about their shape or texture characteristics.

### 121 Performance of Zellige on biological samples.

#### 122 Example 1: Extracting multiples surfaces from an image of a pupal fly specimen.

123 Over the past few decades, the *Drosophila* model has been invaluable to decipher the molecular and cellular  
124 mechanisms underlying organ embryogenesis [30, 31]. Epithelium morphogenesis studies not only revealed  
125 the importance of mechanical stresses (including stress boundary conditions) and planar polarity signaling  
126 on cell dynamics to generate tissues of reproducible sizes and shapes, it also highlighted the importance of  
127 extracellular matrix attachments in constraining the tissue stresses that guide patterning [1, 32]. At the  
128 pupal stage, the fly undergoes dramatic remodeling of its larval organs into adult organs. Large scale tissue  
129 flows initiate at a timing that coincides with molting, when the epithelium contracts away from the  
130 overlying cuticular sac, a protective acellular membrane that imposes mechanical boundary conditions to  
131 the tissue.

132 **Figure 3** shows the results of applying Zellige on a 3D image of a *Drosophila* pupa acquired with a  
133 spinning disk confocal microscope [33]. The sample expresses Ecadherin-GFP, a fluorescent marker of cell-  
134 cell junctions, and encompasses a portion of the pupa's abdomen and a small portion of its wing. Four  
135 surfaces of interest can be identified, with varying signal intensities, noise levels and features (**Figures 3A-**  
136 **B**). The abdomen is formed of an epithelium (surface S2) overlaid by a cuticle (surface S1). Lying just beneath  
137 these two surfaces, one can observe globular structures showing in some places a higher intensity than the  
138 signal coming from the surfaces. The wing also consists of an epithelium of low intensity signal (surface S4),  
139 and an overlying cuticle (surface S3). These two surfaces are relatively flat, except for surface S3 which is  
140 very steep near one of its edges.

141 **Figure 3C** shows a 3D graphical representation of the height-maps reconstructed by Zellige (green)  
142 and those reconstructed by an expert biologist (blue), taken as ground truth (GT). While these height-maps  
143 clearly show greater roughness than those of the synthetic surfaces presented earlier, they could again be  
144 obtained with a single set of control parameters that were adjusted manually with the Zellige interface (see  
145 **Supplementary Table S1**). We observe an excellent match between the four reconstructed and  
146 corresponding GT height-maps, despite the rather complex topography of surfaces S1 and S2 (with slopes  
147 reaching up to 45°), and the near-vertical inclination of surface S3 at its boundary. Yet, small deviations may  
148 be seen in the regions of highest slope of the surfaces. Some of these deviations are likely attributable to  
149 uncertainties in the definition of the GT height-maps, whose accuracy depends on the expert.

150 **Figure 3D** shows the differences between the reconstructed and GT height-maps, plotted as color-  
151 coded error maps. These differences are  $<2$  (in pixel units) for most pixels, while some regions of higher  
152 error values can be seen locally in surfaces S1 and S2, and at the boundary of surface S3. Note that for  
153 surfaces S2 and S4, which contain junctional epithelial meshes composed of larger and smaller cells,  
154 respectively, the GT height-map encompass not only the mesh but also the interior of the cells, where no  
155 junctional signal is detected. The distance calculated inside the cells is thus more subjected to intensity  
156 fluctuations, especially for surface S2. Nonetheless, the RMSEs of surfaces S1, S2 and S4 are less than 1,  
157 showing that on average the reconstructed height-maps match the corresponding ground truth with  
158 subpixel accuracy. The higher RMSE (1.25) of surface S3 is largely due to the region of steep region at the  
159 edge of this surface (yellow region on the error map for this surface, **Figure 3D**). The coverage of the  
160 reconstructed height-maps is excellent ( $\geq 96\%$ ) for surfaces S1 and S2, and slightly lower, but still very good  
161 ( $\geq 93\%$ ) for the smaller surfaces S3 and S4. **Figure 3E** shows the 2D projections of the 3D image obtained  
162 for each of the reconstructed surfaces and for the corresponding ground-truths. The inaccuracies visible on  
163 the error maps (see **Figure 3D**) do not significantly impact these projections, which appear very similar to  
164 the projections obtained with the corresponding ground-truths. Thus, while the biological sample contains  
165 significant noise and shows a much more variable contrast (especially with the presence of high intensity  
166 globular structures near surfaces S1 and S2), Zellige makes it possible to segment these surfaces selectively,  
167 with a quality of segmentation comparable to that obtained by manual expert segmentation.

168 This possibility brings several perspectives that are not offered by single surface extraction  
169 algorithms. First it opens the possibility to systematically study the tissue axial movements (along z) relative  
170 to the cuticle during molting, allowing for example to gain insights into the early tissue contraction of the  
171 wing hinge that acts as a mechanical inducer over the wing blade [8]. Second, Zellige makes it possible to  
172 automatically extract structures such as the abdomen epithelium, which is usually segmented manually  
173 [34], due to the difficulty to separate the large larval cells from the cuticle mesh and from other globular  
174 structures (such as fat bodies or macrophages) present underneath the epithelium. All these structures  
175 become intertwined when using other extracting tools. In this context, Zellige opens new opportunities to  
176 study collective cell behavior during epithelial morphogenesis *in vivo*, and to integrate in the analysis the  
177 surrounding surface-like structures involved in the mechanics of the system.

## 178 **Example 2: Extracting a thin cochlear epithelium surface from a multilayer dataset.**

179 As the first model in which planar cell polarity signalling was shown to be conserved in vertebrates [35], the  
180 mammalian auditory organ, the cochlea, is arguably our most valuable model to study epithelial patterning  
181 and morphogenesis beyond the fly and zebrafish [36, 37]. Cochlear morphogenesis involves complex and  
182 tightly controlled patterning processes during which the cochlear sensory epithelium extends and develops  
183 its characteristic coiled snail shape, while adopting a striking cellular mosaic organization, with graded  
184 changes of morphogenetic parameters along the cochlea [38, 39]. These morphogenetic processes are well  
185 recapitulated in organotypic cultures, on the condition that the mesenchyme that underlies the epithelium

186 be preserved. The cultures are then amenable to live imaging [37, 40], pharmacological [41] and genetic  
187 manipulations.

188 **Figure 4A** shows a confocal swept field microscope acquisition of an embryonic mouse cochlea  
189 [42]. The sample contains only one surface of interest, the cochlear epithelium, but this surface lays on top  
190 of a thick tangled mesh of non-epithelial cells originating from the mesenchyme. The whole biological tissue  
191 is stained for filamentous actin (F-actin) using phalloidin. The epithelium surface presents a non-uniform  
192 signal included in a small z-range ( $6 \leq z \leq 10$ ), and a mesh of very heterogeneous size. Between sections  
193  $z = 10$  and  $z = 14$  one can observe the basolateral region of the epithelial cells, also stained for F-actin. The  
194 particularity of this sample is that the mesenchyme presents an intense and contrasted signal over a wide  
195 range of z-values ( $14 \leq z \leq 43$ ). This makes it challenging to extract the surface of the epithelium, which is  
196 characterized by low intensity and low contrast.

197 **Figure 4B** shows a 3D representation of the height-map reconstructed by Zellige and the  
198 corresponding GT height-map (again reconstructed manually by an expert). On the corresponding error  
199 map (**Figure 4C**), most (~83%) pixels of the reconstructed height-map show subpixel accuracy (with  
200 distances  $< 1$  to the corresponding pixels of the GT height-map). The errors are greater in regions where  
201 the cell size is larger, as well as in the area where the signal intensity is very low. However, they remain  
202 smaller than 2 for  $> 95\%$  of the pixels. This result is consistent with the low value of the RMSE (1.1). The  
203 surface is also reconstructed with an excellent coverage ( $> 99\%$ , **Supplementary Table S1**).

204 As the sample contains a single epithelial surface of interest, we compared the performance of  
205 Zellige with three other software that can extract only a single surface (**Figure 4D**). The projections of  
206 PreMosa, FastSME and LocalZProjector completely miss the epithelium. Only regions of high contrast  
207 corresponding to the mesenchyme are projected. In contrast, Zellige generates a projection very close to  
208 the ground truth. This demonstrates the efficiency of Zellige to selectively extract a low contrast surface,  
209 despite the presence of several structures of higher contrast. Indeed, Zellige detects every structure as a  
210 possible surface seed without any assumption on its contrast, and only extends this seed into a surface if  
211 enough spatial continuity is found in the surrounding signal. This feature allows to separate individual  
212 surfaces from other structures spatially, which should greatly facilitate the analysis of live imaging  
213 experiments.

