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## Studying Muscle Transcriptional Dynamics at Single-molecule Scales in *Drosophila*

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## An optimized method to study muscle transcriptional dynamics at single-molecule scales in *Drosophila*.

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**1 TITLE:**

2 Studying Muscle Transcriptional Dynamics at Single-molecule Scales in *Drosophila*

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**20 SUMMARY:**

21 *Drosophila* is a well-established model for studying key molecules that regulate myogenesis.  
22 However, current methods are insufficient to determine mRNA transcriptional dynamics and  
23 spatial distribution within syncytia. To address this limitation, we optimized an RNA fluorescence  
24 *in situ* hybridization method allowing the detection and quantification of mRNAs at a single-  
25 molecule scale.

26

**27 ABSTRACT:**

28 Skeletal muscles are large syncytia made up of many bundled myofibers that produce forces and  
29 enable body motion. *Drosophila* is a classical model to study muscle biology. The combination of  
30 both *Drosophila* genetics and advanced omics approaches led to the identification of key  
31 conserved molecules that regulate muscle morphogenesis and regeneration. However, the  
32 transcriptional dynamics of these molecules and the spatial distribution of their messenger RNA  
33 within the syncytia cannot be assessed by conventional methods. Here we optimized an existing  
34 single-molecule RNA fluorescence *in situ* hybridization (smFISH) method to enable the detection  
35 and quantification of individual mRNA molecules within adult flight muscles and their muscle  
36 stem cells. As a proof of concept, we have analyzed the mRNA expression and distribution of two  
37 evolutionary conserved transcription factors, Mef2 and Zfh1/Zeb. We show that this method can  
38 efficiently detect and quantify single mRNA molecules for both transcripts in the muscle  
39 precursor cells, adult muscles, and muscle stem cells.

40

**41 INTRODUCTION:**

42 Adult skeletal muscles are made of differentiated multinucleated myofibers whose contractile  
43 properties generate movements. Muscle growth, maintenance, and regeneration rely upon  
44 muscle progenitors and muscle stem cells (MuSCs) that are specified during embryonic

45 development<sup>1</sup>. The myogenic program is finely controlled by a set of core myogenic transcription  
46 factors (TFs) (e.g., Pax3/7, MYOD, Mef2, and ZEB)<sup>2-4</sup>. Deciphering the molecular mechanisms  
47 regulating muscle biology is important for elucidating fundamental myology-related questions  
48 and for possible therapeutic use in treating muscle-degenerative diseases.

49  
50 *Drosophila* has a long history as a genetic model to study myogenesis<sup>5</sup>. It has recently emerged  
51 as a new model to investigate muscle regeneration<sup>6-8</sup>. The muscle structure and core myogenic  
52 programs are highly conserved between flies and mammals. For example, the TFs Mef2 and  
53 Zfh1/ZEB have a conserved function in regulating muscle development and regeneration<sup>3,9-11</sup>.  
54 Adult *Drosophila* muscles, such as the Indirect Flight Muscles (IFMs), are formed from a specific  
55 population of MuSCs, known as Adult Muscle Progenitors (AMPs)<sup>12</sup>. These AMPs are specified  
56 during embryogenesis and associate with epithelial structures such as wing and leg discs.  
57 Throughout embryonic and larval stages, AMPs remain undifferentiated until metamorphosis  
58 when they engage in differentiation and fusion to form the IFMs<sup>13,14</sup>. The TF Spalt major (*spalt*,  
59 *salm*) is expressed during flight muscle development and is necessary to determine their  
60 structural identity<sup>15</sup>. Mef2 is another important myogenic TF that is essential for adult muscle  
61 formation<sup>16,17</sup>. It is expressed in the AMPs and maintained in the adult IFMs<sup>10,18</sup>. While the  
62 majority of AMPs differentiate into functional muscles, a subset escapes differentiation and  
63 forms the adult MuSCs<sup>11</sup>. Similar to vertebrates, the TF Zfh1/ZEB is required to prevent  
64 premature differentiation of the adult MuSCs and to maintain their stemness<sup>9,10</sup>.

65  
66 Gene expression dynamics have been proven to regulate various muscle biological processes<sup>19,20</sup>.  
67 The advent of high-throughput single-cell and single-nuclei RNA sequencing techniques has  
68 enabled a comprehensive exploration of these transcriptional dynamics<sup>21,22</sup>. One notable  
69 limitation of these approaches is their inability to provide the spatial distribution of mRNA  
70 molecules in the multinucleated muscle fibers. These features can be investigated by single-  
71 molecule fluorescence *in situ* hybridization (smFISH) that reveals two types of mRNA bodies: 1.  
72 Individual mRNA molecules spread out in the cytoplasm and representing the mature RNA and  
73 2. A maximum of two bright nuclear foci correspond to the nascent transcript and reveal the  
74 transcriptionally active alleles<sup>23</sup>. Therefore, smFISH is a method of choice for individual mRNA  
75 molecule quantification, investigating their spatial distribution and providing snapshots of the  
76 gene transcriptional dynamics.

