

1 **Live applications of norbormide-based fluorescent probes in**

2 ***Drosophila melanogaster***

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19 Abstract

20 In this study we investigated the performance of two norbormide (NRB)-derived fluorescent probes,
21 NRB^{MC009} (green) and NRB^{ZLW0047} (red), on dissected, living larvae of *Drosophila*, to verify their
22 potential application in confocal microscopy imaging *in vivo*. To this end, larval tissues were exposed
23 to NRB probes alone or in combination with other commercial dyes or GFP-tagged protein markers.
24 Both probes were rapidly internalized by most tissues (except the central nervous system) allowing
25 each organ in the microscope field to be readily distinguished at low magnification. At the cellular
26 level, the probes showed a very similar distribution (except for fat bodies), defined by loss of signal
27 in the nucleus and plasma membrane, and a preferential localization to endoplasmic reticulum (ER)
28 and mitochondria. They also recognized ER and mitochondrial phenotypes in the skeletal muscles of
29 fruit fly models that had loss of function mutations in the atlastin and mitofusin genes, suggesting
30 NRB^{MC009} and NRB^{ZLW0047} as potentially useful *in vivo* screening tools for characterizing ER and
31 mitochondria morphological alterations. Feeding of larvae and adult *Drosophilae* with the NRB-
32 derived dyes led to staining of the gut and its epithelial cells, revealing a potential role in food intake
33 assays. In addition, when flies were exposed to either dye over their entire life cycle no apparent
34 functional or morphological abnormalities were detected. Rapid internalization, a bright signal, a
35 compatibility with other available fluorescent probes and GFP-tagged protein markers, and a lack of
36 toxicity make NRB^{ZLW0047} and, particularly, NRB^{MC009} one of the most highly performing fluorescent
37 probes available for *in vivo* microscopy studies and food intake assay in *Drosophila*.

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41 Introduction

42 Norbormide [5-(α -hydroxy- α -2-pyridylbenzyl)-7-(α -2-pyridylbenzylidene)-5-norbornene-2,3-
43 dicarboximide] (NRB) is a selective rat toxicant that exhibits little or no non-target effects (1), and
44 was developed and commercialized as an ecologic pesticide in the 1980s (Roszkowski, 1965).
45 Evidence suggests that the rat-selective action of NRB is mediated by a generalized vasoconstrictor
46 effect that has only been observed in the rat peripheral blood vessels, both *in vivo* and *in vitro*. In
47 contrast, NRB displays a vasorelaxant action in arteries from non-rat species, as well as in rat aorta
48 and extravascular smooth muscle, that has been proposed to be the result of a reduction of Ca^{2+} entry
49 through L-type Ca^{2+} channels (2,3). The molecular mechanism underlying NRB-induced
50 vasoconstriction is not known, however, it has been proposed that the compound acts on rat vascular
51 myocytes where it activates the PLC-IP3-PKC pathway (2), a signaling cascade stimulated by most
52 receptor-coupled vasoconstrictor agents (4). In an attempt to identify the cellular targets of NRB, we
53 previously developed fluorescent derivatives of the parent compound by linking it to either
54 nitrobenzoxadiazole (NBD) or boron-dipyrromethene (BODIPY FL) fluorophores, and found that
55 both were able to clearly label intracellular structures such as endoplasmic reticulum (ER), Golgi
56 apparatus, mitochondria, and lipid droplets (LDs) in various cell lines, in the absence of cytotoxic
57 effects. Based on these results, we proposed NRB as a scaffold for the development of new, high
58 performing, non-toxic fluorescent probes for live cell imaging (5,6).

59 *Drosophila melanogaster* is an animal model widely used to investigate the biochemical pathways
60 and the cellular/subcellular morphological alterations that characterize human diseases (Aldaz et al.,
61 2010; Chatterjee, 2014; Mushtaq et al., 2016; Musselman and Kühnlein, 2018; Orso et al., 2005; Tan
62 and Azzam, 2017) Confocal fluorescent microscopy live imaging is a particularly informative
63 methodology in this model, especially when fluorescent probes are used in combination with genetic
64 tools (e.g. mutant flies, RNA interference, fluorescently marked proteins, Gal4/UAS activation

65 system) (13,14), allowing the visualization of dynamic biological processes in living systems without
66 the artifacts often generated by sample fixation procedures (15).

67 In this study, we investigated the performance of two NRB-derived fluorescent probes, the
68 previously developed NRB^{MC009} (green fluorescence) and the newly developed NRB^{ZLW0047} (red
69 fluorescence), on dissected, living third instar larvae of *Drosophila melanogaster*, to assess their
70 potential application in confocal microscopy imaging *in vivo*. In particular, we were able to
71 characterize the distribution of NRB^{MC009} and NRB^{ZLW0047} to cellular structures and organelles in
72 tissues of wild-type *Drosophila* larvae, as well as in tissues of mutant lines exhibiting morphological
73 alterations of endoplasmic reticulum and mitochondria. Finally, we explored if these probes could be
74 useful for studying fruit fly feeding and gut morphology.

75

76 **Material and methods**

77 **NRB^{MC009} and NRB^{ZLW0047} synthesis**

78 NRB^{MC009}, a BODIPY FL derivative of norbormide, was synthesized as previously reported (6).
79 Stock solutions 1 mM in DMSO were prepared and maintained at -20 °C and diluted to the desired
80 concentration before each experiment.

81 NRB^{ZLW0047}, a BODIPY TMR derivative of norbormide, was prepared as follows. BODIPY TMR
82 (4,4-difluoro-5-(4-methoxyphenyl)-1,3-dimethyl-4-bora-3a,4a-diaza-s-indacene-2-propionic acid)
83 (16), along with its corresponding N-hydroxysuccinimide ester, BODIPY TMR NHS ester (16), and
84 N-2'-aminoethyl-endo-5-(α -hydroxy- α -2-pyridylbenzyl)-7-(α -2-pyridylbenzylidene)-5-norbornene-
85 2,3-dicarboximide (17) were prepared using literature methods. A solution of N-2'-aminoethyl-endo-
86 5-(α -hydroxy- α -2-pyridylbenzyl)-7-(α -2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide (111
87 mg, 0.20 mmol), BODIPY TMR NHS ester (110 mg, 0.20 mmol) and *N,N*-diisopropylethylamine (35
88 μ l, 0.20 mmol) in dichloromethane (7 ml) was stirred at room temperature for 16 h. The mixture was

89 then diluted with dichloromethane (20 ml), washed with water (10 ml), the separated aqueous phase
90 further extracted with dichloromethane (2 × 10 ml), the combined organic layers washed with brine
91 (3 × 20 ml), dried over anhydrous magnesium sulfate, filtered and the solvent removed in vacuo.
92 Purification by flash chromatography (petroleum ether/ethyl acetate, 1:3) afforded NRB^{ZLW0047} as a
93 mixture of endo stereoisomers (purple solid; 70 mg, 37%). ¹H NMR (400 MHz, CDCl₃) δ 8.64-8.38
94 (2H, m, αPyr), 7.88-7.82 (2H, m, ArH), 7.59-6.77 (20H, m, ArH), 6.53-6.47 (1H, m, C=CH), 6.29
95 (0.2H, br s, OH), 6.28 (0.1H, br s, OH), 6.21-6.20 (0.2H, m, W/H-6), 6.15-6.14 (0.1H, m, U/H-6),
96 5.54-5.53 (0.4H, m, Y/H-6), 5.52-5.51 (0.3H, m, V/H-6), 5.22 (0.4H, br s, OH), 5.15 (0.3H, br s,
97 OH), 4.49-4.46 (0.2H, m, W/H-1), 4.46-4.43 (0.4H, m, Y/H-1), 4.31-4.29 (0.3H, m, V/H-1), 4.03-
98 4.01 (0.1H, m, U/H-1), 3.85-3.84 (3H, m, OMe), 3.84-3.24 (7H, m, H-2, H-3, H-4 and NCH₂CH₂),
99 2.75-2.72 (2H, m, COCH₂CH₂ or COCH₂CH₂), 2.47 (3H, m, Me), 2.26-2.22 (2H, m, COCH₂CH₂ or
100 COCH₂CH₂), 2.20-2.18 (3H, m, Me).
101 Stock solutions 1 mM in DMSO were prepared and maintained at -20 °C and diluted to the desired
102 concentration before each experiment.
103

104 **Fluorescence spectra**

105 Excitation and emission spectra of NRB^{MC009} and NRB^{ZLW0047} were obtained by diluting the stock
106 solution in Milli-Q water to reach the final concentration of 2 μM. To verify that the medium used for
107 *Drosophila* live imaging (HL3) had no effect on the emission spectra, a comparison was made
108 between probes diluted in water and probes diluted in HL3 1 - no variation in fluorescence was
109 observed.
110 Analysis of excitation/emission peaks were evaluated using a Jasco FP6500 spectrofluorometer
111 (temperature 25 °C; b=1 cm; λ ex/em: 470/540 for NRB^{MC009} and 545/580 for NRB^{ZLW0047}; sli: 5/10
112 nm; data pitch 0.2 nm; scanning speed 200nm/min).

113

114 **Fly stocks**

115 *Drosophila melanogaster* strains used: w^[1118] (BL-5905), Tubulin-Gal4 (BL-5138), UAS-Mito-GFP
116 (BL-8443), UAS-mCD8-GFP (BL-5130), were obtained from Bloomington Drosophila Stock Center,
117 and UAS-ATL2^{RNAi} (18) and UAS-Marf^{RNAi} (ID 40478), were resourced from Vienna Drosophila
118 Resource Center. UAS-Lamp-GFP was provided by Helmut Krämer (University of Texas
119 Southwestern Medical Center, Dallas), and UAS-HneuGFP was generated by cloning HNEU-GFP
120 (19) in pUASTattB, and transgenic lines generated by BestGene Inc, (Chino Hills, CA, USA). W^[1118]
121 flies were maintained on standard food at 25 °C, and Gal4/UAS crossings were performed at 28 °C.
122 Starvation was induced by leaving third instar larvae for 6 hours in 20% sucrose dissolved in PBS.

123

124 **Larval dissection**

125 Fly larvae having reached the third instar stage were pinned between the posterior spiracles and
126 above the mouth hooks in a Sylgard dissection dish, and cut along the dorsal midline. Hemolymph-
127 like (HL3) saline (70 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM
128 trehalose, 115 mM sucrose, 5 mM sodium HEPES, pH 7.2, all supplied by Sigma-Aldrich) was
129 added and the lateral flaps were fastened with four needles to stretch the body wall. All larval organs
130 were left on the muscle fillet for whole larval acquisition, whereas for single tissue acquisition the
131 unnecessary organs were removed.

132

133 **Live tissue imaging**

134 To characterize NRB^{MC009} and NRB^{ZLW0047} localization, each were diluted in HL3 medium at
135 concentrations of 500 nM and 1 μM, respectively, and were added to the dissected larva and images
136 acquired after 15 min. For NRB^{MC009} colocalization studies, ER-Tracker™ Red 2 μM (BODIPY™

137 TR Glibenclamide), MitoTracker™ Orange CMTMRos 1 μ M, LysoTracker™ Deep Red 2 μ M, HCS
138 LipidTOX™ Deep Red Neutral Lipid Stain 1:100, or CellMask 1 μ M (all by Thermo Fisher,
139 respectively #E34250, #M7510, #L12492, #H34477, and #10045) were added, together with
140 NRB^{MC009} at 500 nM. To verify NRB^{ZLW0047} colocalization with other organelles, it was added on
141 dissected larvae expressing GFP-tagged proteins (Hneu-GFP, Mito-GFP, Lamp-GFP, and mCD8-
142 GFP), or together with BODIPY 493/503™ dye 10 μ g/ml (#D3922, Thermo Fisher). Whole larvae
143 images and magnifications were acquired using a Zeiss LSM800 Axio Observer Z1 inverted
144 microscope equipped with a Zeiss Plan-Apochromat 5x/0.15 ph1 or 40x/0.95 objectives, all other
145 images were acquired with a Nikon EZ-C1 confocal microscope equipped with a Nikon Plan Apo
146 60 \times /1.40 or a Nikon Plan Apo 40x/1.0 oil immersion objectives.

147

148 **Three-choice preference assay**

149 To test larval food preference, a Petri dish was divided in three quadrants filled with a warm liquefied
150 standard food solution. In two quadrants, the food contained either NRB^{MC009} or NRB^{ZLW0047} (both
151 20 μ M), in the third the food was left probe-free. At the beginning of the experiment, a group of 10
152 larvae was placed in the middle of the assay plate and after 5 min the number of larvae on each
153 quadrant was counted (20). The experiment was repeated five times.