### 214 **Example 3: Extracting a single bronchial epithelial surface rendered abnormally rough by SARS-CoV-2.**

215 Recently, we used Zellige to extract the surface of a primary culture of bronchial epithelial cells following  
216 infection by the SARS-CoV-2 virus [43]. The infection causes the surface of the epithelium to become  
217 abnormally rough due to cell damages as seen from discontinuities within the cell layer. The sample we  
218 chose from this study is a 3D confocal image of the epithelium responding to SARS-CoV-2 infection [44]  
219 (**Figure 5A**). The surface of interest in this image corresponds to the layer of epithelial cells stained for the  
220 tight junction protein Zona Occludens-1 (ZO-1). The surface roughness causes the network of junctions to  
221 extend over the height of the z-stack, with a signal of varying intensity (**Figure 5A**). In addition, the junctional  
222 network remains non-planar even at the level of a single cell, hence violating the smoothness condition  
223 commonly assumed to hold in the context of epithelial surface extraction. We also observe the presence of  
224 nearby punctiform structures of high contrast that are mainly located outside of the epithelium surface.  
225 This sample therefore provides an example of a surface with a complex landscape, interspersed with a  
226 constellation of signals which may interfere with the surface segmentation.

227 The 3D representation of the reconstructed and corresponding GT height-maps (**Figure 5B**) makes  
228 it possible to appreciate the roughness of the surface of interest. The two height-maps overlap quite  
229 satisfactorily. As shown on the error map (**Figure 5C**), a large majority (71.1%) of pixels of the reconstructed

230 height-map show errors smaller than 1 pixel (~ 96% of them showing errors smaller than 2 pixels). The error  
231 is however larger in regions where the cell size is larger, as well as in areas where the ZO-1 signal intensity  
232 is very low, preventing a complete reconstruction of the junctions. Nevertheless, the overall RMSE remains  
233 small, with a value of 0.81 (**Figure 5**). The coverage of the reconstructed surface is also excellent (98% of  
234 the GT height-map), despite the above-mentioned discontinuities. **Figure 5D** shows the comparison of the  
235 projection generated by Zellige to those produced by PreMosa, FastSME, and LocalZProjector. The  
236 projection generated by PreMosa misses many junctions of the epithelial network, but it removes quite  
237 well the punctiform signal originating from other optical sections. FastSME performs better than PreMosa  
238 in reconstructing the junctions, but they produce a projection where the punctiform signal remains strong.  
239 In contrast, Zellige and LocalZProjector manage to both reconstruct the surface well and to filter out the  
240 punctiform signal quite effectively. This result demonstrates the efficiency of Zellige to extract a surface  
241 with complex topography by excluding intense and contrasted spurious signals away from the epithelium  
242 surface.

#### 243 Example 4: Extracting the apical and basal layers of a dome-shaped epithelium (developing inner ear 244 organoid).

245 Organoids are stem cell-derived and self-organizing 3D tissue structures that can mimic certain organ  
246 structures. They have emerged as promising *in vitro* models for developmental biology research, as well as  
247 biomedical translational research applications. Here we take the example of mouse stem cell derived inner  
248 ear organoids that form vesicular structures composed of an epithelium harboring sensory cells. These  
249 organoids are part of a cellular aggregate that also contains other tissues such as the mesenchyme [45]  
250 adjacent to the organoids. The epithelial cells of the forming inner ear organoids acquire a basal-apical  
251 polarity, with their apical side facing the lumen of the organoid, and their basal side facing outwards. The  
252 apical junctional network of the epithelium is difficult to visualize in microscopy images as it is seen from  
253 below, through the basal layer. Another difficulty is the spherical geometry of the vesicle system, which  
254 makes the epithelial surface of interest difficult to extract in regions of high inclination relative to the focal  
255 plane.

256 **Figure 6** shows the result of applying Zellige on a 3D confocal microscopy image of half of a  
257 developing inner ear organoid at 14 days of culture, a stage at which markers characteristic of the mouse  
258 otic vesicle can be detected [46]. The sample was fixed and stained for F-actin to visualize all cellular  
259 structures including the epithelium. Two surfaces of interest can be identified, namely the basal side of the  
260 epithelium and the apical junctional network (**Figure 6A-B**). Both surfaces are mesh-like structures of high  
261 inclination, high signal intensity and high contrast. The vesicle lumen also contains cell debris of high  
262 intensity and contrast that are not part of any surface of interest.

263 **Figure 6C** shows a 3D graphical representation of the height-maps reconstructed by Zellige and  
264 those reconstructed by an expert biologist, taken as ground truth (GT). Due to their dome-shaped  
265 topography, the manual segmentation of these surfaces was rather laborious, and is more likely prone to  
266 errors in the regions of high inclination. Despite this, we observe an excellent match between the two  
267 reconstructed and corresponding GT height-maps (**Figure 6D**). The distance between the two height maps  
268 is <2 for the large majority (> 92%) of pixels, while larger error values occur locally in regions of near-vertical  
269 inclination of the surfaces. The RMSEs of both apical and basal surfaces are close to 1, showing that on  
270 average the reconstructed height-maps match the corresponding GT with about pixel accuracy. The  
271 coverage of the reconstructed height-map is nearly 100% for the basal surface, and ~88% for the apical  
272 surface. **Figure 6E** shows the 2D projections of the 3D image obtained for each of the reconstructed  
273 surfaces, as compared to the projected GT height maps. Thus, for the extraction for these highly inclined



274 surfaces, Zellige produces height maps of a quality comparable to those obtained from manual expert  
275 segmentation.

276 In this example, Zellige could be combined with a 2D cell tracking framework such as TissueMiner  
277 [3] to perform cell dynamics analysis. Note that geometric distortions introduced in the projected surfaces  
278 by the epithelium inclination could be corrected for, using complementary tools such as DProj [11]. This  
279 approach could provide a means to quantitatively address how an inner ear organoid epithelium patterns  
280 at the cellular and organoid scales, while quantifying the epithelial thickness changes due to cellular  
281 intercalation or cell shape changes in the depth of the epithelium. This would also permit to better  
282 characterize the variability of inner ear organoids within in a given aggregate, and it could allow one to  
283 explore how the organoid interacts with surrounding tissues and how these interactions influence the  
284 differentiation of their constituent sensory cells.

## 285 A sensitivity analysis reveals the robustness of Zellige in extracting surfaces from 286 biological images.

287 To evaluate further the quality and robustness of the segmentation obtained by Zellige, we carried out a  
288 sensitivity analysis of the reconstruction on each of the samples tested. This analysis consisted in varying  
289 one control parameter at a time (**Figures S2-S6, Figure 7**, Supplemental note 4), while keeping the other  
290 parameters fixed at a nominal value (Supplementary Table S1). The RMSE and coverage of each of the  
291 reconstructed surfaces were evaluated and plotted as a function of the value of the parameter that was  
292 varied.

293 **Figure S3** shows the results of the sensitivity analysis carried out on the image of **Figure 3**  
294 (Example 1, pupal fly specimen), when varying the parameters controlling step 1, *i.e.* the selection of  
295 putative surface pixels (parameters  $T_A$ ,  $T_{otsu}$ ,  $S_{min}$ ,  $\sigma_{xy}$  and  $\sigma_z$ ). As can be seen, the variations of the two  
296 classification threshold parameters  $T_A$ ,  $T_{otsu}$  and of the minimum island size  $S_{min}$  in their respective intervals  
297 does not substantially modify the RMSE and the coverage of the reconstructed surfaces, whose values  
298 remain roughly constant for  $T_A \leq 8$ ,  $T_{otsu} \leq 12$  and over the entire  $S_{min}$  interval (**Figure S3A**). Within these  
299 intervals, the RMSEs of all the reconstructed surfaces remain  $\leq 1.5$ , while the coverage values are  $> 95\%$  for  
300 surfaces S1 and S2, and  $> 85\%$  for surfaces S3 and S4. The surfaces S1 and S3 show lower signal intensity  
301 and lower contrast than surfaces S2 and S3, making them more difficult to extract. Surface S4 has the lowest  
302 contrast, and fails to be reconstructed if the classification threshold values are too stringent (namely for  
303  $T_{otsu} > 12$ , or  $T_A > 8$ ). Nevertheless, the intervals of stability of  $T_A$  and  $T_{otsu}$  (that is, the intervals of values  
304 over which a high-quality extraction of all surfaces is obtained) remain relatively wide (cf. **Figure 7**). The  
305 smoothing parameters  $\sigma_{xy}$  and  $\sigma_z$  also have some effect on the reconstruction of the surfaces. When  $\sigma_{xy}$  is  
306 less than 3, the RMSE is higher for the mesh-like epithelial surfaces S2 and S4 (formed by the junctional  
307 network of the epithelium). A minimal smoothing along the axial direction is also important to ensure that  
308 the reconstructed surfaces are not too fragmented, preventing their complete reconstruction. Yet,  $\sigma_z$   
309 should not be chosen too large either, to avoid merging nearby surfaces along the z axis. In this case, the  
310 closely positioned surfaces S1 and S2 are well-separated if setting  $\sigma_z$  close to 1, but they become merged  
311 when  $\sigma_z > 2$ . In general, the surface construction parameters have little effect on RMSE and coverage  
312 (**Figure S3B**). The sensitivity analysis on this challenging specimen shows a good robustness of Zellige to  
313 extract the four surfaces with a single set of parameters, each of which can be chosen in a reasonably wide  
314 interval considering the other fixed.