77  
78 The smFISH method relies on a set of short fluorescent oligonucleotide probes specifically  
79 designed to be complementary to the target transcript. Upon annealing, it generates high-  
80 intensity point sources permitting the detection of mRNA at a single-molecule level using  
81 confocal microscopy<sup>24</sup>. This method is increasingly applied for a wide variety of cell types,  
82 including mammalian muscle tissues<sup>19,20</sup>. However, in *Drosophila* like other animal models, most  
83 of the knowledge about adult muscle gene expression is derived from bulk molecular assays,  
84 which lack quantitative information on the spatial location of mRNA molecules. Here we  
85 optimized a method for performing smFISH on muscle precursor cells and adult *Drosophila*  
86 muscles<sup>23,25</sup>. This protocol includes a fully automated analysis pipeline for nuclei segmentation  
87 and mRNA counting and localization.

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**PROTOCOL:**

**1. Wing disc and adult muscle dissection and preparation**

NOTE: Clean up the dissection bench and all the dissection instruments with RNase inhibitor solution (**Table of Materials**). Laboratory gloves should be worn during the whole experimental procedure.

**1.1. Wing disc**

1.1.1. Place the L3 staged larvae in cold 1x phosphate-buffered saline (PBS) (**Figure 1A**).

1.1.2. Rinse the larvae 3x with 1x PBS and keep them in this solution for 5 min.

1.1.3. Using two pairs of sharp forceps, hold the larva from the anterior part and pull and discard the rest of the body tissues (around 2/3).

1.1.4. Hold both the first 1/3 of the larva with one pair of forceps and the mouth hooks with the other pair. Retract the mouth hooks inside the larva until it is fully inverted.

NOTE: Dissect approximately 30 larvae per experiment, to ensure adequate sample size for analysis.

1.1.5. Observe that the wing discs are closely associated with the two primary branches of the tracheae, which run along each side of the larva.

1.1.6. Remove all the other larval components (leg discs and brain) to obtain a clean inverted larval carcass with a pair of wing discs attached to the trachea (**Figure 1B**).

1.1.7. Place the samples in a 1 mL centrifuge tube and fix them in 4% paraformaldehyde (PFA) diluted in 1x PBS for 45 min at room temperature under agitation.

NOTE: The sample agitation is achieved by an oscillating stirrer (**Table of Materials**).

1.1.8. Wash the samples 3 x 5 min with 70% ethanol (EtOH, diluted in RNase-free water) and store them in the same washing solution at 4 °C for at least 2 days and up to 1 week.

**1.2. Indirect flight muscles**

1.2.1. Anesthetize the adult flies on a CO<sub>2</sub> fly pad and remove the heads, abdomens, legs, and wings.

132 1.2.2. Place the dissected thoraxes in cold 1x PBS and continue doing so until all samples are  
133 dissected (**Figure 1D**).

134  
135 1.2.3. Prefix the thoraxes in 4% PFA, (1% Triton) for 20 min at room temperature under agitation.  
136

137 1.2.4. Rinse the samples 3 x 5 min with 1x PBT washing solution (1% Triton in 1x PBS).  
138

139 1.2.5. Position the thoraxes on a double-sided tape (**Table of Materials**) on a glass slide and bisect  
140 them with a sharp microtome blade to produce two hemi-thoraces (**Figure 1E,F**).  
141

142 1.2.6. Fix the samples in 4% PFA (1% Triton) for 45 min at room temperature under agitation.  
143

144 1.2.7. Wash 2 x 20 min with 1x PBT washing solution (1% Triton, 1x PBS) at room temperature  
145 under agitation.  
146

147 1.2.8. Place the hemi-thoraces in cold 1x PBS and use the forceps to gently isolate the IFMs from  
148 the hemi-thorace (**Figure 1G**).  
149

150 NOTE: To achieve this, it is important to remove the cuticle as thoroughly as possible.  
151

152 1.2.9. To permeabilize the samples, store the isolated IFMs in 70% EtOH solution for at least 2  
153 days and up to 1 week at 4 °C.  
154

155 NOTE: Isolate the IFMs from approximately 10 thoraxes per experiment to ensure adequate  
156 sample size for analysis.  
157

## 158 **2. Hybridization, immunostaining, and mounting** 159

160 NOTE: Similar hybridization procedure is applied for both wing discs and IFMs samples. It is critical  
161 to isolate the wing discs from the larval carcasses and put them in 200 µL of Buffer A prior to  
162 starting the hybridization.  
163

164 2.1. Resuspend the probes in 400 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for a final  
165 stock solution of 12.5 µM.  
166

167 2.2. Transfer the samples to a 0.5 mL centrifuge tube containing Buffer A (**Table of Materials**).  
168

169 NOTE: By decreasing the volume of the container, it becomes easier to visualize the samples and  
170 avoid losing them during the washing process.  
171

172 2.3. Wash the samples 2 x 20 min with Buffer A.  
173

174 2.4. Aspirate Buffer A, replace it with 400 µL of the hybridization buffer (**Table of Materials**), and  
175 prehybridize the samples for 30 min at 37 °C in a thermal mixer (**Table of Materials**).

