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Immunostaining of Whole-Mount Drosophila Testes for 3D Confocal Analysis of Large Spermatocytes

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1 **TITLE:**
2 Immunostaining of Whole-Mount *Drosophila* Testes for 3D Confocal Analysis of Large
3 Spermatocytes
4
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18 **KEYWORDS:**
19 *Drosophila Melanogaster*, Testes, Spermatocytes, Immunofluorescence, Confocal microscopy,
20 Image 3D, Colocalization, Nucleoporin, Nuclear Territory, Nuclear pore complex
21
22 **SUMMARY:**
23 We present here an improved protocol for the whole-mount preparation and immunostaining
24 of *Drosophila* testes, suitable for confocal microscopy and also allowing reproducible and
25 reliable labeling. We illustrate this protocol by 3D representation and quantification of
26 colocalization experiments on the large S4-S5 spermatocytes.
27
28 **ABSTRACT:**
29 *Drosophila* testes are a powerful model system for studying biological processes including stem
30 cell biology, nuclear architecture, meiosis and sperm development. However, immunolabeling
31 of the whole *Drosophila* testis is often associated with significant non-uniformity of staining due
32 to antibody penetration. Squashed preparations only partially overcome the problem since it
33 decreases the 3D quality of the analyses. Herein, we describe a whole-mount protocol using
34 NP40 and heptane during fixation together with immunolabeling in liquid media. It preserves
35 the volume suitable for confocal microscopy together with reproducible and reliable labeling.
36 We show different examples of 3D reconstitution of spermatocyte nuclei from confocal
37 sections. The intra- and inter-testes reproducibility allows 3D quantification and comparison of
38 fluorescence between single cells from different genotypes. We used different components of
39 the intranuclear MINT structure (Mad1-containing Intra Nuclear Territory) as well as two
40 components associated with the nuclear pore complex to illustrate this protocol and its
41 applications on the largest cells of the testis, the S4-S5 spermatocytes.
42
43 **INTRODUCTION:**
44 Drosophila testes are a valuable model for the study of nuclear architecture. In mitotic

45 spermatogonial cells, the nuclear volume is largely occupied by chromatin. These cells undergo
46 four rounds of division, producing a cyst of 16 primary spermatocytes. Over the next several
47 days, the spermatocytes pass through 6 developmental stages (S1-S6), greatly increasing in
48 volume, approximatively 20-fold[1,2]. They also change their nuclear morphology. The outline of
49 the nucleus, revealed by the lamin Dm0, becomes irregular and shows deep invaginations[1,3].
50 This is accompanied by extensive gene expression changes and modifications in chromatin
51 organization[1,4-7]. The interphase chromosomes are well-defined in stage S4-S5, forming three
52 distinct masses corresponding to the 2 major bivalent autosomes and the X-Y pair docked with
53 the nucleolus.
54
55 Mad1 was originally isolated as a key component of the mitotic checkpoint (reviewed in[8]). In
56 interphase, it is described as located primarily at the nuclear envelope but also in the
57 nucleoplasm[9-11]. In Drosophila testis, we reported that Mad1 is prominent in the nucleoplasm,
58 intimately associated with the two major autosomal chromatin masses and to a lesser extent
59 with the XY chromatin. We defined this chromatin-associated structure as MINT (Mad1-
60 containing IntraNuclear Territory)[12]. Four other proteins were associated with MINTs in
61 spermatocytes: the nucleoporin Mtor/Tpr, the SUMO peptidase Ulp1, the mitotic checkpoint
62 protein Mad2 and a subunit of a Polycomb-like complex Raf2[12].
63
64 To complete the description of MINTs, we needed to use antibodies and were confronted with
65 the technical problems generally encountered with immunostaining in testis: a poor
66 permeability resulting in a significant non-uniformity of staining in the depth of the testis,
67 particularly in the large spermatocytes. Squashed preparations increased antibody access but
68 led to compressed images, restricting the 3D analyses, and preventing measurement of
69 quantitative fluorescence, including colocalization. The problem of antibody penetration could
70 also be addressed by permeabilizing agents such as 0.3% Na deoxycholate[13], but this procedure
71 did not in our hands yield reproducible results. Because we performed many co-labeling
72 experiments, we looked to improve the permeabilization protocol. The combination of 1%
73 NP40 plus heptane resulted in more reproducibility, particularly in studying the large
74 spermatocytes.
75
76 This protocol performs fixation and immunostaining of testes in suspension, improving the
77 sample quality as well as enhancing reproducibility, and rendering it suitable for confocal
78 microscopy. We used the protocol to better define the spatial relationships of certain nuclear
79 envelope proteins with the nucleoplasmic structures of large spermatocytes. This method will
80 permit better investigations of the changes that accompany spermatocyte development, for
81 example the dynamic structural changes of the nucleus including chromatin, nucleoplasm and
82 the nuclear pores.
83
84 **PROTOCOL:**
85
86 **1.   Drosophila testes collection**
87
88 NOTE: Young males (0-15 h post-eclosion) are necessary for examining diploid germ cells (i.e.,

89 spermatogonia and spermatocytes).

90

91 1.1.   Select young flies as much as possible to minimize interference from developing
92 spermatids.

93

94 1.2.   Anesthetize flies by brief exposure to carbon dioxide and transfer to a fly pad[14,15].

95

96 1.3.   Under a dissecting microscope (40x magnification), use forceps to remove the head.