315 The results of the sensitivity analyses performed with the other biological image stacks  
316 (examples 2, 3 and 4 described above) are shown in **Figures S4, S5** and **S6** (Supplemental note 4). These  
317 results are summarized in **Figure 7**, which show the stability intervals over which the extracted height-maps

318 satisfy the criteria  $RMSE \leq 1.5$  and coverage  $\geq 85\%$ , for which the reconstruction can be considered of high  
319 quality. Overall, the stability intervals for the two classification threshold parameters ( $T_A$  and  $T_{otsu}$ ) are  
320 narrower for specimens containing a surface of low signal intensity and low contrast, while still covering  
321 about 1/4 of their respective width. The graphical user interface of Zellige allows the user to adjust  $T_A$  and  
322  $T_{otsu}$  interactively, making it intuitive to search for reasonable values. We found that  $2 \leq \sigma_{xy} \leq 3$  and  $\sigma_z = 1$   
323 generally give high quality results for all tested specimens (**Figure 7A**). We therefore expect only little  
324 adjustment to be required by the user on the smoothing parameters from their default permissive values  
325 (set to  $\sigma_{xy} = 1$  and  $\sigma_z = 1$ ). Values of  $\sigma_z$  that are too large may lead to the merging of a surfaces with a nearby  
326 structure of high contrast (surface or else), as it happens for the epithelium surface in the cochlea specimen,  
327 which merges with the underlying mesenchyme signal when  $\sigma_z$  is greater than 2 (**Figure 4 and Figure S4**).  
328 The effect is even more pronounced for surfaces of high inclination (**Figures 2, 6 and Figures S2, S6**), or  
329 presenting a particularly rough texture (**Figures 5 and Figures S5**).

330 Regarding the control parameters of step 2 (the assembly step), for the four examples presented  
331 except for example 1, the quality of the reconstructed surfaces is stable and high over the main part of their  
332 respective intervals of variation, deteriorating occasionally only when extreme values ( $\approx 1$ ) are used for  
333 these parameters (see **Figures S2-S6, Figure 7B** and Supplemental note 4). Example 1 poses particularly  
334 stringent constraints on the control parameters of the reconstruction due to the requirement of  
335 reconstructing the 4 surfaces of different contrast and texture present in this sample using the same set of  
336 parameters.

337 Finally, the computation times for running Zellige on a given dataset ranged between a few seconds  
338 and a minute on a standard PC computer (see **Figure S7** and Supplementary note 5), except in a few  
339 exceptional cases corresponding to extreme values of the control parameters. As a safeguard, a stopping  
340 criterion could be implemented so as to exit the run (declaring the current parameter values invalid) if the  
341 surface assembly computation exceeds a user-prescribed duration.

342 Overall, the sensitivity analysis indicates that the surface extraction performed by Zellige is robust  
343 to variations of the control parameters of step 1 (surface pixel selection step). In general, the reconstruction  
344 is more sensitive to the amplitude threshold parameter ( $T_A$ ), and the Otsu threshold parameter ( $T_{otsu}$ )  
345 should be kept sufficiently low for samples containing surfaces of intensity close to the background.  
346 However, in some cases such as in our example 3, the opposite is true. Thus, the two threshold parameters  
347 play somewhat complementary roles, and the possibility to adjust them independently is useful in practice  
348 to be able to cover as many cases as possible. A smoothing along  $xy$  appears necessary to correctly  
349 reconstruct the surfaces supported by a junctional mesh. Not surprisingly, best results are obtained when  
350 the radius of the gaussian filter used for this (parameter  $\sigma_{xy}$ ) is adapted to the mesh (or cell) size. Likewise,  
351 a smoothing along  $z$  is beneficial, but the extent of this smoothing (parameter  $\sigma_z$ ) should not be too large  
352 to avoid causing the fusion of nearby surfaces. With a few exceptions, the values of the RMSE and coverage  
353 show little sensitivity to the values of the parameters controlling step 2 (surface assembly step), at least  
354 once putative surface pixels have been properly selected. In the presence of several surfaces of potentially  
355 very different sizes, the parameter controlling the fraction of OSE sizes allowed for OSE seeds (parameter  
356  $T_{OSE1}$ ) should be relatively large ( $\geq 0.5$  or greater, *i.e.* allowing more than 50% of the largest OSE sizes for  
357 seeds) to allow the extraction of a surface of small size (for example, to extract the surface n° 4 of  
358 example 1, which covers less than 20% of the  $xy$ -field of view,  $T_{OSE1}$  must be larger than 0.6). Extreme values  
359 (close to 1) for the connectivity rates (C1 and C2) are too stringent and lead to a drop in the coverage of the  
360 reconstructed surfaces. To sum up, we see that the most critical parameters for a satisfactory extraction of  
361 the different surfaces are those controlling step 1. In most cases the parameters controlling step 2 do not  
362 need to be adjusted and can be fixed to their default reference values.

363

## 364 Conclusion.

365 We have developed Zellige, a new tool to extract multiple surfaces from 3D fluorescence microscopy  
366 images. Zellige automatically finds surfaces by first identifying putative pixels that are likely to belong to a  
367 biological surface, and second by assembling a surface through connection of adjacent pixels satisfying  
368 natural proximity constraints. By using Zellige on synthetic epithelium images we have shown that it  
369 accurately reconstructs a surface with excellent performances in terms of both the distance to the ground-  
370 truth height-map and the surface coverage (**Figure 2**). Zellige can deal with complex images containing  
371 multiple surfaces, with computation times not exceeding a few tens of seconds on a standard computer.  
372 Importantly, the user is not required to specify the number of surfaces to be extracted. In the *Drosophila*  
373 specimen (**Figure 3**), the software readily extracts the 4 surfaces of interest that could be identified. Since  
374 Zellige detects putative surface pixels in the first step by combining local and global thresholds, it can deal  
375 with images where the multiple surfaces display different features, such as in the mouse cochlear embryo  
376 (**Figure 3**). With this difficult dataset, we could also confirm Zellige's robustness against very low signal-to-  
377 noise levels. The constructive approach of surface region growing used by Zellige in its second step enables  
378 it to circumvent the surface smoothness requirement, that is classically assumed by other surface extraction  
379 tools. For instance, it could reconstruct the highly irregular surface of a bronchial tissue infected by SARS-  
380 CoV-2 (**Figure 5**).

381 The robustness and flexibility of Zellige come at a price, namely, the requirement to specify 12  
382 parameters when running the surface extraction. However, the sensitivity analysis we performed shows  
383 that adjusting only 4 of these parameters is enough in practice to handle a wide range of image types. These  
384 parameters correspond to intuitive notions (*e.g.*, thresholding and smoothing), which makes Zellige  
385 particularly easy to use. The Fiji interface that we implemented to perform this adjustment should make  
386 Zellige even more user-friendly and effective for biological applications.

387 To our knowledge, Zellige is the only open-source tool that can extract an unspecified number of  
388 epithelial surfaces from a 3D volume, possibly larger than two. This unique feature is especially useful in  
389 complex images that could be processed only by specialized tools before. For instance, Zellige can extract  
390 surfaces with projections on the *xy* plane that completely overlap, such as the basal and apical epithelia in  
391 the organoid image of **Figure 6**. Previously, such an image could be processed only by tools that relied on  
392 segmenting a mesh around the object surface, such as MorphoGraphX or ImSAnE [16, 47].

393 The flexibility and robustness of Zellige should allow to considerably relax the constraints that were  
394 previously imposed on the sample preparation and the image acquisition steps by the subsequent analysis.  
395 Indeed, Zellige can accommodate any number of surfaces in the acquired volume, overlapping or not, and  
396 of different contrast features. Zellige also showed excellent robustness against image noise. This should  
397 make it particularly useful in imaging contexts that are not easily amenable to automated analysis, such as  
398 intravital imaging. Finally, it is worth noting that Zellige is a generalist and modular method. With some  
399 adaptation of the surface pixel selection step, it could be used with imaging modalities beyond the scope  
400 of this article, for instance, in extracting the irregular and noisy surfaces of biological objects imaged with  
401 3D electron microscopy images.

402

403

## 404 Methods.

### 405 Implementation.

406 Zellige was devised with the goal of achieving accurate segmentation of multiple biological surfaces from  
407 3D confocal images. Unlike other existing surface extraction tools, it makes no assumption on the number  
408 of surfaces to be extracted and does not require the surfaces of interest to be the structures of highest  
409 contrast in the image. Zellige is written in Java, relying on the ImgLib2 library [48] and is distributed as a Fiji  
410 plugin, with a graphical user interface (GUI) designed to allow users to quickly find a good set of extraction  
411 parameters for a given image.