176  
177 2.5. Dilute the probes to a final concentration of 125 nM for wing discs and 200 nM for adult  
178 muscles, in the hybridization buffer and add the primary antibody.

179  
180 NOTE: The antibody dilution varies depending on the specific antibody being used (**Table of**  
181 **Materials**). Adding antibodies is optional. Users may opt to forego protein staining and instead  
182 use a similar smFISH protocol that does not include the addition of antibodies.

183  
184 2.6. Aspirate the hybridization buffer and replace it with 100  $\mu$ L of hybridization buffer containing  
185 the probe and the antibody.

186  
187 2.7. Incubate the samples in the dark at 37  $^{\circ}$ C for at least 16 h under 300 rpm agitation in a  
188 thermal mixer.

189  
190 2.8. Warm up an aliquot of Buffer A at 37  $^{\circ}$ C.

191  
192 2.9. Wash the samples for 3 x 10 min in the thermal mixer at 37  $^{\circ}$ C under 300 rpm agitation.

193  
194 2.10. During the last wash, dilute the secondary antibody (1/200) and DAPI (1/10,000) in Buffer  
195 A and incubate the samples in this solution at 37  $^{\circ}$ C for at least 1 h.

196  
197 2.11. Wash the samples for 3 x 20 min with Buffer B (**Table of Materials**).

198  
199 2.12. Carefully transfer the samples on a microscope slide, wipe off the residual Buffer B, and add  
200 30  $\mu$ L of mounting medium, and cover with an 18 x 18 mm coverslip. Use a nail polish to seal the  
201 preparation.

202  
203 2.13. Store the samples for up to 1 week at 4  $^{\circ}$ C.

204  
205 NOTE: However, it is recommended to perform the imaging as soon as possible to avoid probe  
206 signal degradation.

207  
208 **3. Imaging**

209  
210 3.1. Acquire images with an xyz acquisition mode using a confocal microscope equipped with 40x  
211 and 63x oil immersion objectives (**Table of Materials**). The smFISH signal is detected by the HyD  
212 detector via a photon counting mode.

213  
214 3.2. Locate the samples using the DAPI signal and UV lamp.

215  
216 3.3. To image the wing disc-associated muscle progenitors and IFMs (**Figure 2** and **Figure 3**), apply  
217 the following setup for laser lines and emission filters: select **DAPI** (excitation (ex.) **405 nm**,  
218 emission (em.) **450 nm**), **Alexa Fluor 488** (ex. **496 nm**, em. **519 nm**), and **Quasar 670** for the  
219 smFISH probes (ex. **647 nm**, em. **670 nm**).

220  
221 3.4. To image the adult MuSCs (**Figure 2D**), apply the following setup for laser lines and emission  
222 filters: select **DAPI** (ex. **405 nm**, em. **450 nm**), **Alexa Fluor 488** (ex. **496 nm**, em. **519 nm**), **Alexa**  
223 **Fluor 555** (ex. **555 nm**, em. **565 nm**), and **Quasar 670** for the smFISH probes (ex. **647 nm**, em. **670**  
224 **nm**).

225  
226 NOTE: For the imaging, the wavelengths can be adapted according to the dyes associated with  
227 secondary antibodies and probes.

228  
229 3.5. Save the confocal images as .TIFF files.

## 230 231 4. Post imaging analysis

232  
233 4.1. To get started, launch ImageJ and navigate to the **Plugins** menu. From there, select **Macros**  
234 **| Edit** to open the macros source code.

235  
236 NOTE: This will enable adjustment of the parameters as needed, and if required, modify the  
237 folder directory for each channel within the macro's source code.

238  
239 4.2. To follow this protocol and quantify *Mef2* smFISH spots in larvae, use the following settings:  
240 **Log\_radius = 3.0** and **Log\_quality = 20.0**. Segment *Mef2*-positive nuclei with **blur = 2**;  
241 **nucleus\_scale\_parameter = 30**; **nucleus\_threshold = -8.0**; **nucleus\_size = 300**.

242  
243 4.3. In muscle fibers, use the following parameters: **Log\_radius = 2.5**; **Log\_quality = 60.0**; **blur =**  
244 **2**; **nucleus\_scale\_parameter = 100**; **nucleus\_threshold = 0.0**; **nucleus\_size = 300**.

245  
246 4.4. To initiate the macro, simply execute the **run** command and wait for the macro to  
247 automatically load all images from the designated folder and quantify them in a sequential  
248 manner.