154

155 **Food intake test**

156 The food intake test was conducted in third instar larvae starved for 1 hour in 20% sucrose dissolved
157 in PBS. The starved larvae were then divided into three groups, separately fed with brilliant blue R
158 dye 0.08%, NRB^{MC009}, or NRB^{ZLW0047} (both at a concentration of 20 μ M) dissolved in liquid food
159 (sucrose 20% and 20% dry yeast in PBS) for 30 min (21), frozen at -80° C and imaged using a Leica
160 MZ 16 FA microscope. To estimate food intake, the area of dye-labeled gut, visible through the

161 cuticle, was quantified relative to larval total body area. 10 larvae for each experiment were used and
162 the experiment was repeated three times. Image analysis was performed with ImageJ 1.52h software.

163

164 **Analysis of gut fluorescence in *Drosophila* chronically fed with**
165 **NRB^{MC009} and NRB^{ZLW0047}**

166 Third instar larvae, grown in NRB^{MC009}- and NRB^{ZLW0047}- enriched food as described in the chronic
167 toxicity assay, were collected and food debris was removed by washing in PBS for 5 minutes and
168 70% ethanol for 1 minute and then dissected, being careful to not cut the digestive tract. Images were
169 acquired using a Zeiss Axio Observer Z1 inverted microscope equipped with a Zeiss Plan-
170 Apochromat 5x/0.15 ph1 or 40x/0.95 objectives.

171

172 **Chronic toxicity assay**

173 The toxicity of NRB-derived probes was investigated by exposing the flies to NRB^{MC009} or
174 NRB^{ZLW0047} 20 μ M over the entire life-cycle (mating, eggs maturation, pupal development, eclosion).
175 Male and female w^[1118] flies were placed in a tube with standard food containing vehicle, NRB^{MC009},
176 or NRB^{ZLW0047} 20 μ M, and grown at 25 °C. After 5-6 days, parent flies were removed and analyzed
177 to verify probe intake. Eggs were left to develop in food containing one of each of the NRB
178 fluorescent probes until the eclosion. Two parameters were considered in the evaluation of toxicity:
179 1) eclosion rate (percent of emerged flies versus the total number of pupae, including the dead ones);
180 2) morphological alterations of eclosed adults. Morphological alterations of male and female adult
181 body, eyes, wings, and legs were evaluated and imaged using a Leica MZ 16 FA microscope.

182

183 **Statistical analysis**

184 Analysis of colocalization was performed using Pearson's correlation coefficient calculated with
185 Coloc2 plugin of Fiji (22). All values are expressed as means \pm standard error of the mean (SEM) of
186 n observations (n \geq 10). Significance was calculated using One-way ANOVA test followed by Tukey's
187 Multiple Comparison Test, using GraphPad Prism 3.03.

188

189 **Results and discussion**

190 **Synthesis and excitation/emission spectra of fluorescent probes**

191 **NRB^{MC009} and NRB^{ZLW0047}**

192 The structures and synthetic routes to generate NRB^{MC009} and NRB^{ZLW0049} are summarized in Figs 1A
193 and 1B, respectively. The excitation and emission spectra of the dyes in water, and in the
194 physiological buffer (HL3) solution which was used to perform live imaging experiments in
195 *Drosophila* tissues, are presented in Figs 1C and 1D, respectively, and summarized in Fig 1E. The
196 excitation spectra analysis showed that the probes can be excited using the common laser lines 561
197 nm (NRB^{ZLW0049}) and 488 nm (NRB^{MC009}), respectively.

198

199 **Fig 1. NRB^{MC009} and NRB^{ZLW0047} synthesis and fluorescent spectra.** Scheme of the synthesis of
200 NRB^{MC009} (A) and NRB^{ZLW0047} (B). Excitation and emission spectra of NRB^{MC009} (C) and
201 NRB^{ZLW0047} (D) diluted in water (light color) or in HL3 medium (dark color). Summary table of
202 physical properties of NRB^{MC009} and NRB^{ZLW0047} (E), MW: molecular weight; λ_{ex} : excitation
203 wavelength; λ_{em} : emission wavelength.

204

205 **Tissue distribution of NRB^{MC009} and NRB^{ZLW0047} in *Drosophila* larvae**

206 To characterize NRB^{MC009} and NRB^{ZLW0047} fluorescence distribution in *Drosophila* tissues, we
207 dissected a wild type larva and exposed the whole body to the fluorescent probes. Figs 2A and 3A are
208 confocal images of dissected third instar larvae in which all tissues were left intact and labeled with
209 500 nM NRB^{MC009} or 1 μ M NRB^{ZLW0047}, respectively. As shown in the pictures, both dyes localized
210 to most of the tissues, with an apparent brighter signal detected in imaginal discs, tracheal system,
211 salivary glands, and fat body (Figs 2B, D, H, J, respectively, and Figs 3B, D, H, J, respectively), and
212 a less intense signal being detected in muscular tissues, oenocytes, the entire digestive tract, the ring
213 gland, and epidermal cells (Figs 2C, E, F, G, I, K, respectively, and Figs 3C, E, F, G, I, K,
214 respectively). NRB^{MC009} and NRB^{ZLW0047} were unable to label the central nervous system i.e.
215 ganglion, brain lobes, and nerves (dotted boxes on Figs 2A and 3A).

216

217 **Fig 2. NRB^{MC009} distribution in *Drosophila* larval tissues.** Confocal live imaging of (A) whole
218 dissected w^[1118] third instar larva labeled with NRB^{MC009} 500 nM. Small letters reveal corresponding
219 magnified tissues, dotted box shows unlabeled CNS. Magnification 5x; scale bar 200 μ m. Detailed
220 images of (B) leg imaginal disc, (C) muscles, (D) trachea, (E) oenocytes, (F) hindgut, (G) midgut,
221 (H) fat body, (I) epidermis and hematocytes, (J) salivary gland, and (K) ring gland; magnification
222 40x; scale bars 20 μ m.

223

224 **Fig 3. NRB^{ZLW0047} distribution in *Drosophila* larval tissues.** Confocal live imaging of (A) whole
225 dissected w^[1118] third instar larva labeled with NRB^{ZLW0047} 1 μ M. Small letters reveal corresponding
226 magnified tissues, dotted box shows unlabeled CNS. Magnification 5x; scale bar 200 μ m. Detailed
227 images of (B) leg imaginal disc, (C) muscles, (D) trachea, (E) oenocytes, (F) hindgut, (G) midgut,
228 (H) fat body, (I) epidermis, (J) salivary gland, and (K) ring gland; magnification 40x; scale bars 20
229 μ m.

230

231 The labeling properties of the two probes were compared by co-loading larval tissues with both dyes,
232 and merging the corresponding images for colocalization analysis. The results, reported in Fig 4A-D,
233 indicate that both dyes were able to penetrate the cells of the tissues, and effectively label the
234 intracellular structures. Cell internalization of the dyes was very rapid, allowing a clear visualization
235 of the intracellular structures in less than 1 min (data not shown). Pearson's coefficient results (Figs
236 4E-F) confirmed that NRB^{MC009} and NRB^{ZLW0047} recognized the same intracellular structures in most
237 of the tissues investigated. However, a clear difference in subcellular expression between NRB^{MC009}
238 and NRB^{ZLW0047} was observed in the fat bodies, in which lipid droplets (LDs) were selectively
239 stained by NRB^{MC009} (Fig 4B). This behavior may reflect a different binding capacity of the two
240 probes to the constituents of LDs, i.e. neutral lipids, mainly triacylglycerols and sterol esters, and
241 phospholipids (23,24).

242

243 **Fig 4. NRB^{MC009} and NRB^{ZLW0047} colocalization.** Confocal live imaging of dissected w^[1118] third
244 instar larval (A) muscle, (B) fat body, (C) salivary gland, and (D) imaginal disc labeled with
245 NRB^{MC009} 500 nM (green) together with NRB^{ZLW0047} 1 μ M (red). Magnification 60x; scale bars 10
246 μ m. Graph (E) and summary table (F) of Pearson's correlation coefficients between NRB^{MC009} and
247 NRB^{ZLW0047} in the evaluated tissues. Data expressed as mean \pm SEM, n \geq 10.

248

249 **Cellular distribution of NRB^{MC009} and NRB^{ZLW0047}**

250 We next characterized the cellular structures labeled by NRB^{MC009} and NRB^{ZLW0047}. In all the tissues
251 investigated, we found that both probes allowed the visualization of intracytoplasmic organelles but
252 did not penetrate the nuclei (S1 Fig). Furthermore, neither probe was able to label plasma membrane
253 as shown by experiments using CellMaskTM Orange dye (S1A-E Figs) in larval tissues expressing
254 mCD8-GFP (S1F-L Figs).

255 The intracellular distribution of NRB^{MC009} and NRB^{ZLW0047} was analyzed in more detail in *Drosophila*
256 larval musculature in which we investigated the binding of these NRB fluorescent derivatives to ER,
257 mitochondria, lysosomes and LDs. The larval body wall muscles provide a relatively simple system
258 to study development of muscles, cytoskeleton dynamics, intracellular trafficking and neuromuscular
259 junction dysfunction. In fact, besides the well-known actin and myosin filaments and their associated
260 proteins, muscles also contain a cytoskeleton, intracellular organelles of the endo-lysosomal pathway,
261 and well-defined endoplasmic reticulum and mitochondrial networks (25). We focused on these
262 particular structures because we recently showed that they contained a significant density of binding
263 sites for NRB^{MC009} (6). To study the intracellular localization of NRB^{MC009} we co-loaded it into larval
264 muscle with organelle-specific red fluorescent dyes. To confirm NRB^{ZLW0047} localization we profiled
265 it in larval muscles labelled with organelle-selective GFPs tagged proteins.
266 The results, showed good co-localization of NRB^{MC009} with ER trackerTM Red (ER probe, Pearson's
267 coefficient 0.65 ± 0.03 , Fig 5A), MitotrackerTM Orange (mitochondrial probe, Pearson's coefficient
268 0.54 ± 0.05 , Fig 5B) and LipidTOXTM (lipid droplets probe, Pearson's coefficient 0.42 ± 0.02 , Fig
269 5C); however, NRB^{MC009} did not colocalize with LysotrackerTM Deep Red (a lysosome probe,
270 Pearson's coefficient 0.04 ± 0.04 , Fig 5D). The localization of NRB^{ZLW0047} was confirmed using
271 green fluorescent-tagged proteins that specifically targeted the ER (UAS-Hneu-GFP), mitochondria
272 (UAS-Mito-GFP) and lysosomes (UAS-Lamp-GFP), and BODIPY 493/503 dye that targeted the
273 LDs. The results, shown in Figs 5E-H, demonstrated that the distribution of NRB^{ZLW0047} partially
274 overlapped that of NRB^{MC009}; that both NRB^{MC009} and NRB^{ZLW0047} exhibited good labeling of ER
275 and mitochondria (Pearson's coefficient 0.51 ± 0.01 and 0.68 ± 0.01 , respectively, Figs 5F and 5G);
276 that both were absent in lysosomes (Pearson's coefficient 0.04 ± 0.02 , Fig 5I); and that they differed
277 in their localization in LDs, where NRB^{MC009} fluorescence was present (Pearson's coefficient $0.42 \pm$
278 0.02 , see also Fig 5C) but NRB^{ZLW0047} fluorescence was absent (Pearson's coefficient 0.16 ± 0.05 ,
279 Fig 5H).

280

281 **Fig 5. NRB^{MC009} and NRB^{ZLW0047} intracellular distribution in larval muscles.** Confocal live
282 imaging of *w^[1118]* third instar larval muscles 6-7 from segment A3 labeled and ER-trackerTM 2 μ M
283 (ER marker, **A**), MitotrackerTM 1 μ M (mitochondria marker, **B**), LipidTOXTM 1:100 (LDs marker,
284 **C**), and LysotrackerTM 2 μ M (lysosomes marker, **D**), all in red, together with NRB^{MC009} 500 nM
285 (green). Magnification 60x; scale bars 10 μ m. Summary table of Pearson's correlation coefficients
286 between NRB^{MC009} and fluorescent organelle-marker probes used in larval muscles (**E**). Data are
287 expressed as mean \pm SEM, $n \geq 10$. Confocal live imaging of third instar larval muscles 6-7 from
288 segment A3 of UAS-Hneu-GFP/Tubulin-Gal4 (ER marker, **F**), UAS-Mito-GFP/Tubulin-Gal4
289 (mitochondrial marker, **G**), *w^[1118]* added with BODIPY 493/503TM 10 μ g/ml (LDs marker, **H**), and
290 UAS-Lamp-GFP/+;Tubulin-Gal4/+ (Lysosomes marker, **I**), all labeled with NRB^{ZLW0047} 1 μ M (red).
291 Magnification 60x; scale bars 10 μ m. Summary table of Pearson's correlation coefficients between
292 NRB^{MC009} and fluorescent organelle-markers in *Drosophila* larval muscles (**L**). Data are expressed as
293 mean \pm SEM, $n \geq 10$.