97

98 1.4.   Transfer 5 to 10 decapitated flies to 100 µL of Ringer's buffer (183 mM KCl, 47 mM NaCl,
99 10 mM Tris-HCl, 1 mM EDTA, complete protein inhibitor, pH 6.8) on a silicone-coated glass slide
100 on a black background support.

101

102 1.5.   Use one #5 forceps to hold the fly between the thorax and the abdomen and with other
103 forceps pull the abdomen away from the thorax. Rapidly isolate the testes and adjacent tissues
104 from the fly carcass[15] and place in a new drop of Ringer's buffer on the same slide.

105

106 1.6.   Once all the testes are ready, clean the testes from the remaining tissues (accessory
107 gland and external genitalia).

108

109 1.7.   Remove excess buffer from the drop containing the dissected testes. Typically, use a
110 p200 pipette with a 10 ⊠L tip fitted on top of a 200 ⊠L tip. This allows the removal of the liquid
111 excess through a small aperture. Place a drop of 4% paraformaldehyde fixative on top of the
112 drop containing the dissected testes, and then rapidly transfer the testes to a tube containing
113 fresh fixative solution.

114

115 2.   **Paraformaldehyde fixation**

116

117 2.1.   Prepare 2 mL of fixative solution just before use: 600 µL of 4% paraformaldehyde
118 diluted in phosphate buffered saline (PBS) with 30 µL of NP40 20% plus 1200 µL of heptane in a
119 2 mL siliconized microcentrifuge tube. Use 4% paraformaldehyde in PBS prepared in 600 ⊠L
120 aliquots and stored at -20°C.

121

122 Caution: Heptane and paraformaldehyde are toxic and should be handled in a fume hood.
123 Dispose of them according to the host institute's regulations.

124

125 2.2.   Transfer the testes using a p200 micropipette with a wide aperture tip to the
126 PFA/NP40/heptane solution. Shake the tube up and down for 30 s by hand. Continue fixing the
127 testes on a rotary mixer at room temperature for 30 min.

128

129 2.3.   Stop the rotary mixer and place the tube in a tube rack to allow phase separation and
130 for the testes to settle to the bottom of the tube.

131

132 2.4.   Remove all the heptane and the fixative solution and rinse the testes quickly three times

133 with 1x PBS.

134

135 2.5.	Transfer to a clean 200 μL individual tube. Perform all incubations in these small tubes
136 to minimize the amounts of antibodies employed.

137

138 2.6.	Remove excess buffer using a 200 μL micropipette with both a P200 and a P10 tip
139 attached. Take care not to aspirate testes.

140

141 2.7.	Keep testes in 1x PBS on ice until all samples are ready.

142

143 3.	**Antibody staining in solution**

144

145 3.1.	Discard the PBS and leave testes in 0.3% PBS-T for 30 min at room temperature to
146 permeabilize cell membranes.

147

148 3.2.	Pre-incubate in the same buffer plus 5% normal goat serum (NGS) for 1 h.

149

150 3.3.	Add 200 μL of the primary antibody diluted in 0.3% PBS-T plus 5% NGS (**Table of**
151 **Materials**).

152

153 3.4.	Incubate the primary antibody overnight at 4 °C (or room temperature) with gentle
154 agitation on a rocker.

155

156 3.5.	Wash 3 times in 0.3% PBS-T for 15 min on a rocker at room temperature. Allow the
157 testes to settle to the bottom of the tube by gravity.

158

159 3.6.	Add 200 μL of secondary antibody diluted 1:500 from the DyLight conjugated series.

160

161 3.7.	Incubate the secondary antibody in a dark chamber for 4 h at room temperature.

162

163 3.8.	Wash in 0.3% PBS-T for 15 min on a rocker at room temperature. Then repeat with 0.2%
164 PBS-T and 0.1% PBS-T, each time for 30 min on a rocker at room temperature. Do a final wash
165 in 1x PBS for 30 min to remove the detergent.

166

167 3.9.	Add 180 μL of DAPI solution at 1 μg/mL in 1x PBS for 15 min. The stock solution is 10
168 mg/mL in H_2O. Avoid making stock solution in PBS, which can reduce the DAPI staining signal on
169 diffuse chromatin in larger spermatocytes.

170

171 3.10.	Wash 3x for 5 min each.

172

173 3.11.	Use a hydrophobic barrier pen to draw a circle on a clean slide.

174

175 3.12.	Aspirate testes and put them on the slide. Pre-rinsing the wide aperture tip with 0.1%
176 PBS-T prevents the testes from sticking to the tip. Remove excess PBS.

177

178   3.13.   Add a drop (approximately 100 µL) of Citifluor AF1.

179

180   3.14.   Gently place a glass coverslip over the tissue, taking care to avoid trapping air bubbles.

181

182   3.15.   Seal the coverslip to the slide using clear nail polish.

183

184   3.16.   Observe slides on a microscope.

185

186   4.   **Colocalization analyses**

187

188   4.1.   Acquire confocal images. We used a Zeiss LSM 710 confocal microscope (63x Plan
189   Apochromatic oil) with a z-resolution of 0.5 or 1 µm.

190

191   4.2.   Select different cells from independent testes.

192

193   4.3.   Import images to processing software (e.g., Imaris).

194

195   4.4.   Define the nucleus by manual segmentation. Use the Coloc software; it collects the
196   Pearson's correlation coefficients (PCCs) inside the entire volume between 2 fluorophores.