249  
250 NOTE: Please note that the duration of this process may vary from a few seconds to several hours,  
251 depending on the volume of data being analyzed.

252  
253 4.5. Look for the results displayed in two new .csv files: **file\_FISH\_results** and **file\_nuclei\_results**.  
254 The first shows the intensities of FISH spots and distances with their nearest nucleus. The second  
255 one gives the number of FISH-detected spots inside and outside the nucleus. It takes into account  
256 the distances between the detected spots and the segmented nucleus.

## 257 258 REPRESENTATIVE RESULTS:

259 In this protocol, we used commercially produced probes targeting *Mef2* and *zfh1* mRNA. We  
260 designed the probes with the manufacturer's FISH probes designer (**Table of Materials**). The  
261 *Mef2* set targets the exons common to all *Mef2* RNA isoforms (from exon 3 to exon 10). The *zfh1*  
262 set targets the third exon common to both *zfh1-RB* and *zfh1-RE* isoforms<sup>10</sup>. Both probes are  
263 conjugated to the Quasar 670 fluorescent dye.

264  
265 We generated a macro in-house that was compatible with ImageJ software to automatically  
266 analyze the raw .tif data. The macro allows the 3D segmentation of the nuclei by Cellpose<sup>26</sup> with  
267 the Fiji plugin BIOP and the transcriptional spot quantification by a Laplacian of Gaussian as  
268 implemented in the Trackmate plugin<sup>27</sup>. MorpholibJ plugin<sup>28</sup> is used for postprocessing  
269 **(Supplemental File 1)**.

270  
271 As a first step, we performed smFISH on wing imaginal discs, where *Mef2* and *zfh1* are known to  
272 be expressed in the AMPs. These data show that both transcripts are uniformly detected in the  
273 AMPs population co-stained with either Mef2 or Zfh1 antibodies **(Figure 2A,B)**. Active  
274 transcription sites (TS) can be revealed and distinguished from mature mRNA by smFISH as they  
275 tend to be larger and have more intense signals than individual cytoplasmic transcripts or mature  
276 nuclear transcripts. Consistently, higher magnifications of AMPs distinguish between TS foci and  
277 mature mRNA scattered in the cytoplasm **(Figure 2A',B')**, validating the sensitivity of this smFISH  
278 protocol. It should, however, be mentioned that we observed one active TS per nucleus for both  
279 *zfh1* and *Mef2* **(Figure 2A',B')**. These data further validate the accuracy of the probe design since  
280 *Mef2* and *zfh1* transcriptional patterns in the AMPs colocalize with the detection of their  
281 respective proteins **(Figure 2)**.

282  
283 In a second step, we examined the transcription site and distribution of *Mef2* and *zfh1* mRNAs in  
284 differentiated adult IFMs and associated stem cells **(Figure 2C,D)**. These data clearly illustrate  
285 *Mef2* TSs in the syncytial muscle nuclei and *Mef2* mRNA distribution throughout the cytoplasm  
286 **(Figure 2C,C')**. Zfh1 specifically marks the MuSCs population<sup>10,11</sup>. Using this smFISH protocol, we  
287 successfully detected *zfh1* transcription in MuSCs marked by Zfh1-Gal4 > GFP expression. Higher  
288 magnification illustrates the detection of both *zfh1* TS and cytoplasmic single mRNAs **(Figure 2D')**.

289  
290 Finally, we used our in-house-built ImageJ macro to quantitatively analyze *Mef2* transcriptional  
291 dynamics and spatial distribution. The computational pipeline can efficiently detect and segment  
292 *Mef2* spots and muscle nuclei **(Figure 3A–C)**. However, due to signal-to-noise ratio issues, some  
293 spots may not be detected in certain cases **(Figure 3A',B')**. We used this automated method to  
294 investigate whether *Mef2* mRNA abundance varies between muscle precursor cells (AMPs) and  
295 differentiated adult IFMs. Our analysis shows that the number of *Mef2* mRNA per nuclei in adult  
296 IFMs and the AMPs are not significantly different **(Figure 3D)**.

297  
298 To gain insight into the spatial distribution of *Mef2* transcripts in adult IFM muscles, we quantified  
299 the fraction of cytoplasmic versus nuclear *Mef2* mRNA spots **(Figure 3E,F)**. Using our program,  
300 we counted both the total number of *Mef2* mRNA spots and the number of *Mef2* mRNA spots  
301 overlapping with nuclei staining (Mef2). Subtracting the nuclei-associated mRNA from the total  
302 mRNA number provides the number of cytoplasmic mRNA spots. Our findings reveal that  
303 approximately 92% of *Mef2* mRNA is present in the cytoplasm, while the remaining 8% is  
304 associated with muscle nuclei, as shown in **Figure 3E,F**.