294

295 These data show that both compounds, but particularly NRB^{MC009}, can be used to visualize and
296 distinguish most organs/tissues of the dissected living larvae, and allow good definition of their
297 intracellular structures. In addition, the co-localization studies revealed that both NRB^{MC009} and
298 NRB^{ZLW0047} labelled subcellular organelles, that they preferentially targeted the ER and
299 mitochondria, and that they were totally absent from the nuclei, plasma membranes and lysosomes,
300 which is in agreement with data reported in mammalian cell studies (6). Moreover, the efficiency of
301 the probes was tested with two different approaches: 1) in combination with commercially available
302 dyes for live imaging, and 2) together with GFP tagged protein markers that bound to specific
303 cellular structures. In both the experimental backgrounds the NRB based probes allowed the
304 identification of endoplasmic reticulum and mitochondria structures, making both probes useful new

305 markers in *Drosophila* studies. Based on the capability of these dyes to recognize the same
306 intracellular structures in both mammalian and fruit fly cells, their potential use in more complex
307 animal models is anticipated. Subsequently, our ongoing work is focused on the development of
308 these probes as tools to allow live imaging studies to be conducted in mouse and rat tissues.

309

310 **NRB^{MC009} and NRB^{ZLW0047} cellular distribution in pathologic mutation-
311 related phenotypes**

312 In consideration of the preferential distribution of NRB-derived fluorescent probes to ER and
313 mitochondria we next verified if they could be developed into tools to highlight phenotypic
314 modifications of the ER and mitochondrial networks in *Drosophila* muscles. A large number of
315 human disease genes are conserved in *Drosophila* and its genome can be easily manipulated to
316 recreate and study human pathologic phenotypes (26); subsequently, *Drosophila* is widely used as a
317 model to study muscle growth, degeneration and correlated diseases (Beckett and Baylies, 2006;
318 Hirth, 2010; Kreipke et al., 2017; McGurk et al., 2015; Rossetto et al., 2011).

319 In this study NRB^{MC009} and NRB^{ZLW0047} were tested on two *Drosophila* pathologic models: Charcot-
320 Marie–Tooth disease (CMTd) and hereditary spastic paraplegia (HSP) (31,32).

321 CMTd *Drosophila* phenotype was obtained by inducing a downregulation of *Marf*, the fruit fly
322 orthologue of the human gene *Mitofusin2*, which encodes for a GTPase that, together with Opa1,
323 fuses mitochondria; mutations of this gene are implicated in CMT disease (33). The depletion of this
324 protein in *Drosophila* is known to cause fragmented and clustered mitochondria in neuronal cell
325 bodies and to disorganize the typical sarcomeric location of mitochondria in the larval muscles,
326 clumping them mainly around the nuclei (34). Fig 6B shows the fluorescent distribution of NRB^{MC009}
327 and NRB^{ZLW0047} in muscles of larvae in which *Marf* had been ubiquitously downregulated (UAS-
328 *Marf*^{RNAi}/Tubulin-Gal4). Since these fluorescent images are comparable with those previously

329 reported with the mitochondrial marker UAS-Mito-GFP (34) in the same model, when considering
330 the mitochondrial labeling properties of NRB^{MC009} and NRB^{ZLW0047} (this study), it can be argued that
331 fluorescent derivatives of NRB are able to also stain altered mitochondria, and be able to highlight
332 pathologic mitochondrial phenotypes.

333 HSP *Drosophila* phenotype was obtained by inducing a downregulation of atlastin. Atlastins are
334 membrane-bound dynamin-like GTPases implicated in ER network morphogenesis, and mutations in
335 *atlastin1* gene are involved in the onset of a common form of HSP (SPG3A). *Drosophila* holds a
336 unique highly conserved *atlastin* orthologue, and its downregulation elicits a fragmented ER in
337 neurons and an enrichment of ER punctae localized in the proximity of nuclei, and visualized using
338 UAS-KDEL-GFP (18). Labeling of larva fillets with NRB^{MC009} and NRB^{ZLW0047} revealed a different
339 pattern between muscles of wild type (Fig 6A) and atlastin-downregulated (UAS-Atl^{RNAi}/Tubulin-
340 Gal4) larvae (Fig 6C), in which a brighter perinuclear signal, compatible with the previously
341 described HSP phenotype, is observed.

342

343 **Fig 6. NRB^{MC009} and NRB^{ZLW0047} highlight pathologic mutation related phenotypes.** Confocal
344 live imaging of *Drosophila* muscles 6-7 from segment A3 of (A) control (Tubulin-Gal4+), (B) Marf
345 downregulation (UAS-Marf^{RNAi}/Tubulin-Gal4), and (C) atlastin downregulation (UAS-
346 Atlastin^{RNAi}/Tubulin-Gal4) labeled with NRB^{MC009} 500 nM (green) or NRB^{ZLW0047} 1 μ M (red).
347 Magnification 60x; scale bars 10 μ m.

348

349 Taken together, these results indicate that NRB^{MC009} and NRB^{ZLW0047} could be useful tools for
350 *Drosophila* live imaging to highlight phenotypes attributable to mutations in, and/or downregulation
351 of genes implicated in mitochondria and/or endoplasmic reticulum network modifications. In
352 addition, the short time it takes for these probes to permeate and label tissue and their general ease of

353 use, means that both could be used as tools in compound screening studies to identify candidates that
354 would help alleviate any network malfunction due to genetic modification.

355

356 **NRB^{MC009} and NRB^{ZLW0047} in food intake tests**

357 Next we explored the possibility of using NRB^{MC009} and NRB^{ZLW0047} as tools to evaluate food intake
358 and to investigate potential gut morphological modifications in *Drosophila* *in vivo*. By adopting a
359 three-choice test (behavioral choice test) we verified that NRB^{MC009} and NRB^{ZLW0047}, when added to
360 the food, were accepted by the flies. As summarized in Fig 7A, there were no substantial differences
361 in food preference between the standard diet and NRB^{MC009}- and NRB^{ZLW0047}-supplemented diets,
362 indicating that the presence of the dyes did not influence the larval food choice. In addition,
363 fluorescence imaging of larvae fed for 30 min with probe-enriched liquid food indicated that both
364 NRB^{MC009} and NRB^{ZLW0047} could be clearly detected in the gut (Fig 7B); a more in depth analysis
365 revealed that gut fluorescence was regulated by the probes contained in the food, since no signal was
366 observed in the gut wall, leading to the conclusion that the strength of the fluorescent signal could be
367 taken as an index of the quantity of ingested food. Fig 7C reports the results of the food intake assay,
368 expressed as a percentage of the gut stained area relative to the total body area - no significant
369 difference was observed between larvae fed with NRB^{MC009}, NRB^{ZLW0047}, or brilliant blue dye, a
370 commonly used dye for the evaluation of food intake in *Drosophila* (21).
371 The lack of gut labeling by NRB^{MC009} and NRB^{ZLW0047}, although a useful outcome for the food intake
372 test, was somewhat unexpected, particularly considering the results obtained in the dissected larvae
373 (see Figs 2 and 3) where the dyes were clearly localized to the intestinal tract. To explain this
374 inconsistency, we hypothesized that the time of exposure (30 min) of the larvae to the probe-
375 supplemented food in the food intake assay was potentially too short to allow an internalization of the
376 dyes to the gut epithelial cells. Therefore, we analyzed the gut wall of larvae grown for 5-7 days in

377 food enriched with NRB^{MC009} or NRB^{ZLW0047}. Figs 7D-F show the clear difference between vehicle-
378 fed (Fig 7D) and NRB^{MC009}- and NRB^{ZLW0047}-fed larvae (Figs 7E and 7F, respectively). The bright
379 fluorescent signal in the digestive tract (mainly midgut and hindgut) indicates that *Drosophila* larvae
380 readily eat the probe-containing food. The digestive tract of NRB^{MC009}- and NRB^{ZLW0047}-fed larvae
381 were clearly labeled by the dyes, a result that was accentuated by the absence of fluorescence in the
382 rest of the body. The *Drosophila* intestinal tract is formed by a monolayer of epithelial cells,
383 intestinal stem cells and enteroendocrine cells, surrounded by visceral muscles, nerves and tracheae.
384 Ingested food from the proventriculus is pushed into the midgut, the main region of digestion and
385 absorption, and then to the hindgut where the final absorption process takes place (13,14). A deeper
386 investigation on dissected *Drosophila* gut revealed that NRB^{MC009} and NRB^{ZLW0047} did not only label
387 the food that was present and visible in the intestinal tract (Figs 7I and 7L), but they also bound to the
388 gut external muscular cells (Figs 7G and 7J) and enterocytes (Figs 7H and 7K).

389

390 **Fig 7. Use of NRB^{MC009} and NRB^{ZLW0047}as dyes for food intake tests.** Quantification of larval
391 dispersal after 5 minutes of three-choice preference assay of vehicle-added food, NRB^{MC009}-added
392 food (20 μ M), or NRB^{ZLW0047}-added food (20 μ M). Data were expressed as percent of total larvae
393 number and represent mean \pm SEM of five different experiments (A). Representative images of
394 larvae fed with liquid food supplemented with Brilliant blue R 0.08%, NRB^{MC009} 20 μ M, or
395 NRB^{ZLW0047} 20 μ M, where gut was labeled by the three dyes (B) and quantification of gut stained
396 area versus total larval area (C). Data were expressed as mean of percent \pm SEM of 30 larvae.
397 Confocal live imaging of whole dissected *Drosophila* third instar larva fed with vehicle-
398 supplemented food (D), NRB^{MC009} 20 μ M-supplemented food (E), or NRB^{ZLW0047} 20 μ M-
399 supplemented food (F); magnification 5x; scale bar 200 μ m. p: proventriculus, m: midgut, h: hindgut.
400 Detailed images of mid gut external muscular cells (G, J), enterocytes (H, K) and intestinal food (I,
401 L), labeled with the two NRB fluorescent derivatives; magnification 40x; scale bars 20 μ m.

402

403 The bright fluorescence of NRB^{MC009} and NRB^{ZLW0047} make these probes eminently suitable for use
404 in food intake tests and chronic feeding assays; as monitoring tools for abnormal gut morphology;
405 and identifying defects in gut functionality during development or screening tests.

406

407 **NRB^{MC009} and NRB^{ZLW0047} toxicity**

408 In an attempt to validate the use of the NRB-derived fluorescent probes for use in chronic assays, we
409 next verified their lack of toxicity in *Drosophila* by exposing the flies to NRB^{MC009} or NRB^{ZLW0047}
410 over their entire life-cycle. The results indicate that male and female flies readily ingested NRB^{MC009}-
411 and NRB^{ZLW0047}-supplemented food, mated and laid eggs normally, from which embryos hatched and
412 larvae developed, grew, underwent pupation and eclosed in a similar fashion to non-treated flies. In
413 addition, no difference in eclosion rate and lethality of adult flies was observed between probe-
414 exposed and control flies (Fig 8A). Finally, we could not detect any apparent macroscopic
415 morphological alteration in adult flies treated with either NRB^{MC009} or NRB^{ZLW0047} (Fig 8B).

416

417 **Fig 8. NRB^{MC009} and NRB^{ZLW0047} chronic toxicity.** Evaluation of toxicity after exposition to
418 standard food added with vehicle, NRB^{MC009} (20 μ M) or NRB^{ZLW0047} (20 μ M) over the *Drosophila*
419 entire life-cycle. **(A)** Quantification of eclosion rate. Data were expressed as mean of percent of
420 emerged flies versus to the total number of pupae. **(B)** Representative images of emerged male (♂)
421 and female (♀) $w^{[1118]}$ flies, where morphological alterations of body, eyes, wings, and legs were
422 evaluated.

423

424 The absence of toxicity and the high level of palatability supports NRB^{MC009} and NRB^{ZLW0047} as
425 potential monitoring tools for long term feeding assays; as markers of intestinal epithelia; and their

426 use in studying *Drosophila* digestive tract functionality e.g. monitoring the effect of compounds or
427 diet on intestinal performance (Apidianakis and Rahme, 2011; Gasque et al., 2013; Storelli et al.,
428 2018)These attributes also make these probes potentially useful as mammalian gastrointestinal (GI)
429 tract markers; for example, the GI tract is one of the most studied tissues in many pathological rodent
430 models (38,39) and the availability of easy-to-use, high performance fluorescent probes to detect
431 intracellular structure abnormalities could be of great benefit.