197

198   **REPRESENTATIVE RESULTS:**
199   The main obstacle to obtaining adequate staining of Drosophila testes is the limited
200   penetrability of antibodies, which has been partially resolved by using squashed preparations
201   but at the cost of inducing a restriction of the 3D analyses (for example 3D representation or
202   measure of colocalization). The procedure permits uniform labeling and preserves the volume
203   of all the cells of the testis. Here we have focused the illustration of the method on the 3D
204   representation of S4-S5 spermatocytes, as they are the largest cells and thus more sensitive to
205   deformation from squashing. We used different nuclear markers, including nucleoporins and
206   the labeling of the intranuclear MINT domain (Mad1-containing Intra Nuclear Territory)
207   described by the mitotic protein Mad1[12]. As MINTs could be revealed by several different
208   antibodies, we used them to test the robustness of the protocol for quantitative 3D
209   colocalization, a method that necessitates a correct spatial resolution.

210

211   The protocol described above permits the acquisition of confocal images of sufficient quality to
212   then be treated for 3D reconstitution. Four adjacent serial sections of a stage S5 spermatocyte
213   are depicted in **Figure 1A**, showing a representative immunostaining of MINT (Mad1
214   immunolabeling using GFP booster in red) together with DAPI (in cyan). The chromosomes are
215   organized into the characteristic 3 major distinct clusters and the MINT structure is shown in
216   fine detail around the DNA, intimately associated with--but distinct from--the autosomal
217   chromatin masses. The resolution also permits detection of the presence of Mad1 at the
218   nuclear periphery as well as between the autosomal masses of chromosomes 2 and 3. We used
219   Imaris software for the 3D representation. The nuclear surface was defined by the low level
220   presence of Mad1 at the nuclear envelope (in grey) and MINT was defined by high level

accumulation of Mad1 inside the nucleus (in red) together with DAPI staining (in blue) (**Figure 1A**). The 3D reconstitution movie well illustrates the volume and the relationship of Mad1 (at the nuclear envelope and in the MINT) together with the chromosomes in stage S4–S5 spermatocytes.

The distribution of nucleoporins has been only poorly described in these cells, probably due to the difficulty detecting them in these large misshapen nuclei. The distribution of Nup154 has only been reported once previously[16] and Nup153 and Nup98 were only studied in the spermatogonia and small, early spermatocytes[17]. The 3D reconstitutions of large S5 spermatocytes stained by the nucleoporin Nup62 and by importin β (product of the *ketel* gene) were feasible and are represented in **Figure 1C** and **Figure 1D**, respectively. These nuclear pore-associated components appear non-uniformly distributed over the nuclear rim as dots of irregular size. For example, the importin β gathers around the autosomal chromatin similarly to where a lamin network has been already described[3]. This kind of distribution with pore-rich areas and large pore-free areas has been described by electron microscopy in rodent spermatocytes[18]. Understanding the changes in lamin and nucleoporin distribution would help to understand the nuclear envelope structure in these G2 cells just prior to male meiosis.

While conserving the 3D volume of the cells this protocol also permits antibodies to penetrate uniformly into the depth of every cell, allowing quantitative analysis such as colocalization. The Pearson correlation coefficient (PCC) is a well-established metric. It can range from +1 (denoting perfect positive correlation) to −1 (perfect negative correlation)[19]. To evaluate the localization of MINT partner proteins relative to Mad1 in spermatocytes, we have previously estimated the PCC on a single section of 0.5 μm using ImageJ in S5 spermatocytes[12] (**Figure 2A,B**). We tested the protocol more deeply by using 3D colocalization on the whole nuclei instead of just a surface. The volume of the nuclei was defined by manual segmentation and the PCC was measured using the Colocation tool. We analyzed eight S5 spermatocyte nuclei, from the same testis or different testes. **Figure 2B** shows a 3D representation of a nucleus that served to measure the PCC. **Figure 2D** shows the measurement of the PCC on eight different S5 spermatocyte nuclei from three different testes. PCC values are not different, varying from 0.6 and 0.75, indicating a high value for colocalization. These are indistinguishable from the PCC defined on a single section. Thus, the procedure permits a reproducible uniform labeling and preserves a good volume of all the cells of the testis.

Because the protocol allows reliable measurement of colocalization from independent testes, it permitted comparison of staining from different fly genotypes. This was illustrated here by comparison of WT flies and flies RNAi depleted for Mtor (generated by expression of dsRNA UAS lines and the Bam-Gal4 driver that achieve substantial depletion in late spermatogonia and early spermatocytes[20]). As described for the colocalization experiments, Mtor-RNAi-depleted testes and wild types testes were dissected and fixed together and the confocal images were acquired under constant settings. Respecting these conditions, the comparison of individual cells becomes possible. We observed that the MINT is more affected than the nuclear envelope by the loss of Mtor as shown by representative Mtor RNAi-depleted (upper) and wt (lower) cells (**Figure 3**)[12].