412 Zellige extracts each surface present in the image in the form of a height-map (or z-map), that is, a  
413 mapping

$$414 \quad z: (x,y) \rightarrow z(x,y),$$

415 which associates to each point  $(x,y)$  over which the surface projects, the z-coordinate of the unique pixel  
416  $(x,y,z)$  belonging to the surface. Each extracted height-map is then used to produce a 2D projection of the  
417 3D stack restricted to a small sub-volume (of user-selected width) centered around the corresponding  
418 surface. To achieve a robust extraction, Zellige proceeds in two algorithmic steps, which are only outlined  
419 below (see Supplementary note 1 for implementation details).

420 In the first step, or *surface pixel selection* step, a segmentation is applied to the 3D image to select  
421 pixels that likely belong to a surface of interest (**Figure 1 and Figure S1A**). These putative surface pixels are  
422 detected as local maxima of image intensity along the z-axis, after using two independent binary classifiers,  
423 one based on pixel contrast and the other one on pixel intensity. Five adjustable parameters control the  
424 selection step: two threshold parameters ( $T_A$  and  $T_{otsu}$ ) control the strength of the binary classifiers applied  
425 on contrast and intensity, respectively, and three parameters ( $S_{min}$ ,  $\sigma_{xy}$  and  $\sigma_z$ ) control clean-up operations  
426 applied at the end of the classification (removal of small isolated spots, and local averaging along the  $xy$   
427 plane and the  $z$  axis, respectively).

428 In the second step, or *surface assembly step*, an iterative algorithm is used to extract the height-  
429 maps of each of the surfaces present in the image (**Figure 1 and Figure S1A**). The assembly starts by  
430 grouping neighboring putative surface pixels together within each orthogonal ( $xz$  or  $yz$ ) section of the 3D  
431 image, in order to form a set of building blocks referred to as *orthogonal surface elements* (OSEs). These  
432 building blocks are then used to assemble the surfaces, in a process analogous to jigsaw puzzles, where  
433 OSEs adjacent to the surface boundary are added if they match this boundary, and rejected otherwise, until  
434 no matching OSE can be found (**Figure S1**). In order to increase the robustness of the assembly step, Zellige  
435 applies it in two rounds, proceeding along different axes during the first and second rounds. Each round is  
436 controlled by 3 adjustable parameters: a threshold parameter ( $0 \leq T_{OSE} \leq 1$ ) sets a minimum size for the  
437 building blocks that can be used as seeds to initiate the assembly of a surface; and two other parameters  
438 ( $0 \leq R \leq 50$  and  $0 \leq C \leq 1$ ) set the matching constraints used to accept or reject the addition of OSEs to a  
439 surface. The assembly step is thus controlled overall by 6 parameters, *i.e.* two groups of 3 parameters ( $T_{OSE1}$ ,  
440  $R_1$ ,  $C_1$ ) and ( $T_{OSE2}$ ,  $R_2$ ,  $C_2$ ) controlling the first and second assembly rounds, respectively.

441 Finally, the height-maps of each of the reconstructed surfaces are used to obtain a corresponding  
442 2D projection (**Figure 1**). In practice a maximum projection restricted to a subvolume of width  $\delta z$  ( $\delta z$  being  
443 a user-defined parameter) centered around the surface of interest is performed.

#### 444 Availability of data and code.

445 The project homepage below contains the source code, installation instructions, and documentations.

#### 446 Zellige

- 447 • Project name: Zellige.
- 448 • Project homepage: <https://gitlab.pasteur.fr/ida-public/zellige-core>
- 449 • URL for the Fiji plugin (Update > Manage update sites): <https://sites.imagej.net/Zellige/>
- 450 • Gitlab Branch: master
- 451 • Operating systems: Platform independent.
- 452 • Programming language: Java.
- 453 • Compiled in Java8
- 454 • Other requirements : Runs from Fiji [28].
- 455 • License: BSD 2
- 456 • Any restrictions to use by non-academics: None.

#### 457 Scripts to create Phantoms

- 458 • Project name: Phantoms.
- 459 • Project homepage: <https://doi.org/10.5281/zenodo.6414596>
- 460 • Operating systems: Platform independent.
- 461 • Programming language: MATLAB.
- 462 • License: BSD 2
- 463 • Any restrictions to use by non-academics: None.

#### 464 Data sets.

465 Image 3D stacks, ground-truth height map and height maps produced by Zellige are available on Zenodo  
466 under the CC-BY license: <https://zenodo.org/communities/zellige/> [29, 33, 42, 44, 46].

#### 467 *Human* bronchial epithelium imaging.

468 The data used in Figure 5 were taken from the recent study [43] to which we refer for the preparation and  
469 imaging of human bronchial epithelium cultures. In brief, MucilAir<sup>TM</sup> were purchased from Epithelix (Saint-  
470 Julien-en-Genevois, France) and cultured for at least 4 weeks to reconstruct a differentiated human  
471 bronchial epithelium *in vitro* and stained as previously described. Images of the cultures were acquired  
472 using an inverted Zeiss LSM 710 confocal microscope controlled by the ZEN pro 2.3 software. Z-stack images  
473 of whole-mount samples were acquired with a Zeiss Plan Apochromat 63x oil immersion lens (NA=1.4). The  
474 image used here was published in Robinot *et al.* [43] under the CC-BY-4 license.

#### 475 *Drosophila* imaging.

476 Flies were raised at 25°C under standard conditions. Pupae were collected for imaging as described  
477 previously [49]. Ecad::GFP flies [50] were used for live imaging as previously described [1]. In brief, images  
478 were acquired with a spinning disk microscope from Gataca Systems driven by the MetaMorph software.  
479 The system is equipped with an inverted Nikon TI2E stand, a motorized XYZ stage, and a Nikon Plan Apo 60x  
480 oil immersion (NA=1.4) lens and with a Prime95B camera.

## 481 Cochlea imaging.

482 The inner ears from wild-type (C57BL/6) mice were rapidly dissected from temporal bones at embryonic  
483 stages E14.5 in HEPES-buffered (10 mM, pH 7.4) Hanks' balanced salts solution and fixed in 4%  
484 paraformaldehyde, 1 hour at room temperature. Specimen were permeabilized and stained for phalloidin-  
485 Atto 565 (Sigma) as previously described [39]. Fluorescence images were obtained with a swept-field  
486 confocal microscope (Opterra II) from Brucker. This system is equipped with a Nikon Plan Fluor 60x oil  
487 immersion lens (NA=1.4).

488 Biologists in this study hold a designer certificate of animal experimentation (level 1), allowing them  
489 to perform experimental work on animals in strict accordance with the European directive 2010/60/EU, and  
490 French regulations. The Ethics Committee of the Institut Pasteur (Comité d'Ethique en Experimentation  
491 Animale - CETEA) has approved this study with the project identifier dha170006. This approval is based on  
492 careful compliance to the 3Rs principle in the care and use of animals (Annex IV - 2010/60/EU).

## 493 Inner ear organoid imaging.

494 ESCs derived from blastocyst-stage embryos of R1 mice (mESCs) (ATCC, SCRC-1036) were maintained in  
495 feeder-free culture on 0.1% w/v gelatin (Sigma) coated substrates using LIF-2i medium as established  
496 previously [51]. The organoids were generated following the previously published protocol [45, 51].  
497 Aggregates were harvested at day 14 and fixed in 4% v/v PFA (Electron Microscopy Sciences) overnight at  
498 4°C. After blocking (PBS; 10% v/v normal goat serum; 0.1% v/v Triton X-100), the aggregates were stained  
499 for phalloidin Atto 565 (1:1000) (Sigma) overnight at RT on a shaker, and washed three times with PBS  
500 containing 0.1% v/v Triton X-100 for 1 h each at RT. Prior to imaging, the aggregates were incubated in a  
501 modified version of ScaleS solution containing 4 M Urea (Sigma), 40% w/v D-Sorbitol (Sigma), and 0.1% v/v  
502 Triton X-100, for 3-5 days to clarify the tissue. Finally, the aggregates were whole-mounted using the ScaleS  
503 solution and imaged using a confocal laser scanning microscope (A1R HD25, Nikon) equipped with a Nikon  
504 25x silicon oil immersion lens (NA=1.05).

## 505 Acknowledgements.

506 We thank Maia Brünstein of the Hearing Institute Bioimaging Core Facility - C2RT/C2RA and Florian Ruckerl  
507 of the Photonic Biolmaging - C2RT/C2RA, for sharing their expertise on light microscopy. We thank Romain  
508 Levayer for sharing flies and lab space with us. We thank Gilles Trébeau ([https://fr.linkedin.com/in/gilles-  
509 trebeau-427601212](https://fr.linkedin.com/in/gilles-trebeau-427601212)) for helpful discussions regarding the Java frontend deployment of Zellige. We thank  
510 Lisa Chakrabarti, Rémy Robinot and Vincent Michel for sharing data. This work was supported by *Fondation  
511 pour l'Audition* (FPA-IDA-STARTING-GRANT); *Institut Pasteur* (PTR#272); DIM ELICIT's grant from *Région Ile-  
512 de-France* (QP-IDF-DIM-ELICIT-2019) ; The French *Agence Nationale de la Recherche* (ANR-21-CE13-0038-  
513 013-01 and LabEx LIFESENSES ANR-10-LABX-65).