432 Overall, this study has investigated the imaging applications of NRB^{MC009} and NRB^{ZLW0047} in
433 *Drosophila melanogaster* *in vivo*. The analysis of the fluorescent signals of these compounds reveal
434 that both can label subcellular specific organelles (preferentially ER and mitochondria), in both wild
435 type and pathological phenotypes. The absence of toxicity and the minimal effect on palatability also
436 allows them to be used as potential monitoring tools in feeding assays, and as markers for intestinal
437 epithelia that could be useful in *Drosophila* digestive tract studies. In summary, the characteristically
438 bright signals of NRB^{MC009} and NRB^{ZLW0047}, in combination with their capacity to permeate tissues
439 rapidly, makes them eminently suitable for confocal imaging applications. Our future studies will
440 focus on investigating whether these compounds, with their enhanced attributes, may have potential
441 to be used in invertebrate animal models.

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551

552

553 Supporting information

554 **S1 Fig. NRB^{MC009} and NRB^{ZLW0047} do not label plasma membranes nor nuclei in larval tissues.**

555 Confocal live imaging of $w^{[1118]}$ larval (A) peripodal membrane cells of a leg imaginal disc, (B)

556 salivary gland, (C) fat body, and (D) central nervous system labeled with NRB^{MC009} 500 nM (green)

557 and CellMaskTM Orange (cell membrane marker) 1 μ M (red). Magnification 60x, scale bars 10 μ m

558 (A-C); magnification 40x, scale bar 100 μ m (D). N: nucleus, G: ganglion, Nv: nerves. Summary table

559 of Pearson's correlation coefficients between NRB^{MC009} and CellMaskTM in the evaluated tissues (E).

560 Data expressed as mean \pm SEM, $n \geq 10$. Confocal live imaging of UAS-mCD8-GFP/Tubulin-Gal4

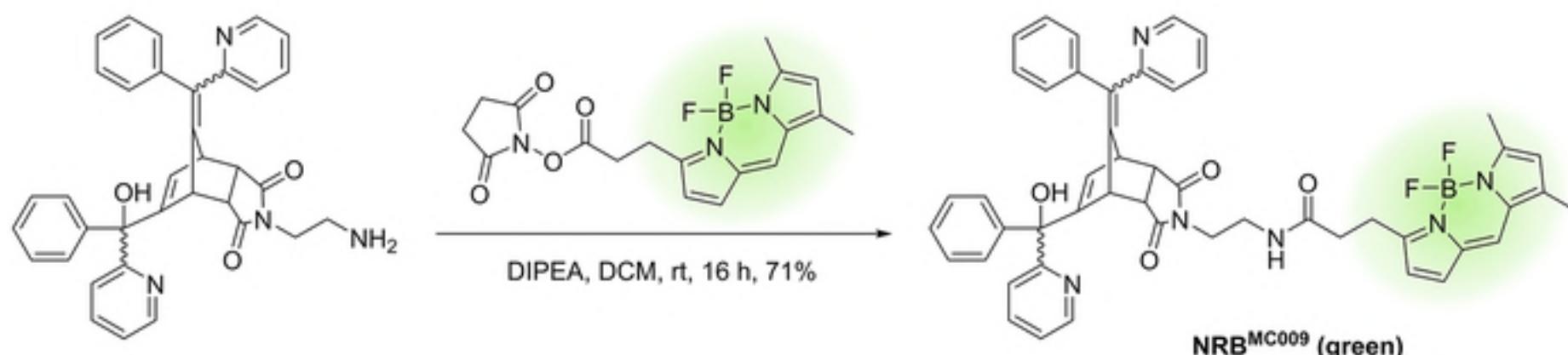
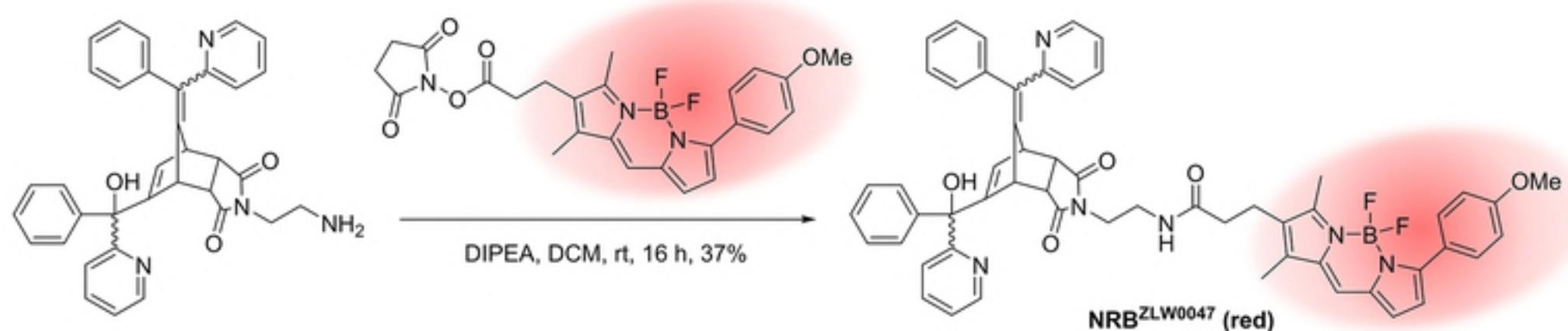
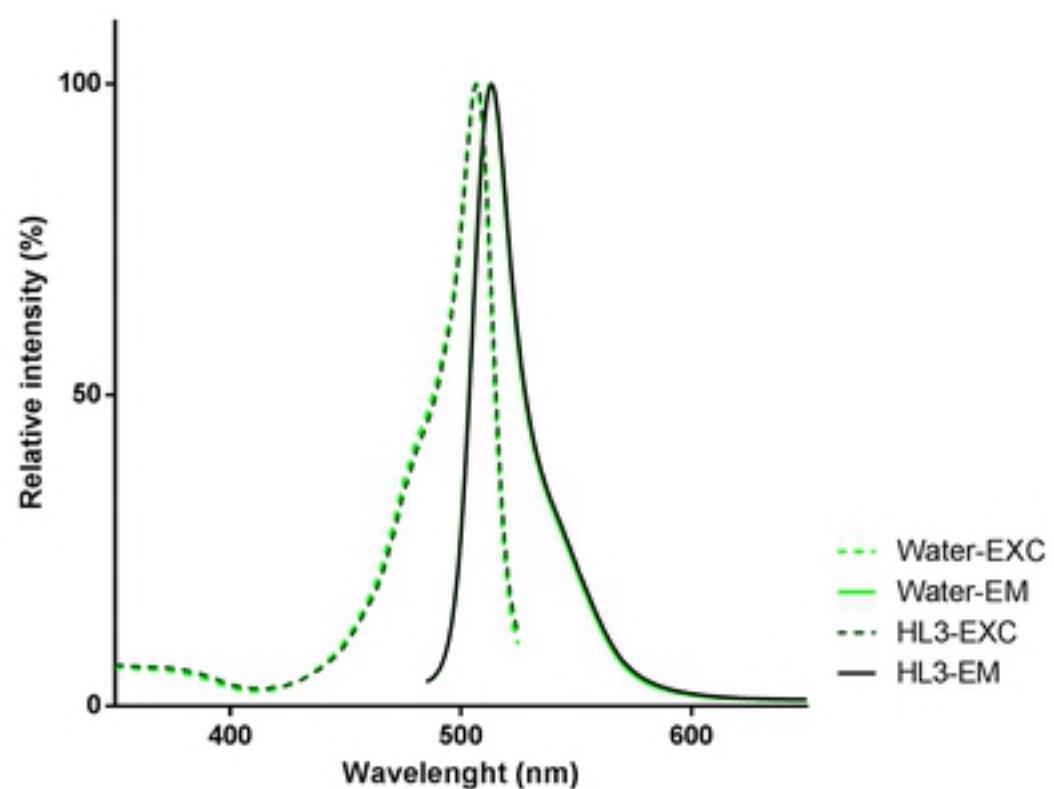
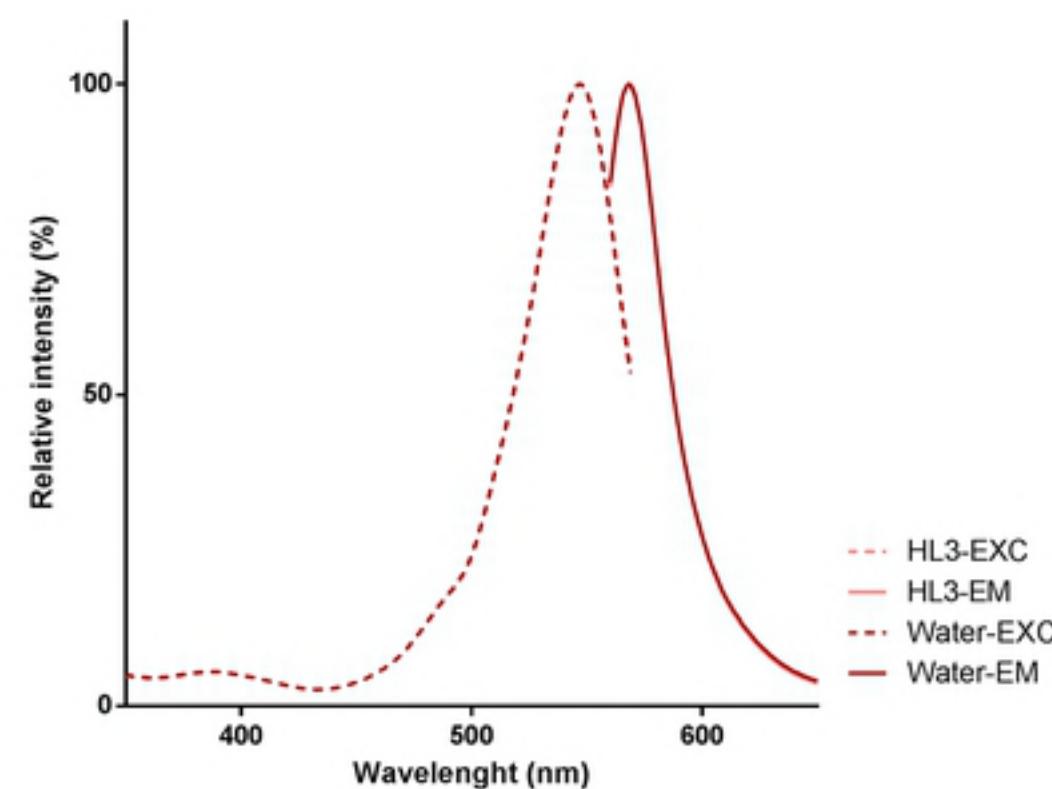
561 (cell membrane marker) larval (F) peripodal membrane cells of a leg imaginal disc, (G) salivary

562 gland, (H) fat body, and (I) central nervous system labeled with NRB^{ZLW0047} 1 μ M (red).

563 Magnification 60x, scale bars 10 μ m (A-C); magnification 40x, scale bar 100 μ m (D). N: nucleus, G:

564 ganglion, Nv: nerves. Summary table of Pearson's correlation coefficients between NRB^{ZLW0047} and

565 mCD8-GFP signal in the evaluated tissues (L). Data expressed as mean \pm SEM, $n \geq 10$.