265

Figure 1: Illustration of immunostaining of *Drosophila* S4-S5 spermatocytes nuclei using different probes. (**A**) Images of a single stage 5 spermatocyte nucleus immunostained with α-GFP antibody (GFP booster) detecting the MINT structure by Mad1-GFP (red) together to DAPI staining (blue). It shows with precision the relationship between Mad1 and the two autosomal chromatin masses (represented as asterisks). Mad1 is also faintly detected at the periphery of the nucleus. All images are serial Z-projections of a stack of 3 (0.5 µm) successive confocal slices. Confocal images were acquired on a confocal microscope (63x, Plan Apochromatic oil DIC objective lens). Scale bars are 5 µm. (**B**) Frame from a movie rendering of the same spermatocyte nucleus. The nuclear surface was defined by low expression of Mad1 at the nuclear envelope (in greys) and MINT was defined by high expression of Mad1 inside the nucleus (in red). (**A**, **B**) Reprinted with permission from previously published figure (from[12]). (**C**) 3D image of a stage 5 spermatocyte nucleus with simultaneous immunostaining of Nup62 (yellow) and MINT (GFP booster in red). Scale bar is 4 µm. (**D**) 3D image of a stage 5 spermatocyte nucleus with simultaneous immunostaining of the importin-β Ketel (yellow) together with DNA (DAPI in blue). Scale bars are 5 µm.

Figure 2: 2D and 3D Colocalization quantification of Mad1 together with Ulp1. (**A**) Merged images of a stage 5 spermatocyte nucleus immunostained with GFP booster antibody detecting Mad1 (green) and with anti-Ulp1 antibody (red) represented as a 1 µm thick stack of a spermatocyte nucleus. On the right is indicated the Pearson intensity correlation coefficient calculated using the Coloc-2 ImageJ plugin. The PCC was estimated to 0.73 (Reprinted with permission from Raich[12]). (**B**) Merged image of stage 5 spermatocyte nucleus (corresponding to nucleus G in the adjacent table) immunostained with GFP booster antibody detecting Mad1 (green) and with anti-Ulp1 antibody (red) represented in a 3D projection. The volume of the nucleus was defined by manual segmentation. On the right, the table lists the Pearson intensity correlation coefficients calculated on 8 different spermatocytes (A to H) from 3 different testes (1 to 3).

Figure 3: **Immunofluorescence analysis of Mtor RNAi-treated spermatocytes compared to wild type.** Cells depleted of Mtor using *Mtor*[RNAi] (ID 110218) cells (upper panels) and wild type cells (lower panels) were stained for Mad1, Raf2 and Mtor proteins. The testes were fixed and stained together, mounted on the same slide and images acquired with the same parameters. The RNAi cells could be distinguished from WT by the absence or presence respectively of Mad1-GFP. The figure is representative of maximum projections of 4 x 0.5 µm stacks. Scale bar is 5 µm (Reprinted with permission from previously published figure in[12]).

DISCUSSION:
This protocol provides a method for successful immunolabeling of whole-mount adult Drosophila testes. As reported in other procedures young males must be used (we even shortened the age to less than 20 hours after eclosion) to minimize the presence of developing sperm, which risks obstructing the spermatocytes. The dissection should be rapid and the antibody dilution must be adjusted to optimize the signal-to-noise ratio[21-23]. Because using other protocols, we did not observe staining throughout the depth of the testis, we developed

309 a fixation method combining PFA together with NP40 and heptane. We now routinely use 600
310 µL of 4% paraformaldehyde in PBS with 30 µL of 20% NP40 plus two volumes of heptane. This is
311 critical to allow for sufficient permeabilization. Reducing the amount of NP40 to 10 µL visibly
312 reduces the labeling. All the subsequent steps were carried out in suspension and the excess
313 buffer was removed by allowing the testes to descend gently to the bottom of the tube by
314 gravity. For the last step transferring samples to a slide, we rinse the tip in 0.1% PBS-T to
315 prevent the testes sticking to the cone. According to observations, the immunohistological
316 staining protocol works well with a variety of nuclear proteins, including the markers used here,
317 as well as probes of epigenetic histone modifications, lamin and nucleoporins. We note that the
318 protocol can also be used for larval testes, in which case the heptane should be omitted, as it
319 makes the testes more difficult to recover intact.
320
321 The protocol is especially designed to maintain undamaged stacks of the spermatocytes
322 together for an efficient and uniform labeling of the testis. We illustrated the protocol with the
323 large late stage spermatocytes, whose misshapen nuclei make them among the most difficult
324 cells to preserve. Furthermore, in these spermatocytes the nuclear labeling often becomes
325 dilute probably because of the size of the nuclei. For example, it is commonly observed that
326 DAPI or lamin labeling is stronger in the cells at the testis apex and becomes progressively
327 weaker and more disperse as the spermatocytes are growing[3,24]. This also could be one of the
328 reasons that the distribution of nucleoporins has only been described in earlier stage cells
329 (spermatogonia and early spermatocytes)[17].
330
331 Excellent images of immunostaining of Drosophila S5 spermatocytes have been published,
332 particularly for the testis-specific t-BRD and dany proteins[25,26] using either 0.3% sodium
333 deoxycholate protocol or following squashing of the tissue[21,22]. In our hands, squashes lead to a
334 significant non-uniformity of staining in the depths of the organ and as well sacrificing the 3D
335 structure. Even if the use of 0.3% sodium deoxycholate gave better results, we still observed
336 non-uniformity. Thus, we prefer NP40 for its reproducibility and quality of labeling. The
337 confocal images permit reconstruction of 3D images for nuclear proteins. For example, we have
338 successfully applied this method using many different probes, illustrated here by the
339 intranuclear MINT domain, intimately associated with the autosomal chromatin masses and the
340 nuclear pore network. Intriguing 3D distribution of nuclear pores of these spermatocyte nuclei
341 is now available for further studies. The images permit reproducible quantification of
342 colocalization from different spermatocytes from the same testis and from different testes. To
343 our knowledge this is the first time that accurate quantification by Pearson correlation
344 coefficient has been measured on such samples. It gives the confidence required for accurate
345 immunolabelled protein levels comparison of different testes.
346

350
351 **DISCLOSURES:**
352 The authors have nothing to disclose.