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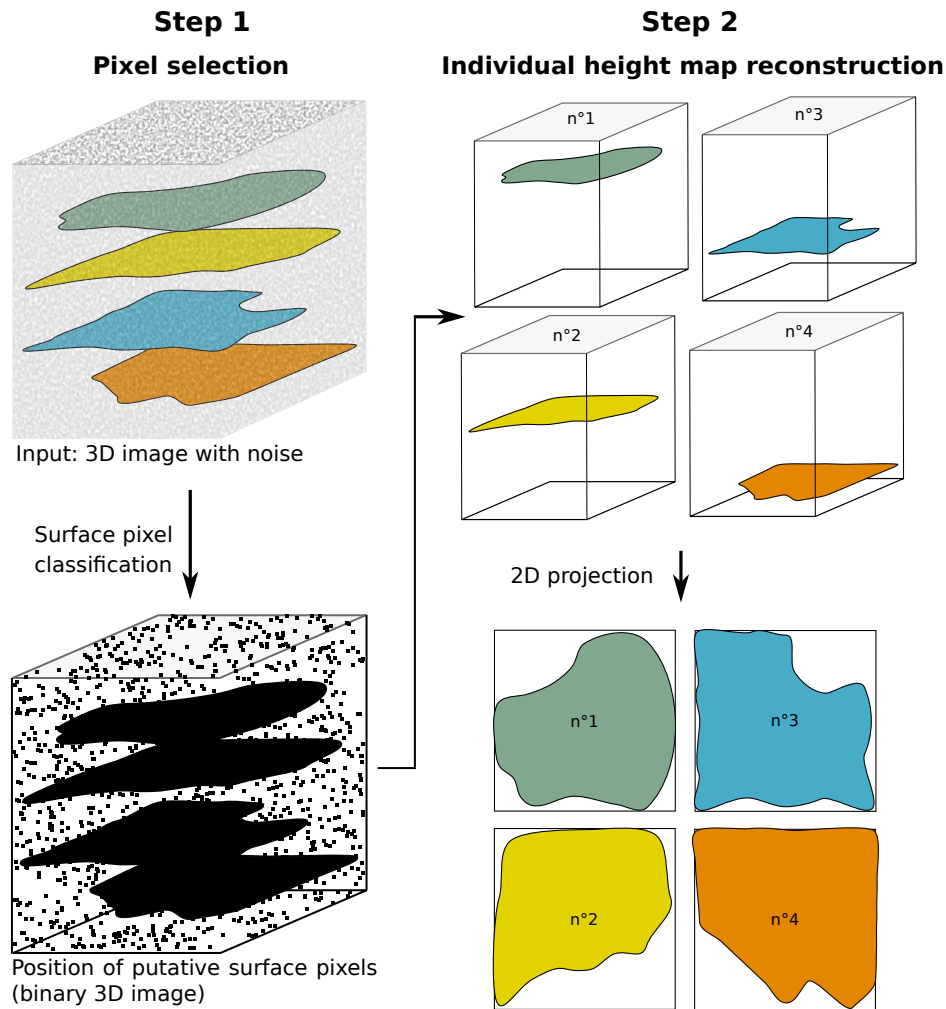
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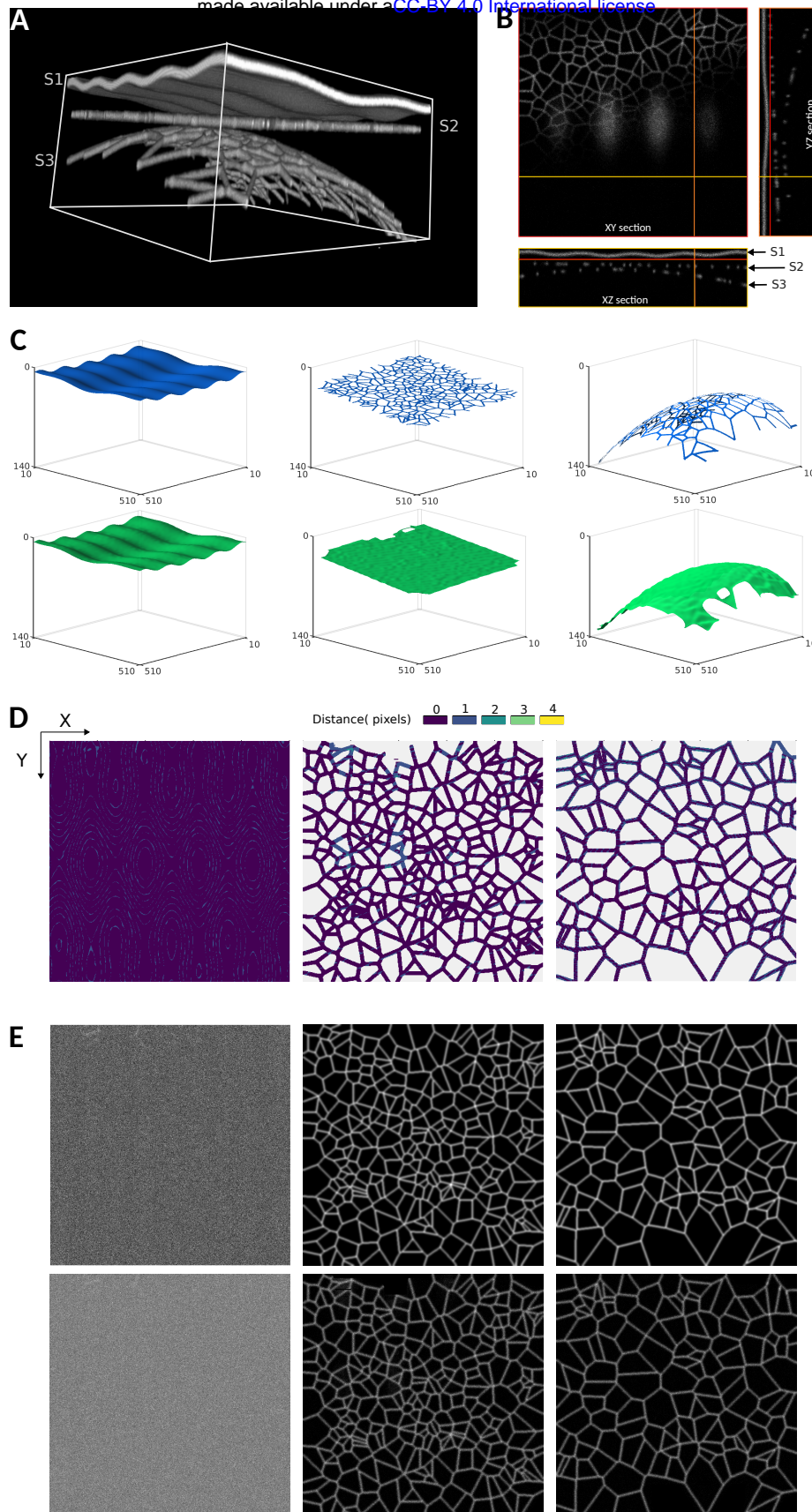
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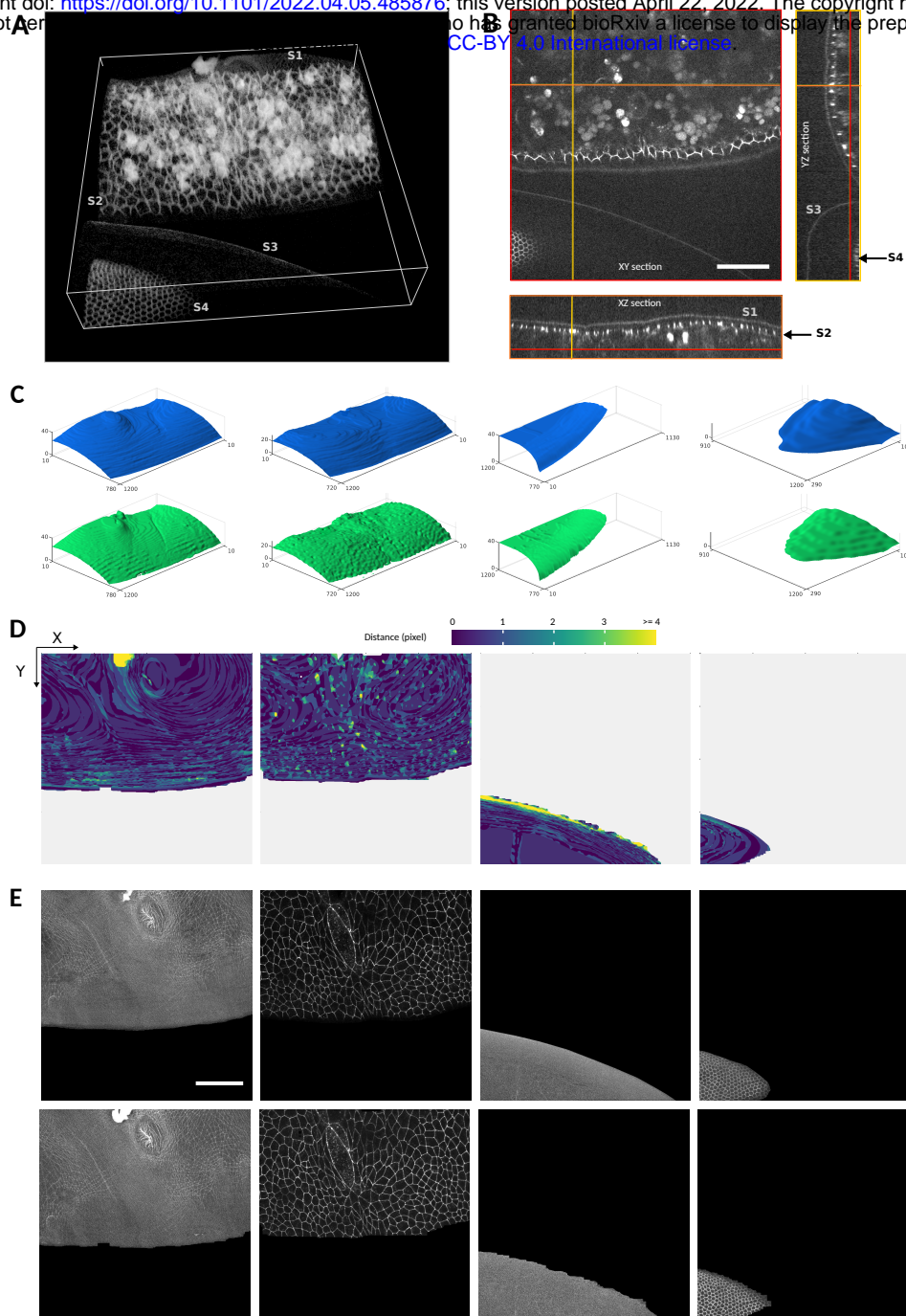
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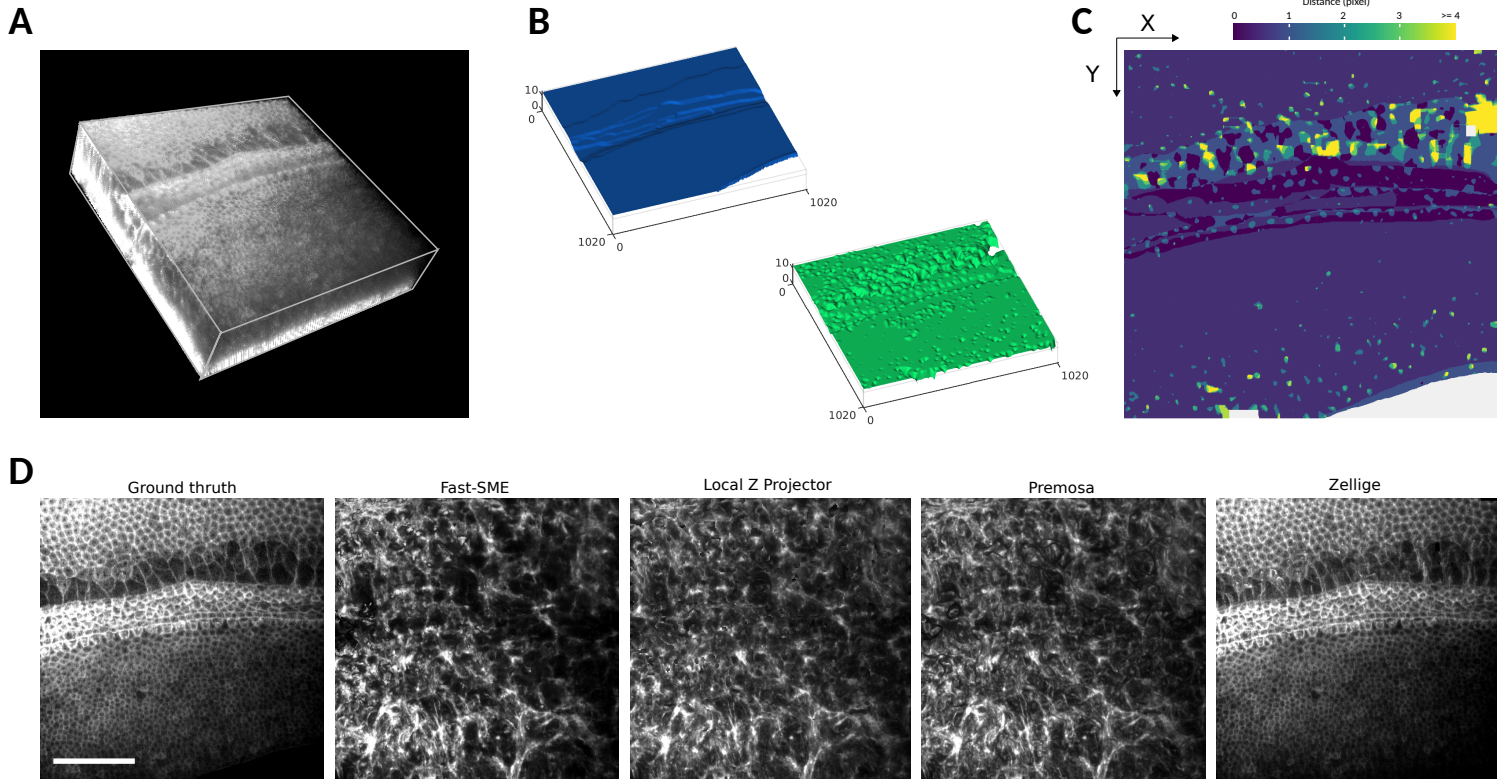
**Figure 1. Flowchart of Zellige's algorithmic steps.** Surface pixel selection (step 1), surface assembly in the form of a height map (step 2), and subsequent projection localized to the height map, are schematically depicted in the case of a 3D image containing 4 surfaces of interest.