A**B****C****D****E**

	MW (g/mol)	λ_{ex} (nm)	λ_{em} (nm)
NRB^{MC009}	828.7	507	513
NRB^{ZLW0047}	934.8	547	568

Fig 1

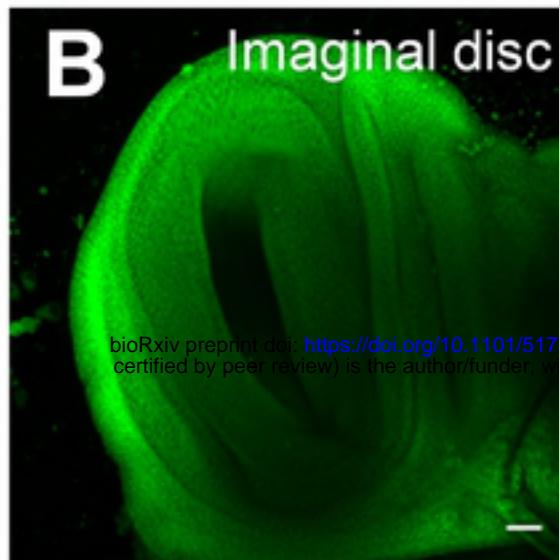
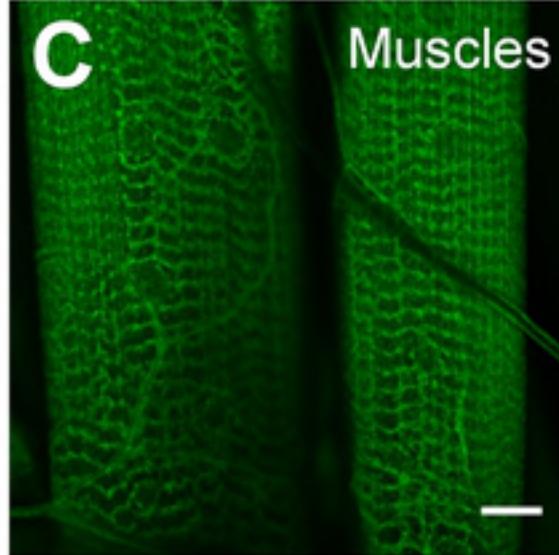
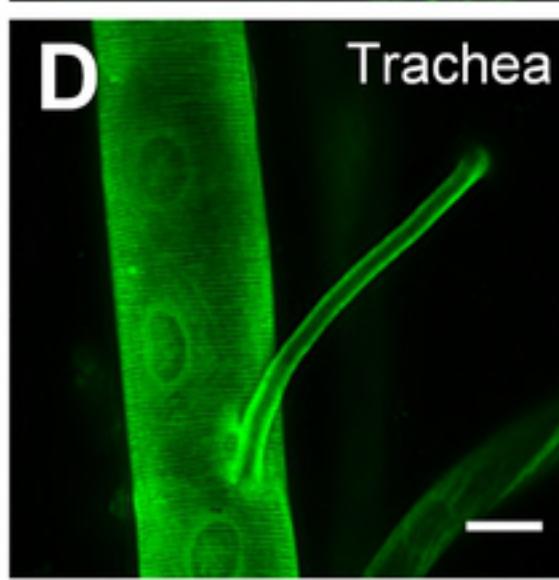
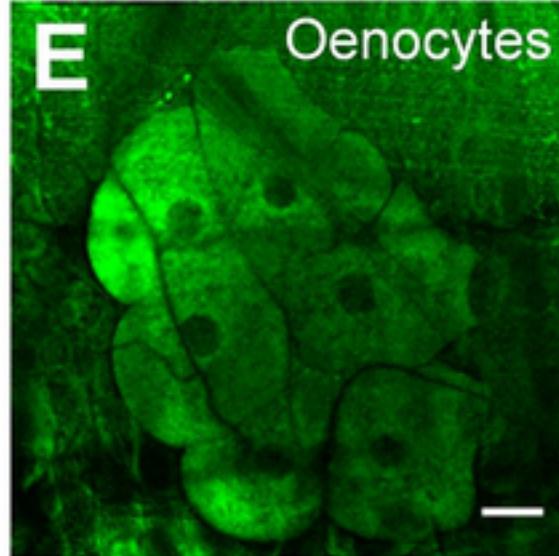
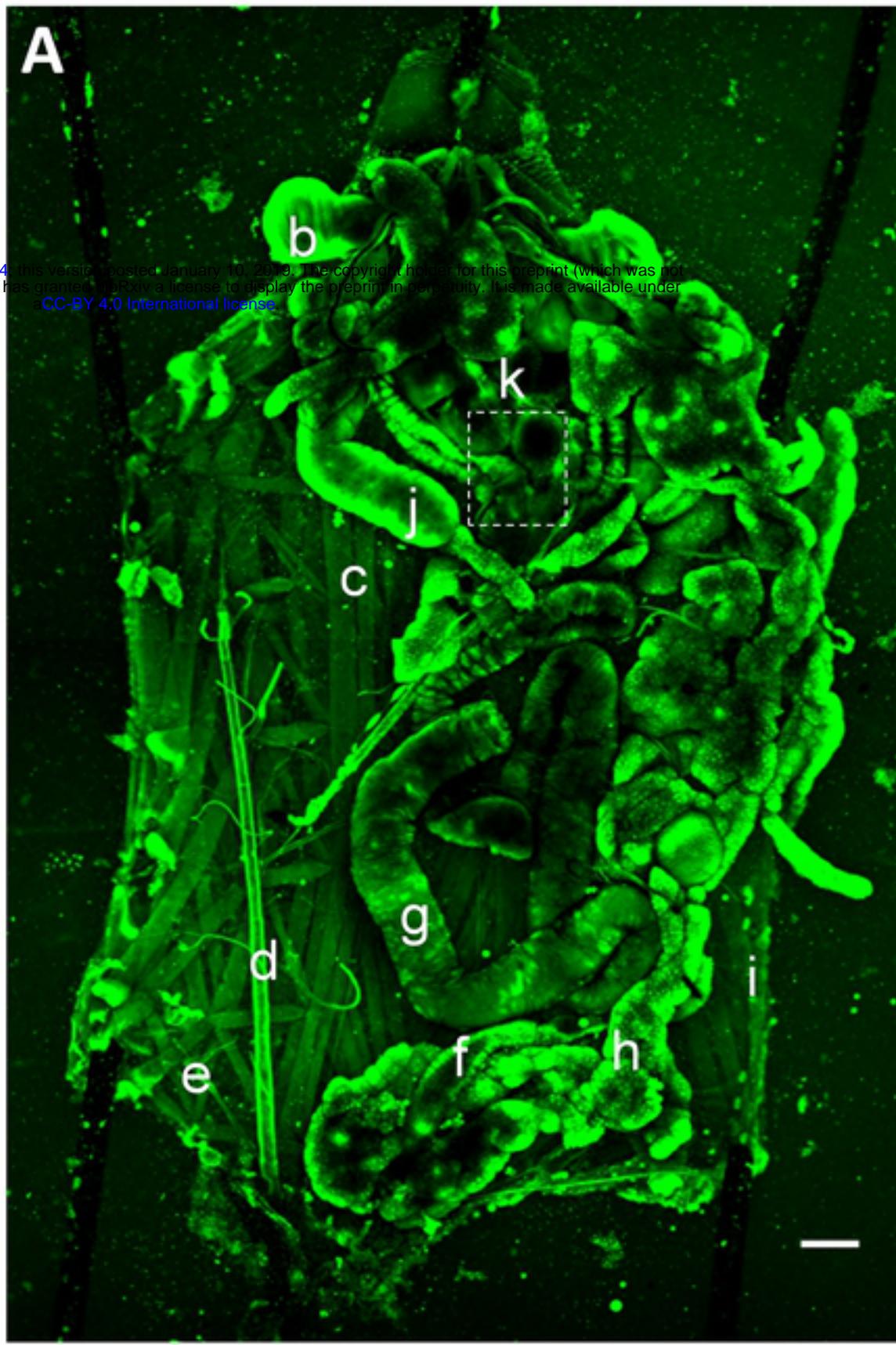
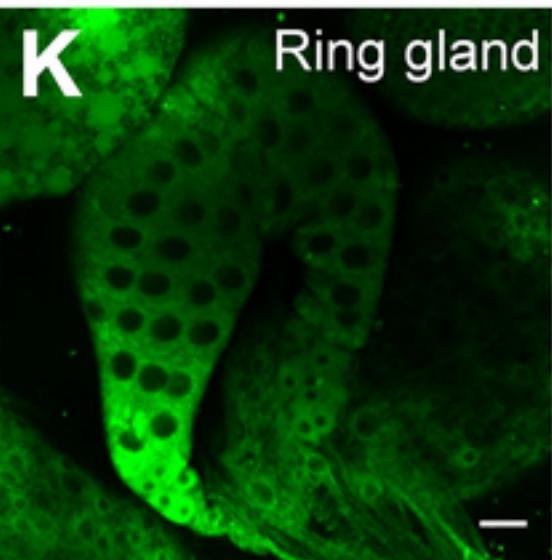
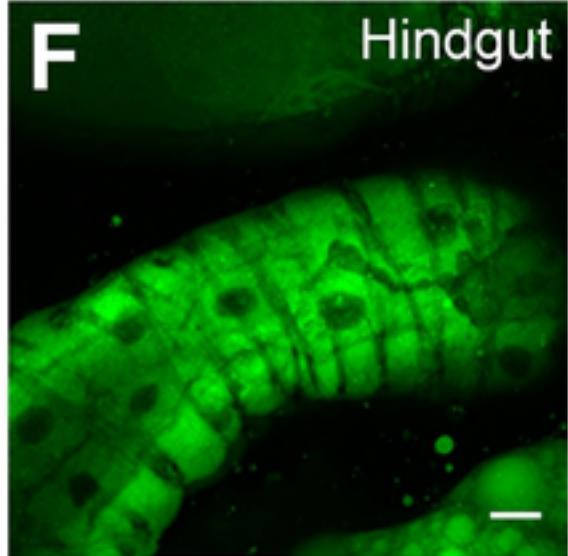
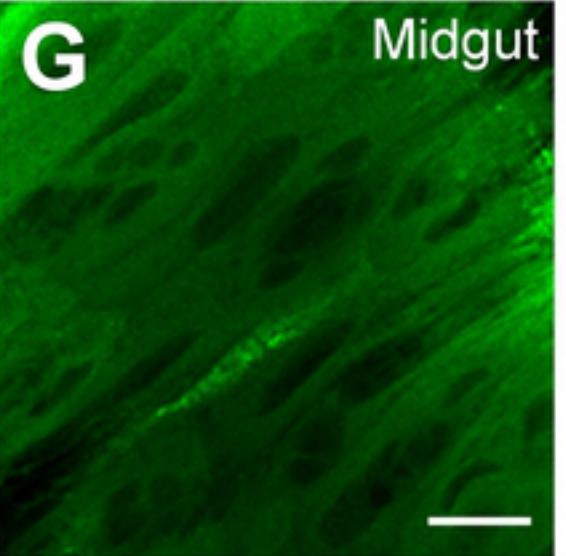
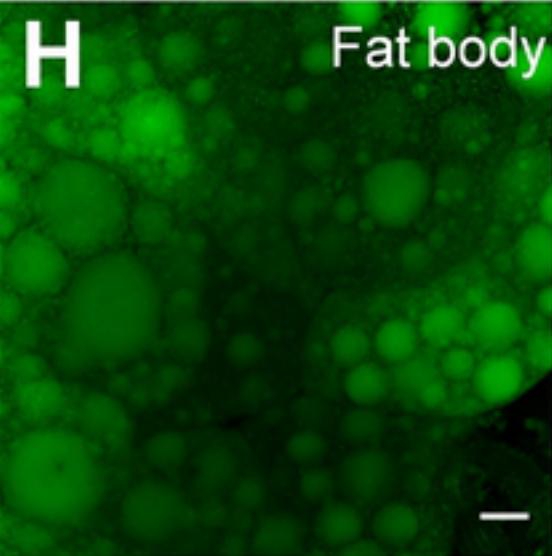
B Imaginal disc**C** Muscles**D** Trachea**E** Oenocytes