353

REFERENCES:

1   Cenci, G., Bonaccorsi, S., Pisano, C., Verni, F., Gatti, M. Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of Drosophila melanogaster spermatogenesis. *Journal of Cell Science*. **107**, 3521-3534 (1994).

2   Bonaccorsi, S., Giansanti, M. G., Gatti, M. Spindle assembly in Drosophila neuroblasts and ganglion mother cells. *Nature Cell Biology*. **2** (1), 54-56 (2000).

3   Fabbretti, F. et al. Confocal Analysis of Nuclear Lamina Behavior during Male Meiosis and Spermatogenesis in Drosophila melanogaster. *PLoS One.* **11** (3), e0151231 (2016).

4   Fuller, M. T. in *The Development of Drosophila melanogaster* Vol. 1 eds M. Bate & A. Martinas-Arias) Ch. 2, 71-148 (Cold Spring Harbor Laboratory Press, 1993).

5   Hiller, M. et al. Testis-specific TAF homologs collaborate to control a tissue-specific transcription program. *Development*. **131** (21), 5297-5308 (2004).

6   Chen, X., Hiller, M., Sancak, Y., Fuller, M. T. Tissue-specific TAFs counteract Polycomb to turn on terminal differentiation. *Science*. **310** (5749), 869-872 (2005).

7   White-Cooper, H., Davidson, I. Unique aspects of transcription regulation in male germ cells. *Cold Spring Harbor Perspectives in Biology*. **3** (7) (2011).

8   Luo, Y., Ahmad, E., Liu, S. T. MAD1: Kinetochore Receptors and Catalytic Mechanisms. *Frontiers in Cell and Developmental Biology*. **6**, 51 (2018).

9   Chen, R. H., Shevchenko, A., Mann, M., Murray, A. W. Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores. *Journal of Cell Biology*. **143** (2), 283-295 (1998).

10   Campbell, M. S., Chan, G. K., Yen, T. J. Mitotic checkpoint proteins HsMAD1 and HsMAD2 are associated with nuclear pore complexes in interphase. *Journal of Cell Science*. **114** (Pt 5), 953-963 (2001).

11   Katsani, K. R., Karess, R. E., Dostatni, N., Doye, V. In vivo dynamics of Drosophila nuclear envelope components. *Molecular Biology of the Cell*. **19** (9), 3652-3666 (2008).

12   Raich, N., Mahmoudi, S., Emre, D., Karess, R. E. Mad1 influences interphase nucleoplasm organization and chromatin regulation in Drosophila. *Open Biology*. **8** (10) (2018).

13   Chang, Y. J., Pi, H., Hsieh, C. C., Fuller, M. T. Smurf-mediated differential proteolysis generates dynamic BMP signaling in germline stem cells during Drosophila testis development. *Developmental Biology*. **383** (1), 106-120 (2013).

14   Stocker, H., Gallant, P. Getting started: an overview on raising and handling Drosophila. *Methods in Molecular Biology*. **420**, 27-44 (2008).

15   Zamore, P. D., Ma, S. Isolation of Drosophila melanogaster testes. *Journal of Visualized Experiments*. 10.3791/2641 (51) (2011).

16   Gigliotti, S. et al. Nup154, a new Drosophila gene essential for male and female gametogenesis is related to the nup155 vertebrate nucleoporin gene. *Journal of Cell Biology*. **142** (5), 1195-1207 (1998).

17   Chen, H., Chen, X., Zheng, Y. The nuclear lamina regulates germline stem cell niche organization via modulation of EGFR signaling. *Cell Stem Cell*. **13** (1), 73-86 (2013).

18   Fawcett, D. W., Chemes, H. E. Changes in distribution of nuclear pores during differentiation of the male germ cells. *Tissue Cell*. **11** (1), 147-162 (1979).

19   Bolte, S., Cordelieres, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy*. **224** (Pt 3), 213-232 (2006).

397   20   White-Cooper, H. Tissue, cell type and stage-specific ectopic gene expression and RNAi
398   induction in the Drosophila testis. *Spermatogenesis*. **2** (1), 11-22 (2012).

399   21   Bonaccorsi, S., Giansanti, M. G., Cenci, G., Gatti, M. Immunostaining of Drosophila
400   testes. *Cold Spring Harbor Protocols*. **2011** (10), 1273-1275 (2011).

401   22   Bonaccorsi, S., Giansanti, M. G., Cenci, G., Gatti, M. Paraformaldehyde fixation of
402   Drosophila testes. *Cold Spring Harbor Protocols*. **2012** (1), 102-104 (2012).

403   23   White-Cooper, H. Spermatogenesis: analysis of meiosis and morphogenesis. *Methods in*
404   *Molecular Biology*. **247**, 45-75 (2004).

405   24   Kibanov, M. V., Kotov, A. A., Olenina, L. V. Multicolor fluorescence imaging of whole-
406   mount Drosophila testes for studying spermatogenesis. *Analytical Biochemistry*. **436** (1), 55-64
407   (2013).