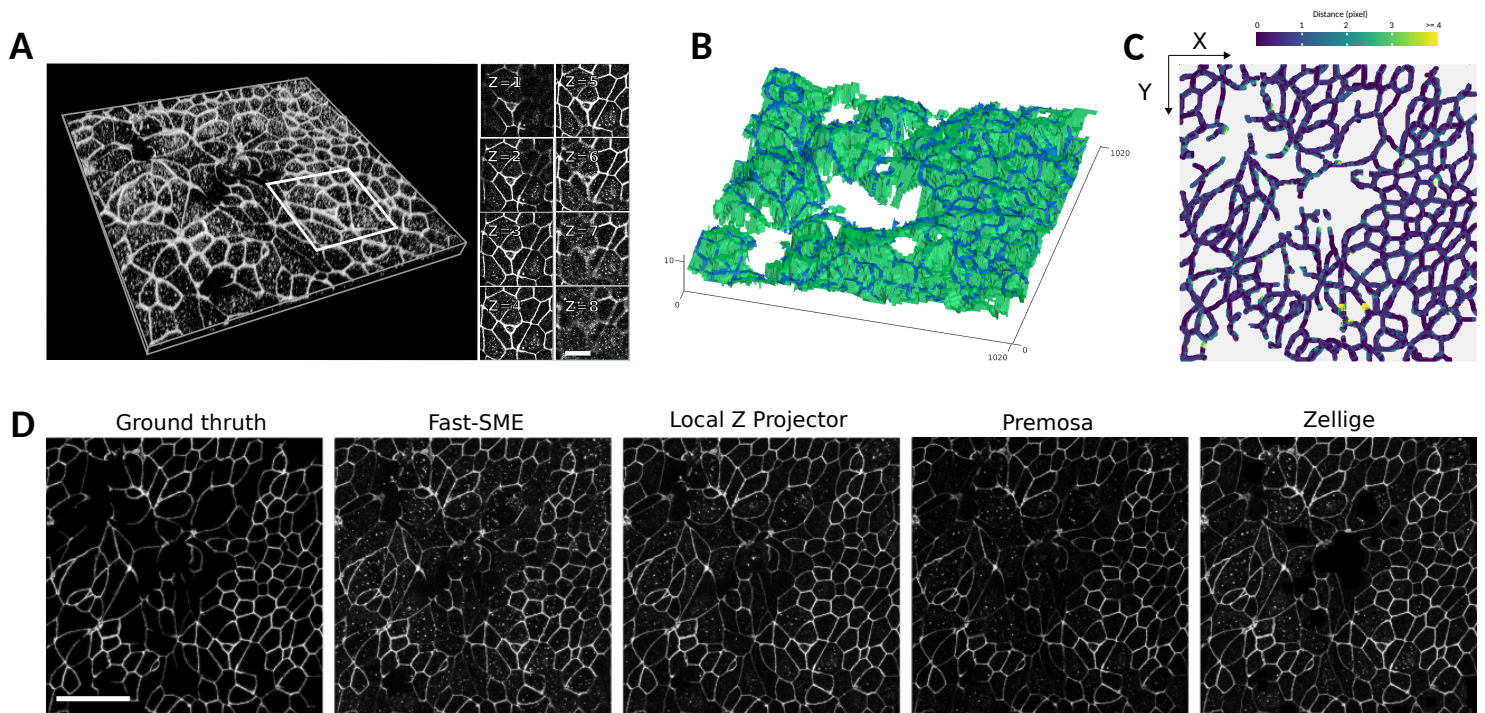
**Figure 2. Multiple surface extraction on a synthetic 3D image. (A-B)** The image contains 3 phantom surfaces (S1, S2, S3) of different shapes (sinusoidal, flat, and paraboloidal, respectively), and different textures (surface S1 has constant intensity, while surfaces S2 and S3 are supported by Voronoi meshes of different cell-sizes). **(C)** 3D representations of the height maps extracted by Zellige (in green) and of the ground truth (GT, in blue) height maps of surfaces S1, S2, and S3. **(D)** Error maps displaying the distance along the z-axis between the reconstructed and GT height maps for surfaces S1, S2, and S3. **(E)** Projections of the 3D image localized to the different surfaces S1-S3 (maximum intensity projections over a subvolume of a width  $\delta z=1$  pixel above or below the corresponding height-maps). Upper and lower panels show the projections based on the GT and the reconstructed height-maps, respectively.