Fig 2

A**K****F****G****H**

Ring gland

Salivary gland

Epidermis

Fat body

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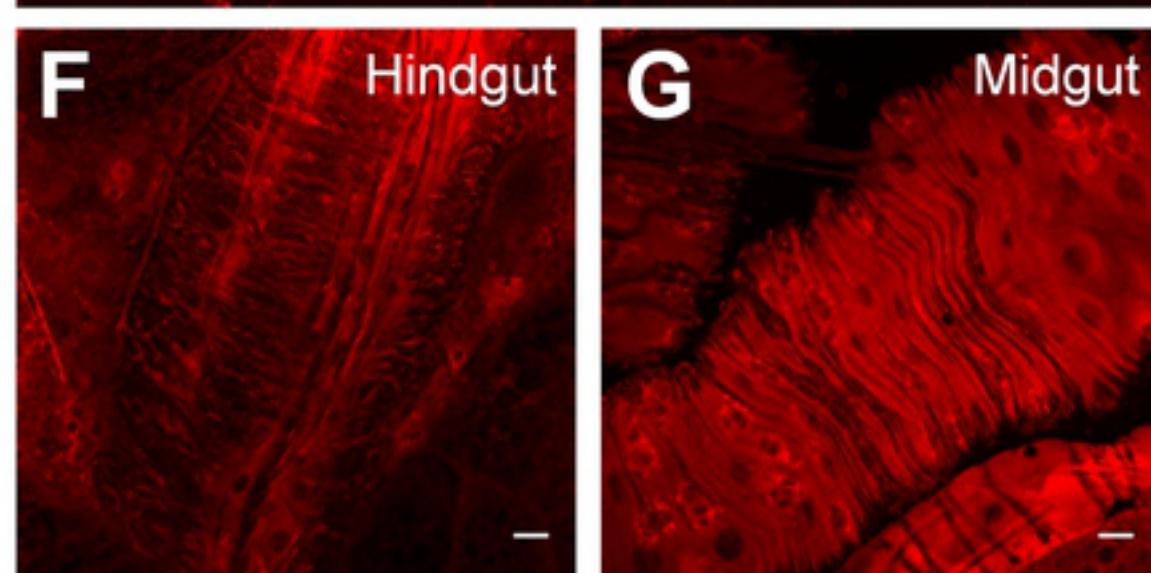
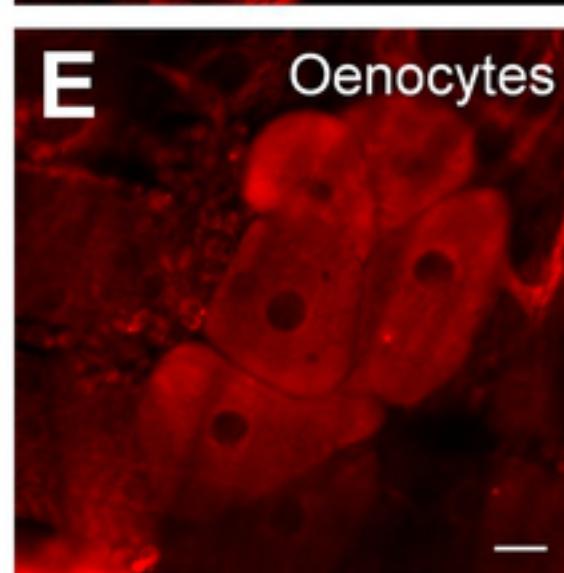
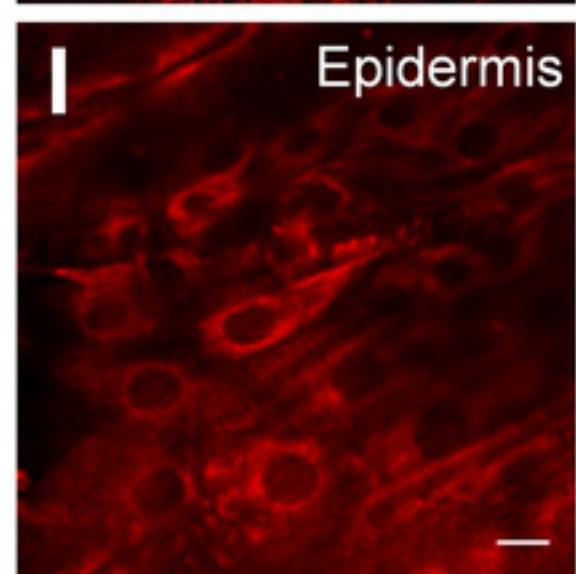
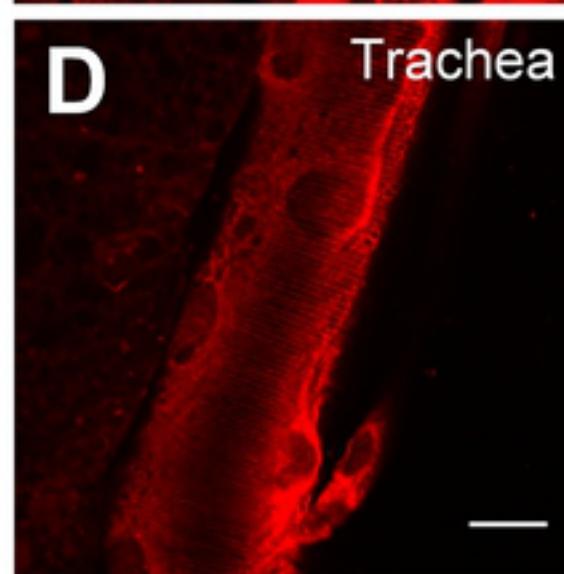
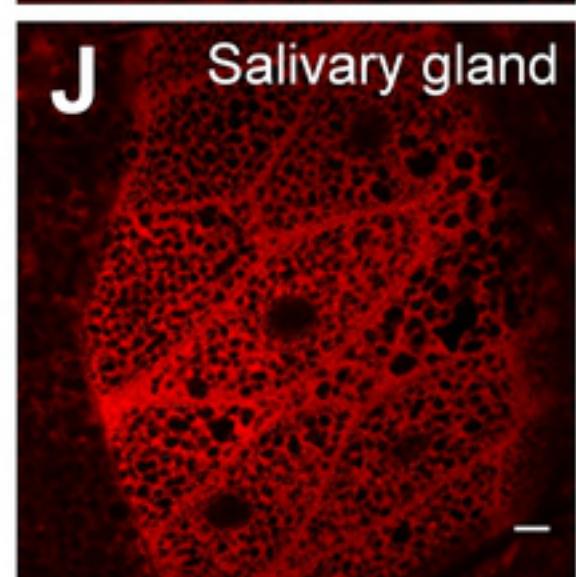
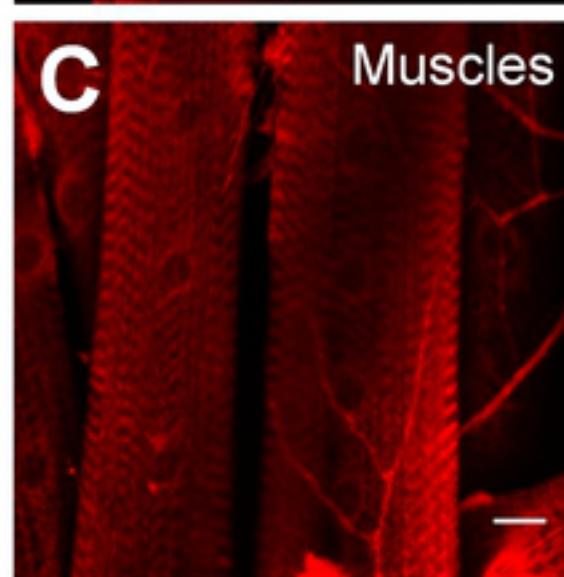
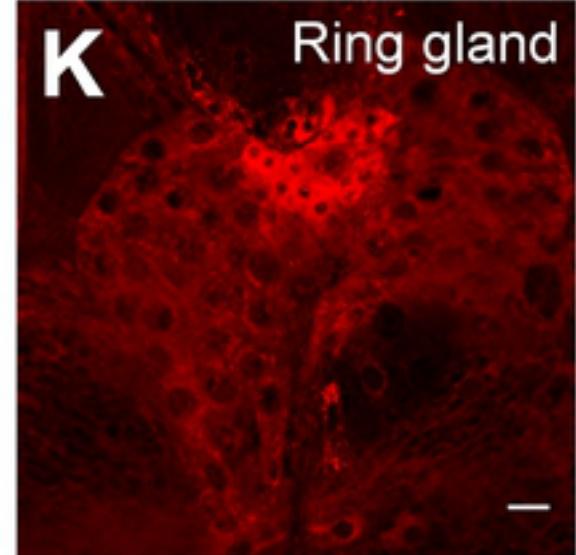
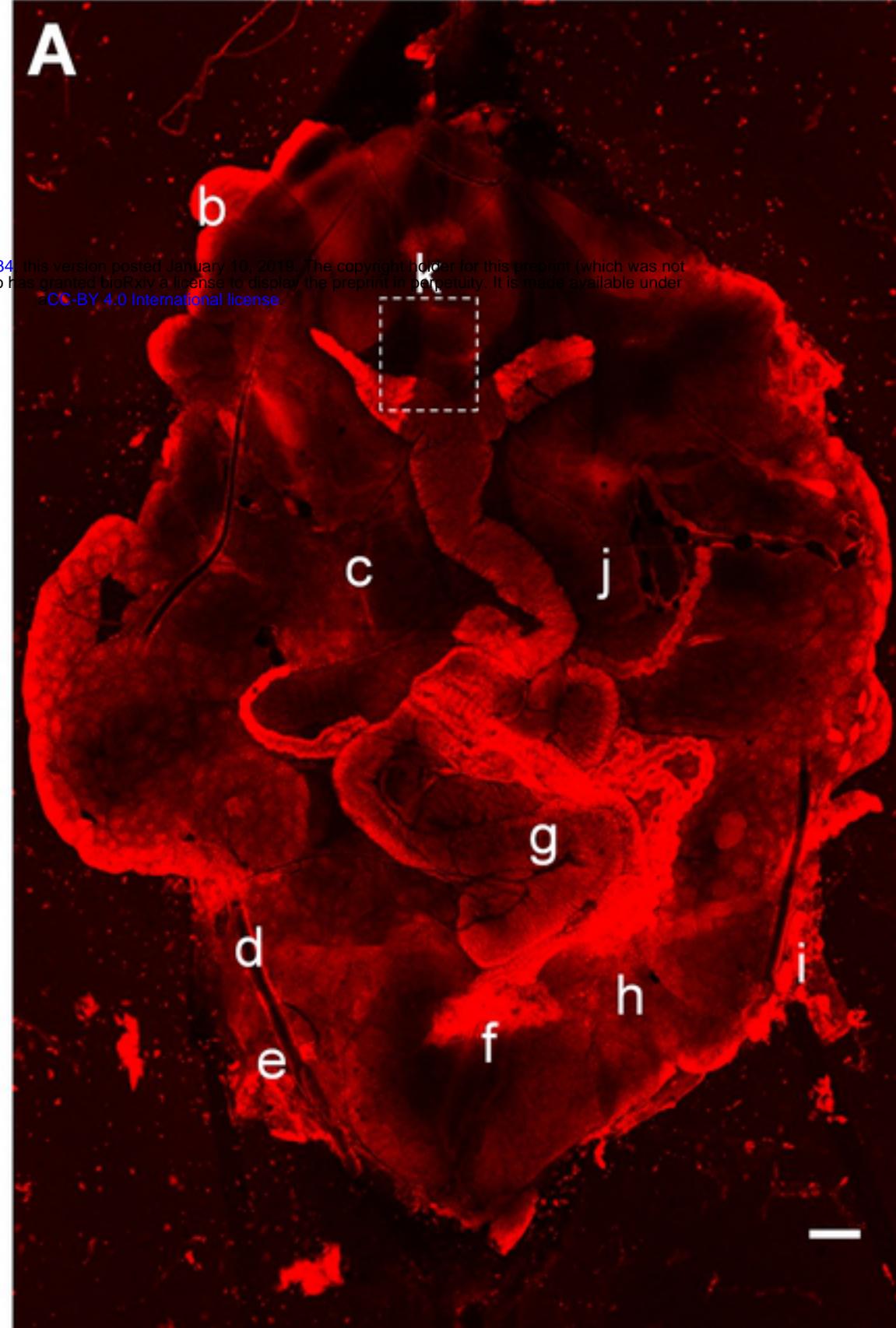
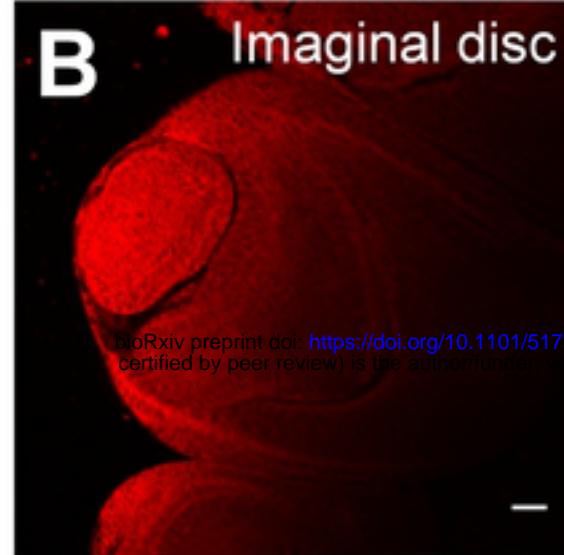
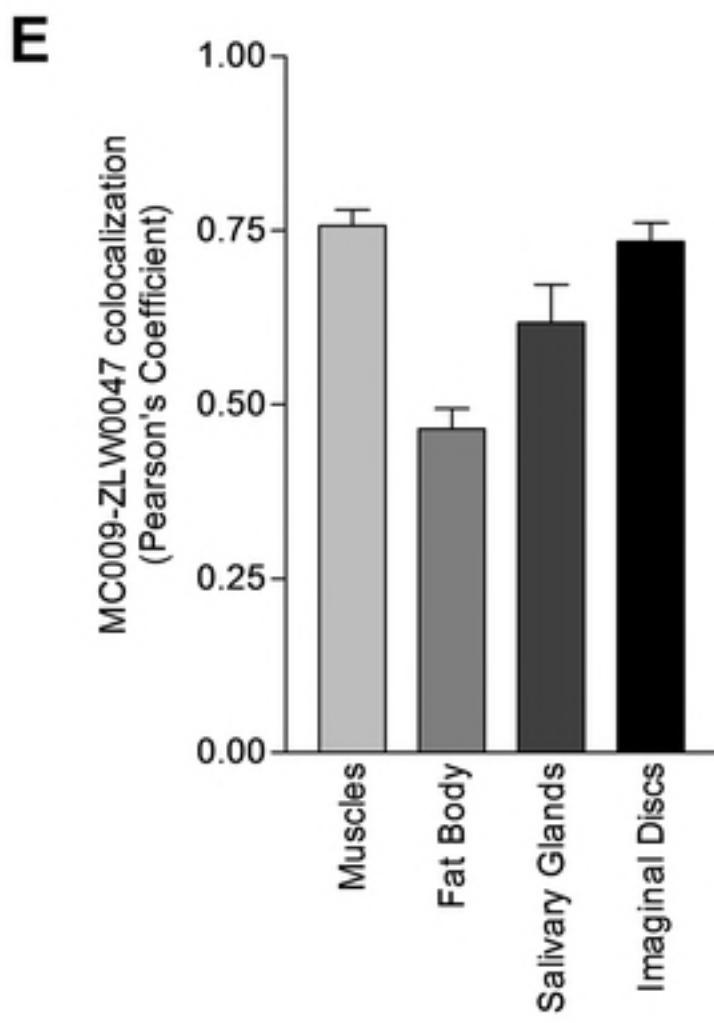
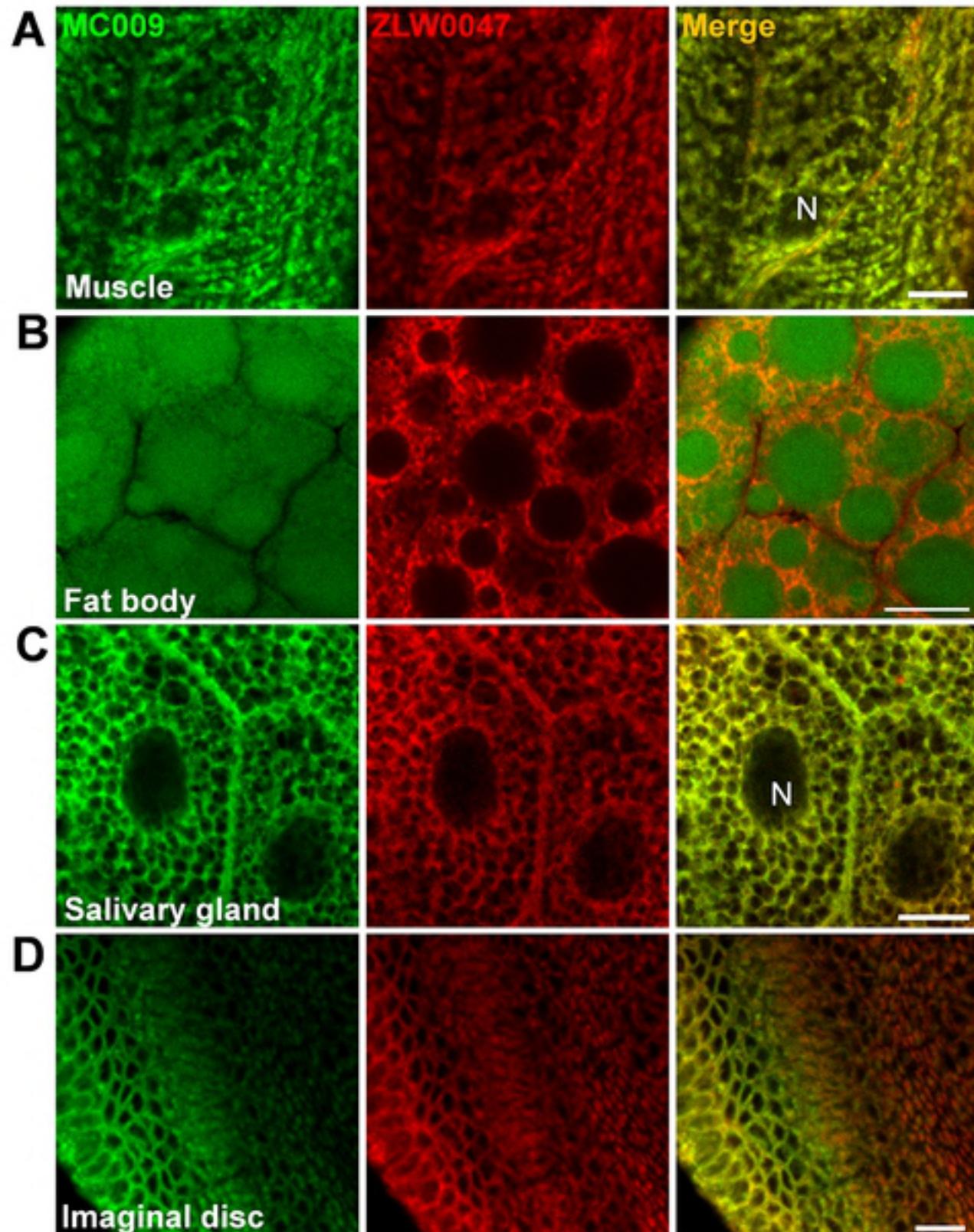