305  
306 **FIGURE AND TABLE LEGENDS:**

307

308 **Figure 1: Procedure to dissect and prepare wing discs and IFM samples for smFISH.** (A) L3 staged  
309 larvae. (B) Dissected and inverted anterior end of the larva. Arrows indicate the wing discs. (C)  
310 Isolated wing disc. (D) Dissected adult thoraxes. (E) Adult thorax oriented on a doublesided tape  
311 prior to bisection (dotted line). (F) Adult hemithorax. (G) Isolated IFMs. (H) Workflow of the  
312 smFISH protocol. Abbreviations: IFMs = indirect flight muscles; smFISH = single-molecule RNA  
313 fluorescence *in situ* hybridization; EtOH = ethanol.

314  
315 **Figure 2: Representative images of *Mef2* and *zfh1* smFISH in wing discs and adult IFMs.** (A,B)  
316 *Mef2* and *zfh1* mRNAs (purple) are detected uniformly in AMPs and co-localize with (A,A') *Mef2*  
317 and (B,B') *Zfh1* proteins (green), respectively. (A'',B'') Higher magnification of the AMPs. (C) *Mef2*  
318 mRNAs and *Mef2* protein are detected in adult IFMs. (C') Higher magnification of the boxed  
319 region in C. (D) *Zfh1-Gal4 >UAS-mCD8GFP* expression (green) labels adult MuSCs. *zfh1* RNA is  
320 detected in adult MuSCs. (D') Higher magnification of the boxed region in D. *Zfh1* transcription  
321 start sites and single mRNAs are indicated by arrows and arrowheads, respectively. DAPI staining  
322 (blue). Scale bars = 50  $\mu\text{m}$  (A,B,A',B'), 10  $\mu\text{m}$  (A'',B'',C,D), 5  $\mu\text{m}$  (C',D'). Abbreviations: IFMs =  
323 indirect flight muscles; smFISH = single-molecule RNA fluorescence *in situ* hybridization; AMPs =  
324 adult muscle progenitors; MuSCs = muscle stem cells; DAPI = 4',6-diamidino-2-phenylindole; GFP  
325 = green fluorescent protein.

326  
327 **Figure 3: Quantification of *Mef2* transcription and cytoplasmic mRNA accumulation by smFISH.**  
328 (A) Distribution of *Mef2* transcripts (purple) and *Mef2* protein (green) in wildtype adult IFMs. (A')  
329 Higher magnification of boxed region in A. Transcription start sites and single mRNAs are  
330 indicated by arrows and arrowheads, respectively. (B,C) Automatic *Mef2* spots finding and  
331 nuclear segmentation. Cytoplasmic and nuclear *Mef2* mRNAs are indicated in green and  
332 magenta, respectively. (B',C') Higher magnifications of boxed regions in B and C. Asterisks  
333 indicate spots that are not counted by the macro. (D) Example of *Mef2* spot quantification in  
334 AMPs and adult IFMs, relative to the total number of nuclei. ( $p = 0,0785$ , Student's *t*-test, wing  
335 discs ( $n = 4$ ), IFMs ( $n = 11$ )). (E) Example of quantification of *Mef2* spot distribution in adult IFMs.  
336 (\*\*\*)  $p < 0.0007$ , Student's *t*-test,  $n = 5$ ). (F) Percentage of *Mef2* mRNA spots detected inside and  
337 outside the nuclei ( $n = 5$ ). Scale bars = 10  $\mu\text{m}$  (A,A'). Abbreviations: IFMs = indirect flight muscles;  
338 smFISH = single-molecule RNA fluorescence *in situ* hybridization; AMPs = adult muscle  
339 progenitors.

340  
341 **Supplemental File 1: MorpholibJ plugin used for postprocessing.**

## 342 343 **DISCUSSION:**

344  
345 The application of the smFISH method has gained popularity in recent times, with its use  
346 extending to various cell types and model organisms. However, in the case of *Drosophila*, the  
347 majority of knowledge about adult muscle gene expression is derived from bulk molecular assays,  
348 which fail to provide quantitative information on the precise spatial location of mRNA molecules.  
349 To address this gap, we have optimized a method for performing smFISH on muscle precursor  
350 cells and adult *Drosophila* muscles. This approach has been adapted from a previously published  
351 protocol<sup>23</sup> and optimized for *Drosophila* muscle tissues.

352  
353 The major obstacle in obtaining high-quality smFISH images is the thickness of adult muscles,  
354 which hinders the optimal penetration of probes. Therefore, it is crucial to separate the adult  
355 muscles from the other tissues of the animal and carry out the hybridization process with mild  
356 agitation. This particular step ensures effective permeabilization of the tissues.

357  
358 Another critical aspect is that the signal-to-noise ratio can cause the computational pipeline to  
359 fail in detecting certain mRNA spots. We found that increasing the signal-to-noise ratio can be  
360 achieved by finding the optimal concentration of probes. The optimal dilution may differ  
361 depending on the composition of each probe set, including the conjugated dye and  
362 oligonucleotide composition. We recommend trying different dilutions; a final dilution of 200 nM  
363 yielded the best signal-to-noise ratio for adult tissues in these experiments.