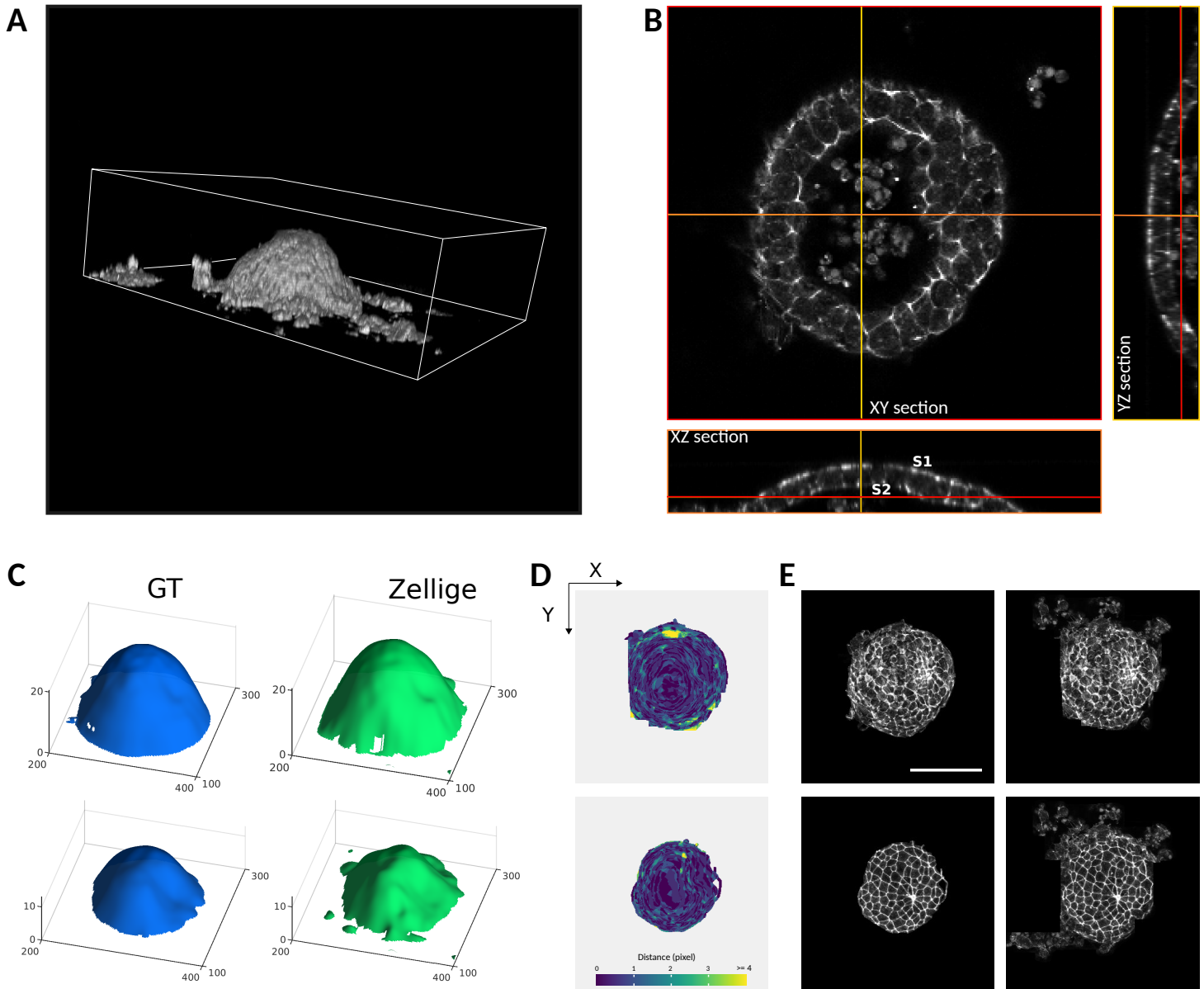
**Figure 3. Fly specimen. (A,B)** Volume rendering (A) and orthogonal sections (B) of a 3D image of fly embryo taken around 24h after puparium formation, covering a portion of the abdomen (showing histoblast cells and larval cells), and a portion of the developing wing. Scale bar 50  $\mu$ m. Four surfaces of interest may be identified in the dataset (of dimensions 1200  $\times$  1200  $\times$  51 pixels): surfaces S1 and S2 are relatively close to one another and located within overlapping z-ranges ( $8 \leq z \leq 50$  and  $20 \leq z \leq 50$ , respectively). Surfaces S3 and S4 (located in the z-ranges  $42 \leq z \leq 50$  and  $9 \leq z \leq 50$ , respectively) are relatively far from each other and can nearly be separated by a plane. **(C)** 3D representations of the height maps extracted by Zellige (in green) and of the ground truth height maps (GT, in blue) of surfaces S1-S4. The reconstructed height-maps of all surfaces S1-S4 cover >93% of the area of the corresponding GT (cf. Figure S2 and Table S1). To reduce the staircase artifacts (more or less visible depending on the surface) due to the digitization of the GT and reconstructed height-maps, all height-maps were smoothed with a 2D gaussian filter with a standard radius of 5 pixels (cf. Supplemental note 1). **(D)** Error maps (color-coded distance along the z-axis between the reconstructed and the GT height-maps) plotted for each of the reconstructed surfaces. The large majority of pixels on the reconstructed height-maps (98%, 96%, 91%, and 99% for surfaces S1 to S4, respectively) display errors of <2 pixels. The height-maps of surfaces S1, S2, S4 show subpixel accuracy on average (RMSE < 1), while that of surface S3 is slightly less accurate (RMSE = 1.25). **(E)** Projections of the 3D image localized to the different surfaces S1-S4 (in this and all subsequent figures, these are maximum intensity projections over a subvolume of width  $\delta z = \pm 1$  pixel above or below the corresponding height-maps). Upper and lower panels show the projections based on the GT and the reconstructed height-maps, respectively. Scale bar 50  $\mu$ m.



**Figure 4. Cochlea specimen.** (A) Volume rendering of a 3D confocal swept-field image of the mouse cochlear embryo on embryonic day E14.5. The dataset (of dimensions  $1024 \times 1024 \times 45$  pixels) shows a portion of the sensory epithelium (at the topmost sections of the stack) and the underlying non-cellular layer of mesenchyme on which the organ develops. Both structures are stained with phalloidin to reveal F-actin. Scale bar  $40 \mu\text{m}$ . The surface of interest is the epithelium surface, harboring the sensory and supporting cells under differentiation. The mesenchyme layer is not (strictly speaking) assimilable to a surface, but it produces a strong background signal nearby the surface of interest, hampering its extraction. (B) 3D representations of the height map extracted by Zellige (in green) and the GT height map (in blue), of the epithelium surface. (C) Color-coded error map of the reconstructed height-map, which shows subpixel accuracy (errors  $<1$ ) over a large majority (83%) of pixels, as well as on average (RMSE  $\sim 1.1$ ). (D) Projections localized to the GT height-map of the epithelium surface (left most panel), and to the height-maps extracted with the four different algorithms: FastSME, LocalZProjector, PreMosa, and Zellige. Only Zellige correctly extracts the surface of the epithelium in this example. Scale bar  $40 \mu\text{m}$ .