408   25   Theofel, I. et al. tBRD-1 and tBRD-2 regulate expression of genes necessary for
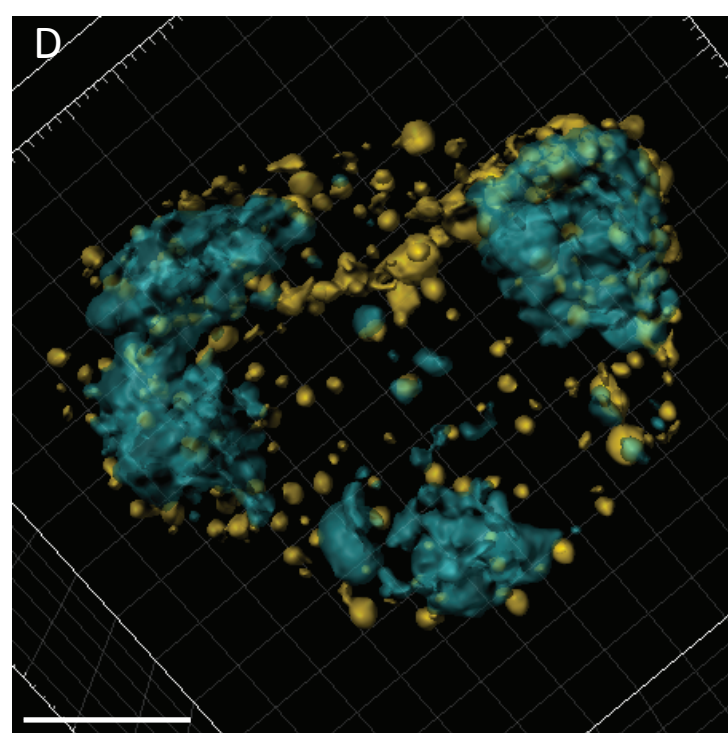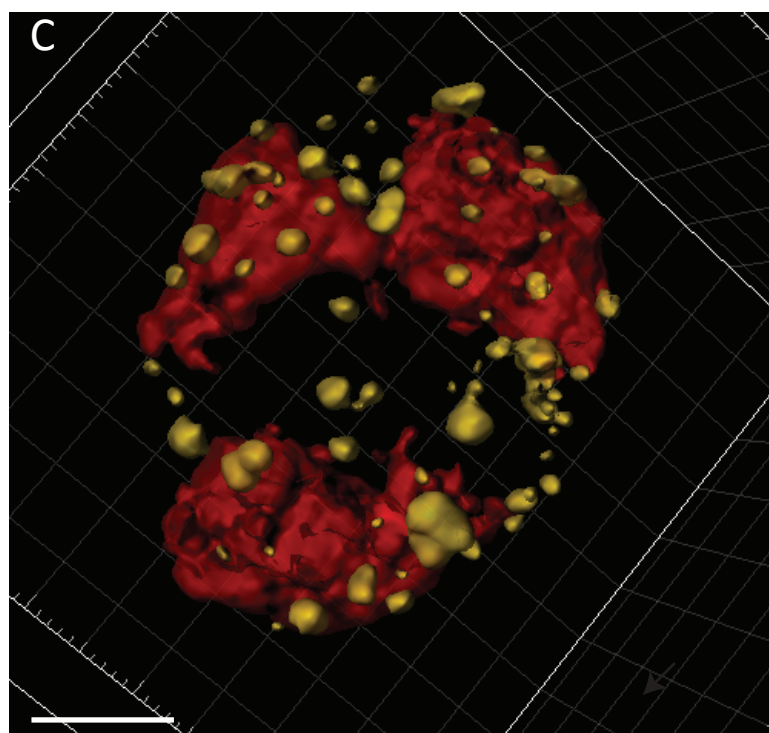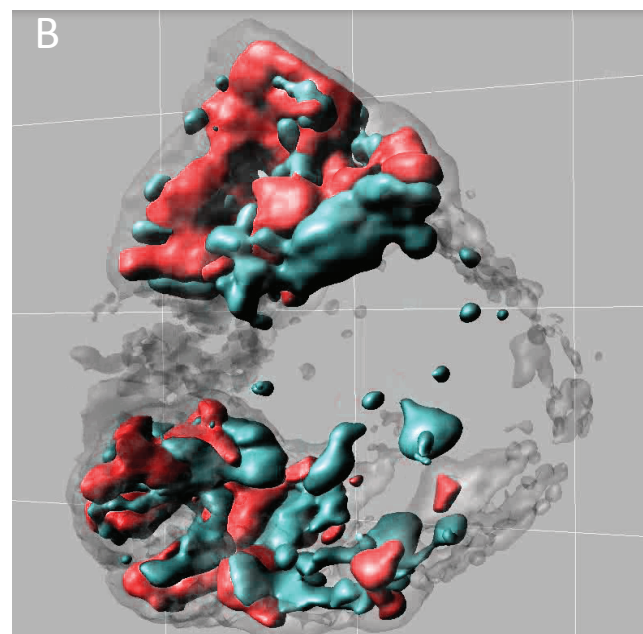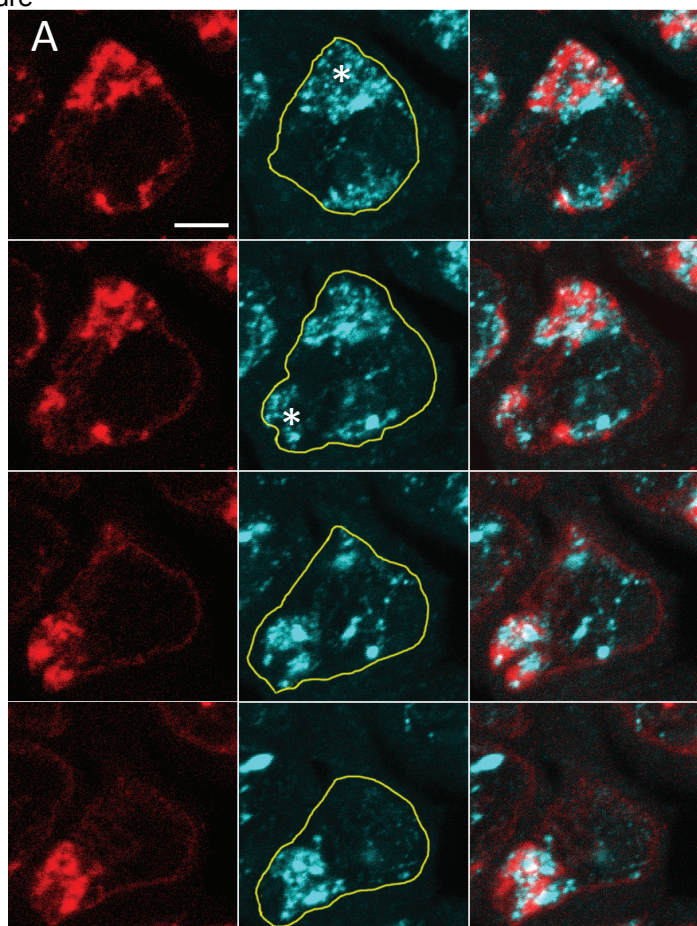409   spermatid differentiation. *Biology Open*. **6** (4), 439-448 (2017).