364  
365 With the smFISH method, it is possible to quantify the number of newly synthesized RNAs at the  
366 TS foci. When a gene is actively transcribed, multiple nascent RNAs are produced concurrently at  
367 the TS. As a result, the TS's intensity will surpass that of mature cytoplasmic RNA, and this feature,  
368 combined with its nuclear localization, can differentiate the TS from individual cytoplasmic RNAs.  
369 In the context of muscle biology, TS detection and quantification are particularly important for  
370 determining the synchronization of transcription of a specific gene among muscle nuclei within  
371 the same syncytium. However, this computational pipeline is not designed to differentiate  
372 between cytoplasmic mRNA and TS signals. As an alternative, we suggest combining this smFISH  
373 method with other well-established smFISH analysis tools, such as BayFISH or FISH-quant<sup>29,30</sup>.  
374 These tools have been proven to facilitate automated segmentation and fluorescence intensity  
375 calculations of RNA aggregates with remarkable precision.

376  
377 Finally, while smFISH detects mRNA molecules with high spatial resolution, it is limited to  
378 analyzing a small number of mRNA at the same time. Multiscale methods like merFISH  
379 (multiplexed error-robust fluorescence *in situ* hybridization) enable the simultaneous analysis of  
380 a large number of different mRNA<sup>31</sup>. By combining oligonucleotide probes and error-correcting  
381 codes, this method facilitates the detection of hundreds of RNA species in single cells.

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### 391 392 **DISCLOSURES:**

393 The authors have no conflicts of interest to disclose.

### 394 395 **REFERENCES :**

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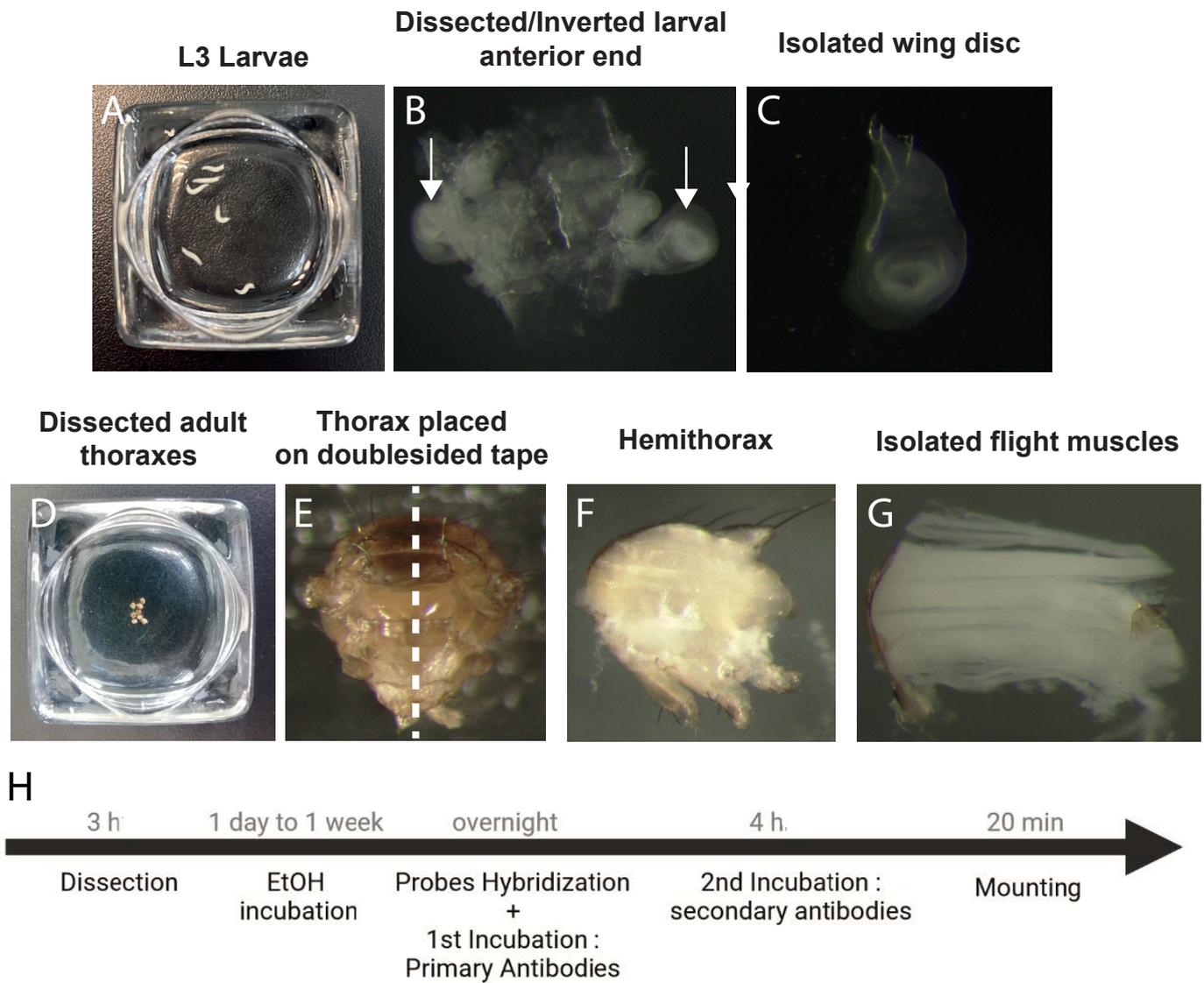