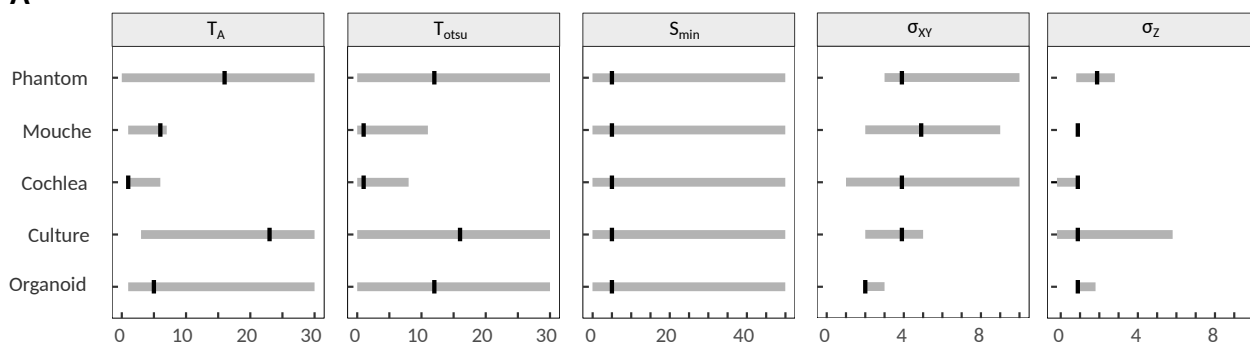


**Figure 5. Human bronchial epithelial cells infected by SARS-CoV-2.** (A) Volume rendering and individual sections of a confocal 3D image of a primary culture of bronchial epithelial cells 4 days after it was infected by the SARS-CoV-2 virus. The dataset (of dimensions  $1024 \times 1024 \times 15$  pixels) covers a portion of the epithelium immunostained for the tight junction protein ZO-1. Notice the roughness of the epithelium surface and the presence of anomalous bulges (arrows) resulting from the SARS-CoV-2 infection. Scale bar  $10 \mu\text{m}$ . (B) 3D representations of the height map extracted by Zellige (in green) and the GT height map (in blue), of the epithelium surface. (C) Color-coded error map of the reconstructed height-map. Despite its roughness, the surface of interest is reconstructed with subpixel accuracy over the majority (71%) of pixels, as well as on average (RMSE  $\sim 0.8$ ). (D) Projections localized to the GT height-map of the epithelium surface (leftmost panel), and the height-maps extracted with the four different algorithms: FastSME, LocalZProjector, PreMosa, and Zellige. Scale bar  $30 \mu\text{m}$ .

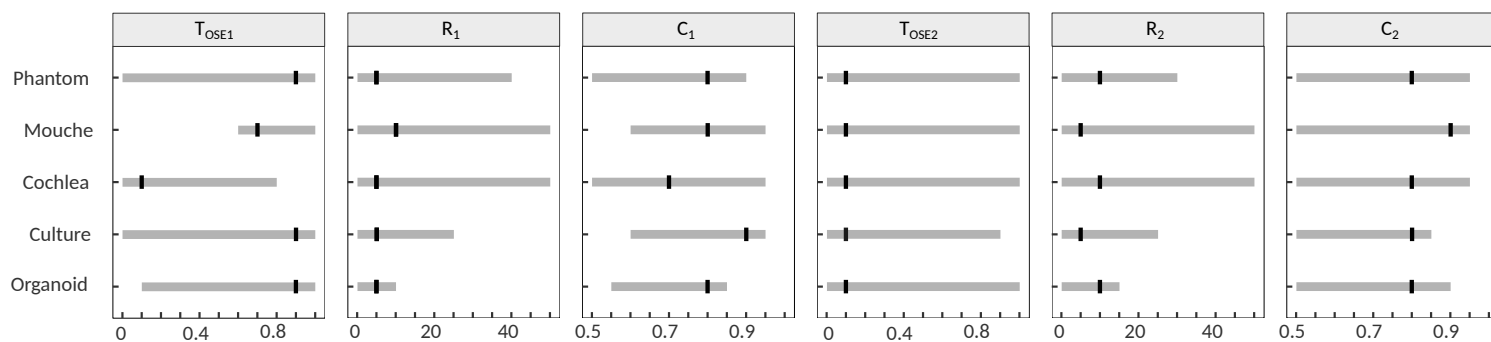


**Figure 6. Organoid specimen. (A-B)** Volume rendering (A) and orthogonal sections (B) of a confocal 3D image of a (half of) inner ear organoid, which has been fixed and stained with phalloidin to reveal F-actin. The dataset (of dimensions  $520 \times 465 \times 35$  pixels) includes two dome-shaped epithelial surfaces of interest, forming the apical (inward) and basal (outward) sides of the organoid. **(C)** 3D representations of the height map extracted by Zellige (in green) and the GT height map (in blue), of the epithelium surface. **(D)** Color-coded error maps of the reconstructed height-maps for the apical (left) and basal (right) epithelial surfaces of the organoid. The surfaces of interest are reconstructed with an error of  $< 2$  pixels over a large majority (96% and 93% for the apical and basal surfaces, respectively) of pixels, as well as on average (RMSE  $\sim 0.8$  and  $1.1$  for the apical and the basal surfaces, respectively). **(E)** Projections localized to the GT height-maps of the epithelium surface (panels on the left), and the height-maps extracted by Zellige (panels on the right). Scale bar  $100 \mu\text{m}$

**A**



**B**



**Figure 7. Summary of the sensitivity analysis.** The intervals indicated in grey for each parameter and each of the images tested correspond to the parameter values for which the reconstruction satisfies high quality criteria defined by  $RMSE \leq 1.5$  and coverage  $\geq 85\%$ . Black marks indicate the reference value obtained by manual adjustment for each image (cf. Supplemental note 2). **(A)** Parameters of the surface selection step. **(B)** Parameters of the surface assembly step.



## FIGURE 1

Flowchart of Zellige's algorithmic steps. Surface pixel selection (step 1), surface assembly in the form of a height map (step 2), and subsequent projection localized to the height map, are schematically depicted in the case of a 3D image containing 4 surfaces of interest.

## FIGURE 2

Multiple surface extraction on a synthetic 3D image. (A) The image contains 3 phantom surfaces (S1, S2, S3) of different shapes (sinusoidal, flat, and paraboloidal, respectively), and different textures (surface S1 has constant intensity, while surfaces S2 and S3 are supported by Voronoi meshes of different cell-sizes). (B) 3D representations of the height maps extracted by Zellige (in green) and of the ground truth (GT, in blue) height maps of surfaces S1, S2, and S3. (C) Error maps displaying the distance along the z-axis between the reconstructed and GT height maps for surfaces S1, S2, and S3. (E) Projections of the 3D image localized to the different surfaces S1-S3 (maximum intensity projections over a subvolume of a width  $\delta z=1$  pixel above or below the corresponding height-maps). Upper and lower panels show the projections based on the GT and the reconstructed height-maps, respectively.

## FIGURE 3

(A,B) Volume rendering (A) and orthogonal sections (B) of a 3D image of fly embryo taken around 24h after puparium formation, covering a portion of the abdomen (showing histoblast cells and larval cells), and a portion of the developing wing. Scale bar 50  $\mu\text{m}$ . Four surfaces of interest may be identified in the dataset (of dimensions  $1200 \times 1200 \times 51$  pixels): surfaces S1 and S2 are relatively close to one another and located within overlapping z-ranges ( $8 \leq z \leq 50$  and  $20 \leq z \leq 50$ , respectively). Surfaces S3 and S4 (located in the z-ranges  $42 \leq z \leq 50$  and  $9 \leq z \leq 50$ , respectively) are relatively far from each other and can nearly be separated by a plane.

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(C) Color-coded error map of the reconstructed height-map. Despite its roughness, the surface of interest is reconstructed with subpixel accuracy over the majority (71%) of pixels, as well as on average (RMSE  $\sim 0.81$ ).

(D) Projections localized to the GT height-map of the epithelium surface (leftmost panel), and the height-maps extracted with the four different algorithms: FastSME, LocalZProjector, PreMosa, and Zellige. Scale bar 30  $\mu\text{m}$

## FIGURE 6

(A-B) Volume rendering (A) and orthogonal sections (B) of a confocal 3D image of a (half of) inner ear organoid, which has been fixed and stained with phalloidin to reveal F-actin. The dataset (of dimensions  $520 \times 465 \times 35$  pixels) includes two dome-shaped epithelial surfaces of interest, forming the apical (inward) and basal (outward) sides of the organoid.

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(E) Projections localized to the GT height-maps of the epithelium surface (panels on the left), and the height-maps extracted by Zellige (panels on the right). Scale bar 100  $\mu\text{m}$ .

## FIGURE 7

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