410   26   Trost, M., Blattner, A. C., Leo, S., Lehner, C. F. Drosophila dany is essential for
411   transcriptional control and nuclear architecture in spermatocytes. *Development*. **143** (14),
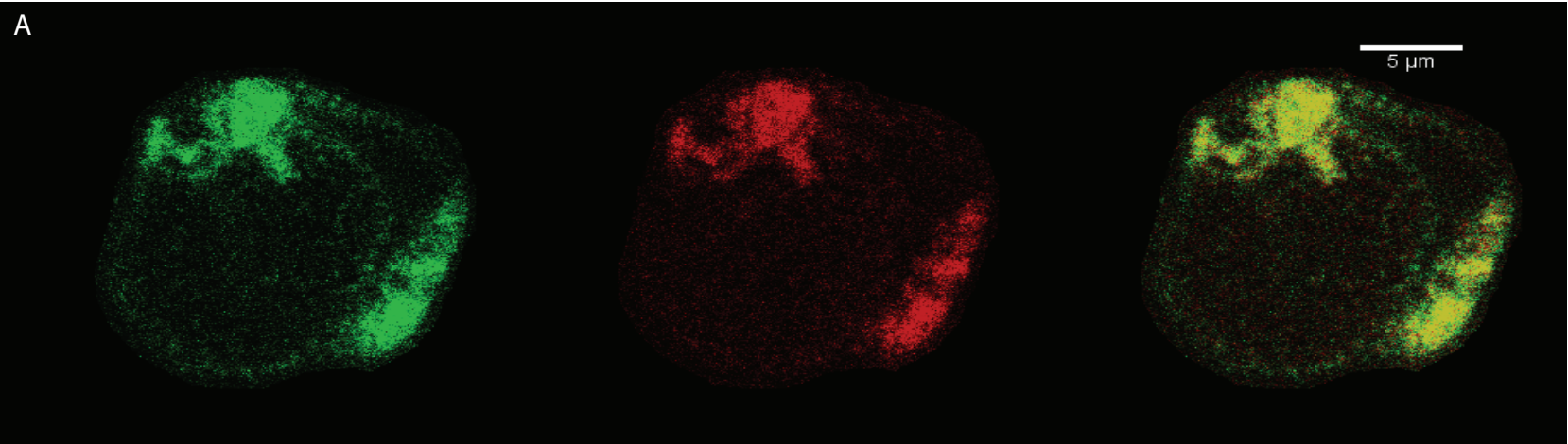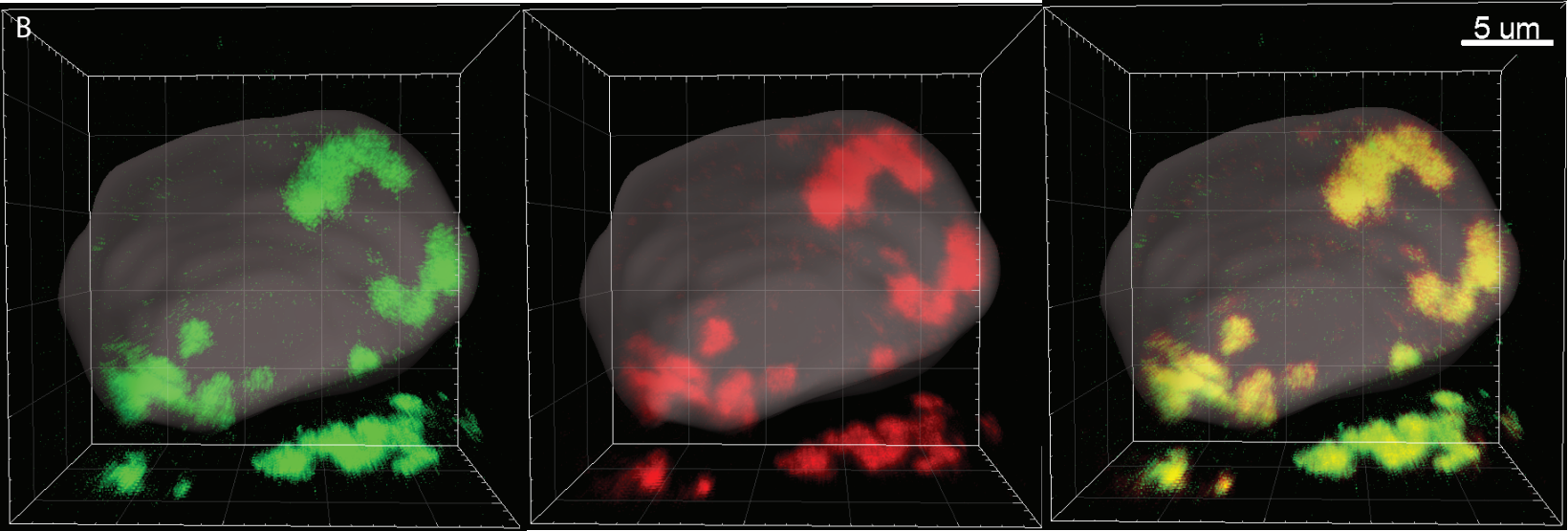412   2664-2676 (2016).