Figure 1

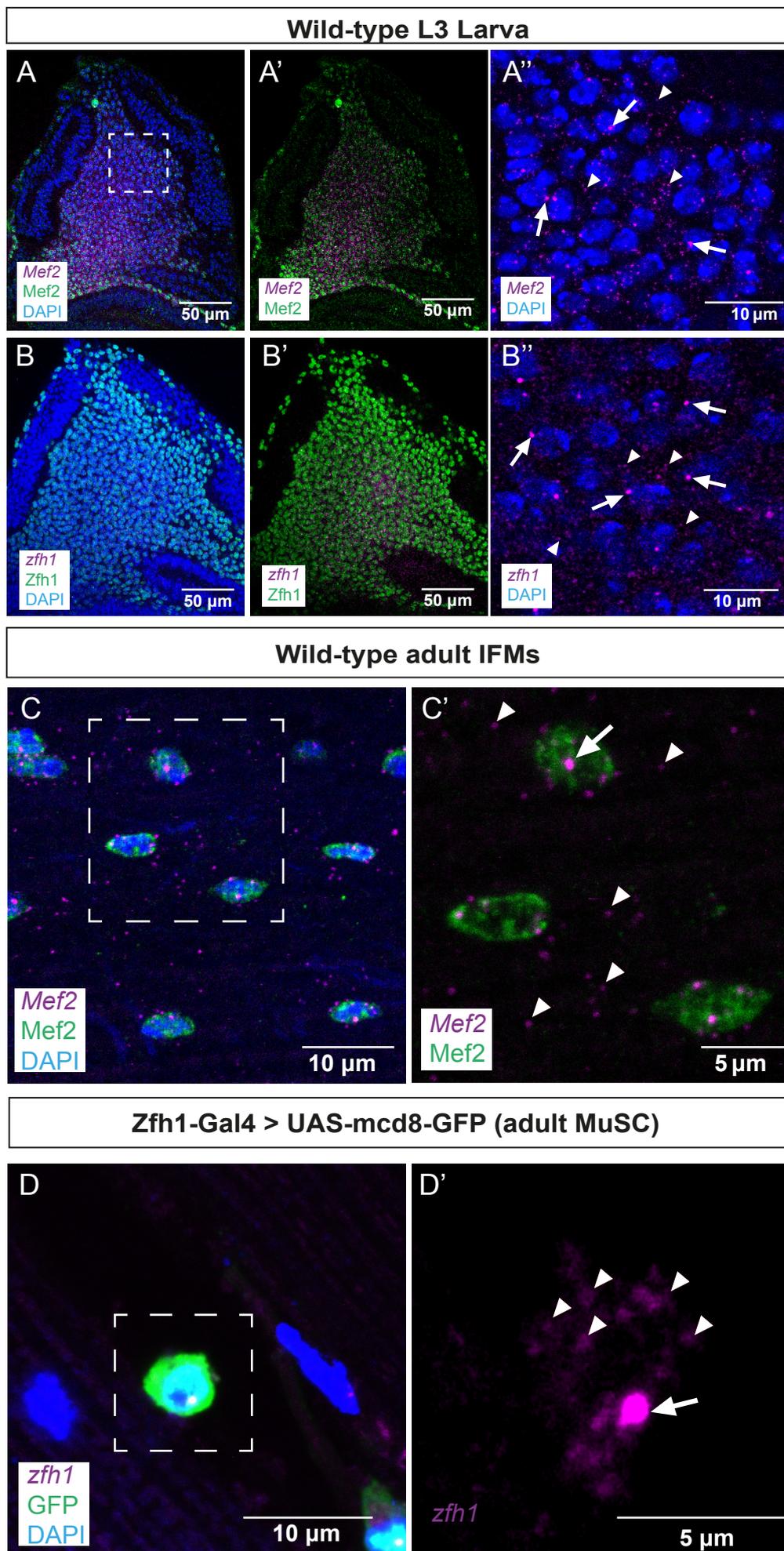


Figure 2

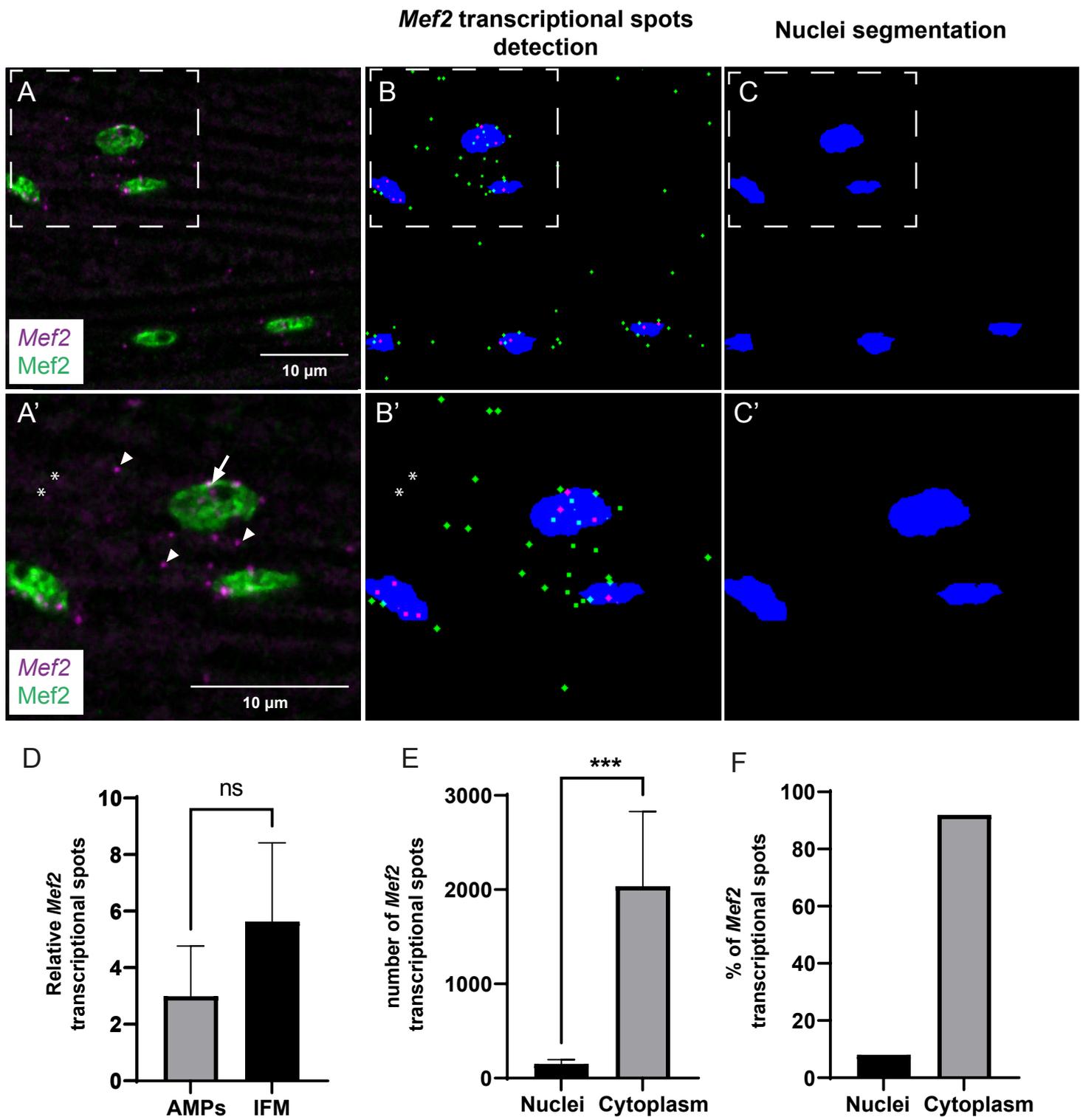


Figure 3

<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
Confocal microscope SP8	Leica	SP8 DMI 6000
DAPI	ThermoFisher	62248
Double-sided tape	Tesa	57910-00000
Dumont #55 Forceps	Fine Science Tools	11255-20
Formaldehyde 16%	Euromedex	EM-15710
Mef2 antibody	DHSB	NA
PBS 10x	Euromedex	ET330
RNaseZAP	Sigma-Aldrich	R2020
Stellaris probes	LGC	NA
Stellaris RNA FISH Hybridization Buffer	LGC	SMF-HB1-10
Stellaris RNA FISH Wash Buffer A	LGC	SMF-WA1-60
Stellaris RNA FISH Wash Buffer B	LGC	SMF-WB1-20
Swann-Morton Blades	Fisher scientific	0210
ThermoMixer	Eppendorf	5382000015
Triton X-100	Euromedex	2000
UAS-mCD8::GFP line	Bloomington	RRID:BDSC_5137
Ultrapure water	Sigma-Aldrich	95284
Watch Glass, Square, 1 5/8 in	Carolina	742300
Zfh1 antibody	Gifted by Ruth Leahman	
Zfh1-Gal4	Bloomington	BDSC: 49924, FLYB: FBtp0059625



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