Fig 3

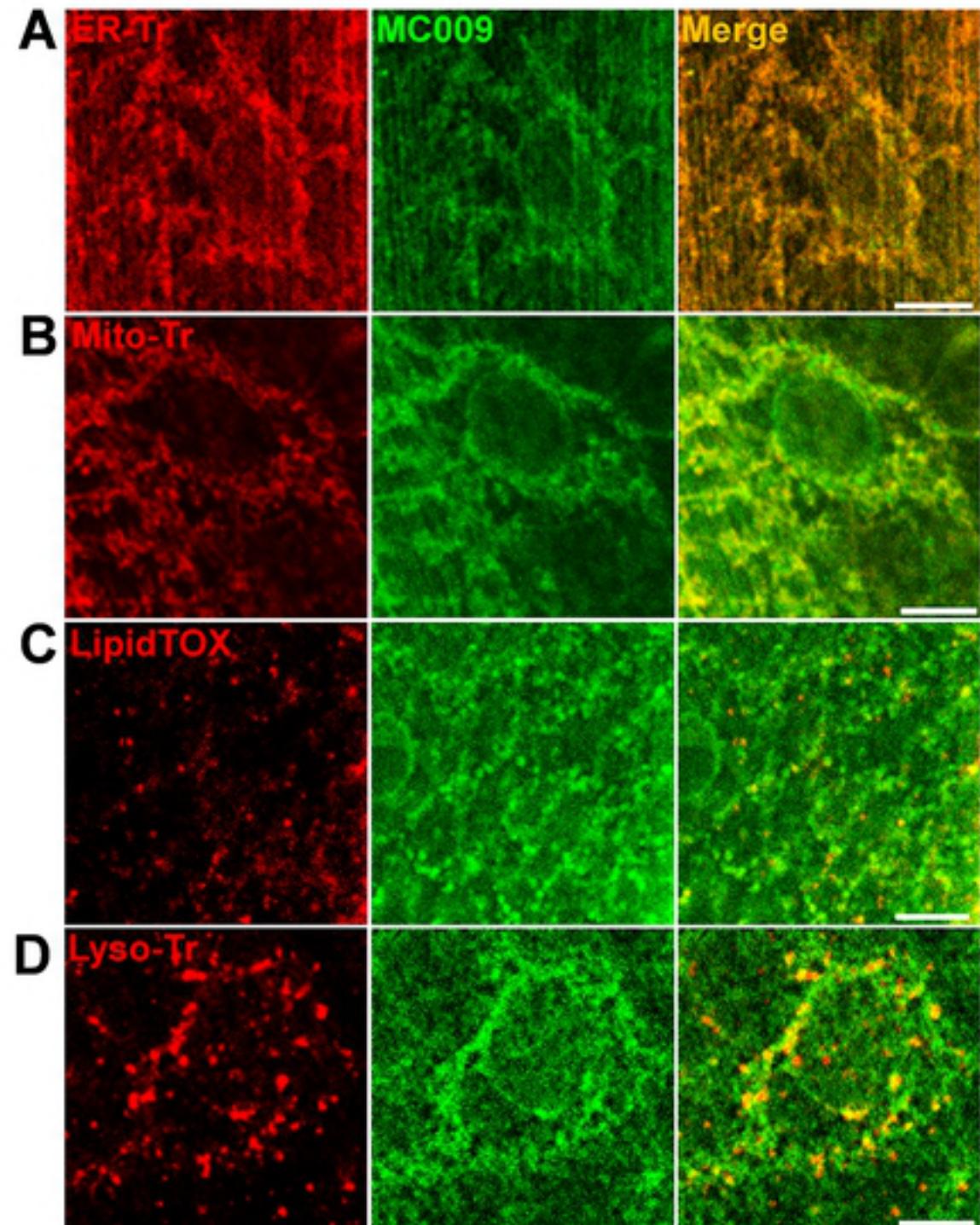


F

NRB^{MC009} vs NRB^{ZLW0047}

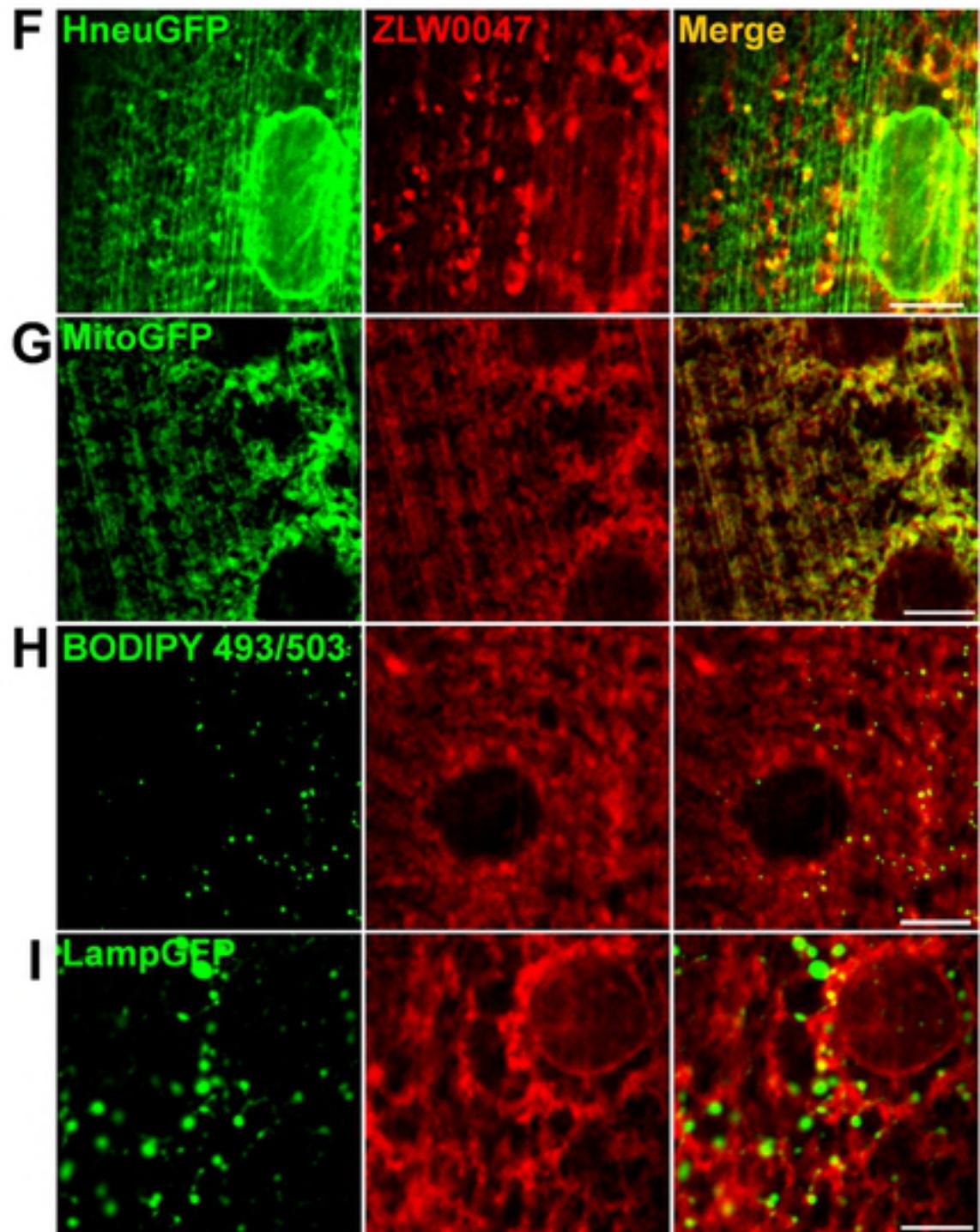
Muscles	0.76 ± 0.02
Fat body	0.47 ± 0.03
Salivary glands	0.62 ± 0.06
Imaginal discs	0.73 ± 0.03