413
414

Testis	Nucleus	PCC
		0,73

Testis	Nucleus	PCC
1	A	0.63
1	B	0.69
1	C	0,6
2	D	0.75
2	E	0.75
3	F	0.66
3	G	0.71
3	H	0.72

Figure
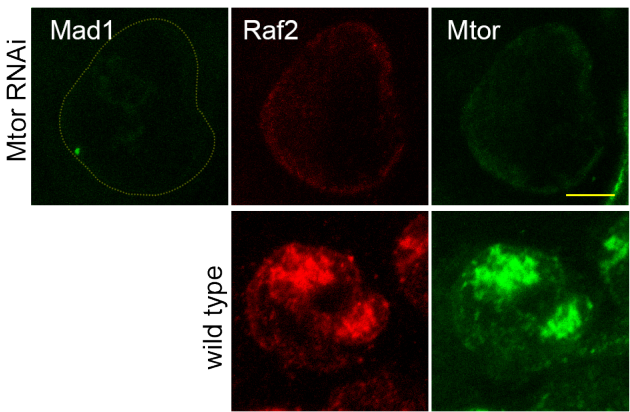
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Dear Dr. Anna Nam Nguyen,

We are returning our manuscript, JoVE61061, initially titled "Immunofluorescence staining protocol of whole-mount Drosophila testes suitable for 3D confocal analyses of large spermatocytes," in its revised form.

1. In response to Comment 3 of the Editor, we propose the more concise title: "Immunostaining of whole-mount *Drosophila* testes for 3D confocal analysis of large spermatocytes."

2. We thoroughly proofread the manuscript and corrected the spelling and grammar issues (comment 1 from Editor and Reviewers comments). We tracked the changes and identified all of the edits; these are most of minor concerns raised by Reviewers 1, 2 and 4 within the manuscript.

3. We corrected the Table and we sorted the "Materials Table" both by category (Drosophila strains and Materials and the antibodies) and alphabetically, for greater clarity. (Comment 2 from Editor)

4. We now explain how the anesthesia is done and we added a reference (line 88); we also indicate the magnification used for dissection (40x) (line 90) and the dilutions of the antibodies (line 139). We also indicate the approximate volume of the drop of Citifluor (line 153). We include two additional references for steps 1-2 and 1-5 (Comments 5-9 from Editor).

5. We corrected the major concerns of Reviewer 1:

a. We explain more clearly that there are two steps to the procedure by adding a second phrase to the sentence: "Place a drop of 4% paraformaldehyde fixative on the slide adjacent to the drop containing the dissected testes, then rapidly transfer the testes to the fixative solution. " (Line 101).

b. We define more clearly the 4% paraformaldehyde solution in the Materials Table.

c. We define the composition of the PBS/triton (PBS-T) in the Materials Table and we harmonized the text.

d. We corrected the error about the DyLight antibodies in the Reagents Table.

e. We mention the incubation period for the DAPI (line 145).

6. We added a line explaining a modification of the protocol when larval testes are used*:* "When preparing larval testes for fixation, the heptane should be omitted as the testes become more difficult to recover" (line 251).

7. Reviewer 4 suggested a color code for the Figures. We used a color code in Figure 1: The MINT structure represented by Mad1 is always in red, the DAPI is in in blue and the nucleoporin is in gold (Figure 1). In Figures 2 and 3 we used the classical green and red colors for MINT-associated proteins as they are the most easily distinguishable.

8. We did not ignore that the "old" Bonaccorsi protocol has generated and no doubt will continue to produce a vast collection of high-quality images in the literature. We have cited these protocols directly but also we added these references again in the Discussion (lines 265) instead citing only their squash method.

9. Figures 1A and B and Figure 3 are now cited appropriately on lines 294 and 322, respectively as "Reprinted with permission from previously published figure in [12]".

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We hope you will now find the manuscript acceptable.

Sincerely yours,
Natacha Raich