Fig 4



E

	NRB ^{MC009}
ER Tracker™	0.65 ± 0.03
Mitotracker™	0.54 ± 0.05
LipidTOX™	0.42 ± 0.02
Lysotracker™	0.04 ± 0.04



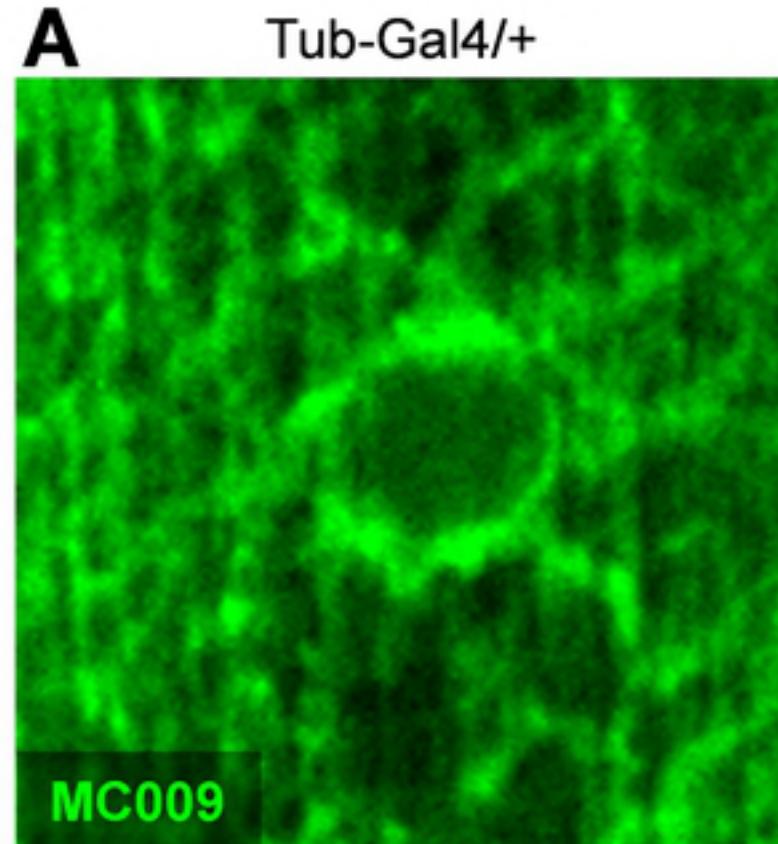
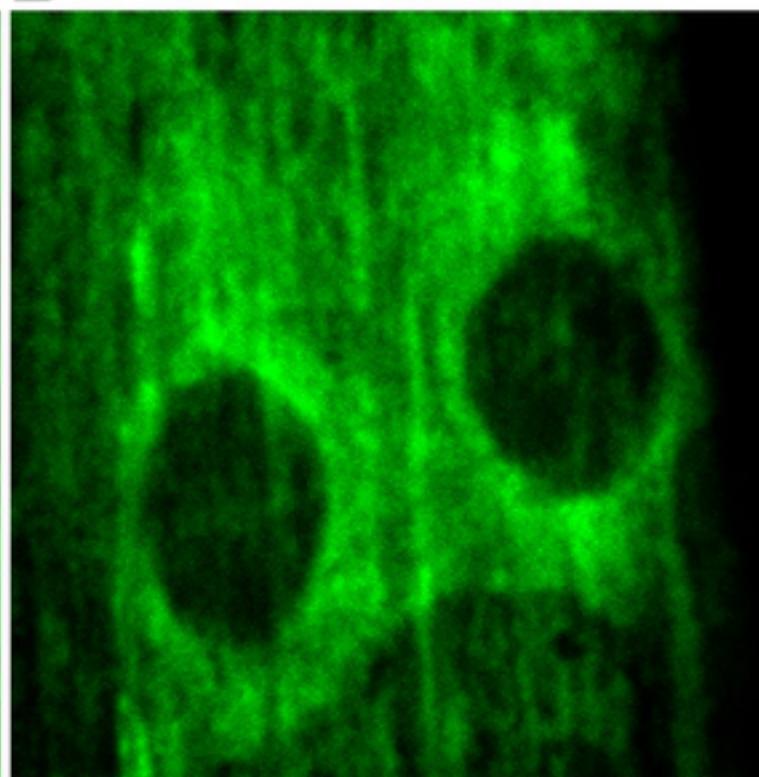
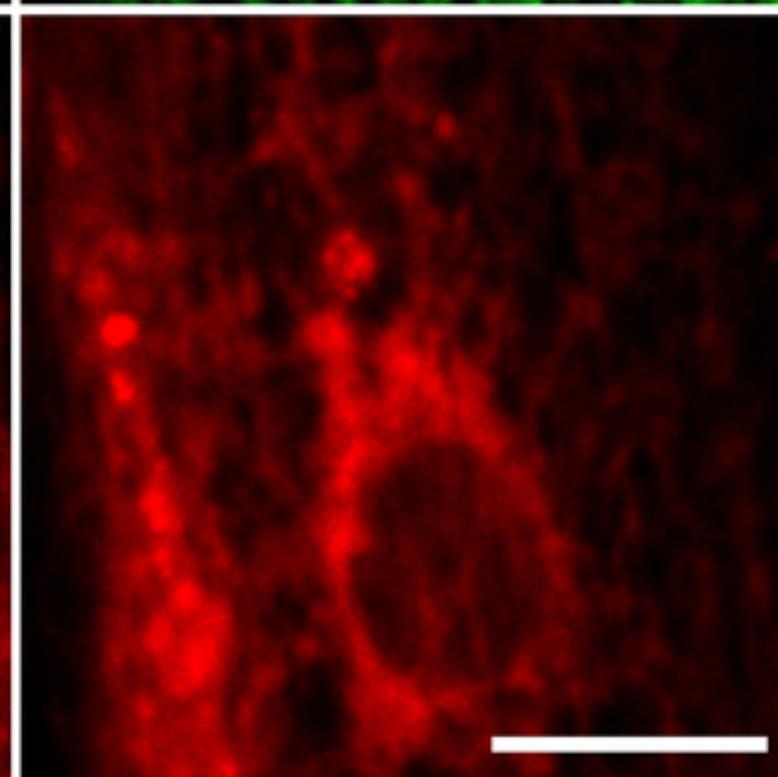
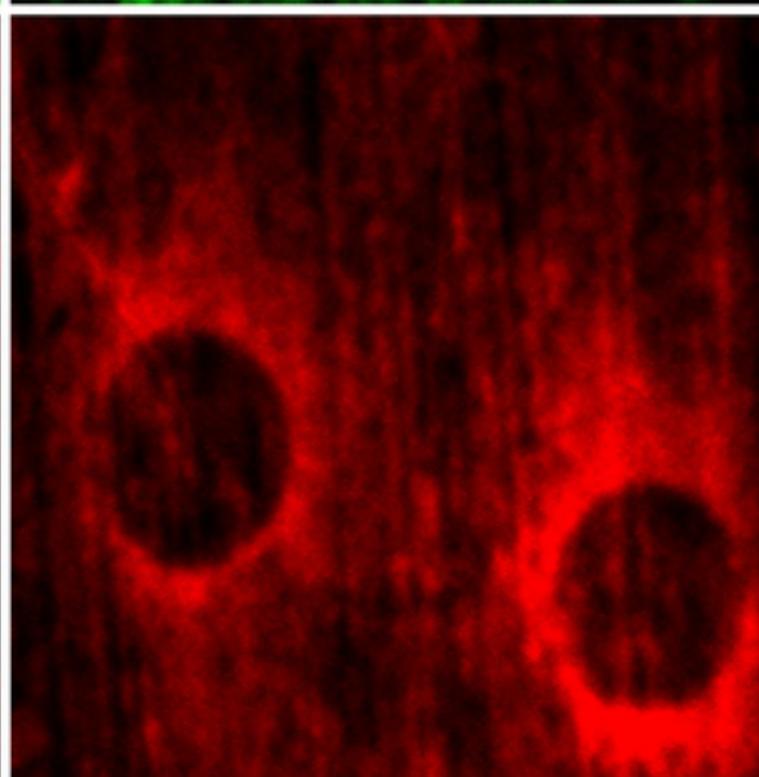
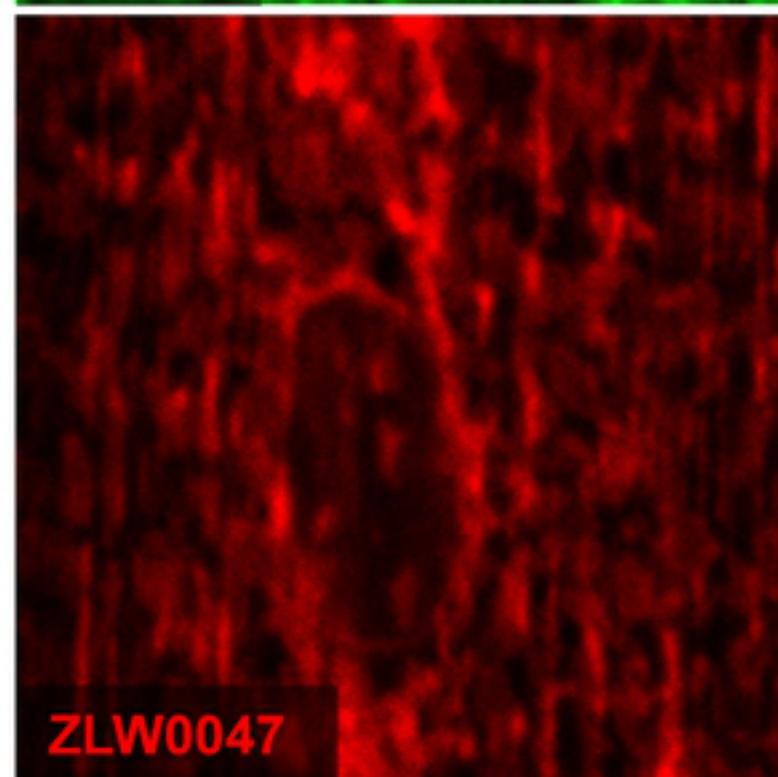
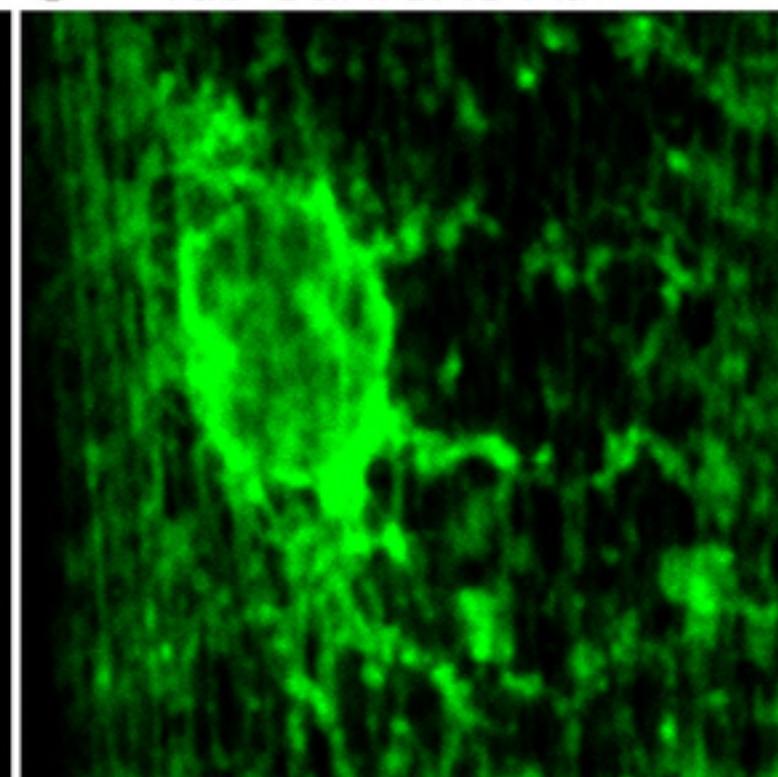
L

	NRB ^{ZLW0047}
Hneu-GFP	0.51 ± 0.01
Mito-GFP	0.68 ± 0.01
BODIPY 493/503	0.16 ± 0.02
Lamp-GFP	0.04 ± 0.05

Fig 5

Control

Tub-Gal4/+

**Marf RNAi**Tub-Gal4/UAS-Marf^{RNAi}**Atl RNAi**Tub-Gal4/UAS-Atl^{RNAi}**Fig 6**

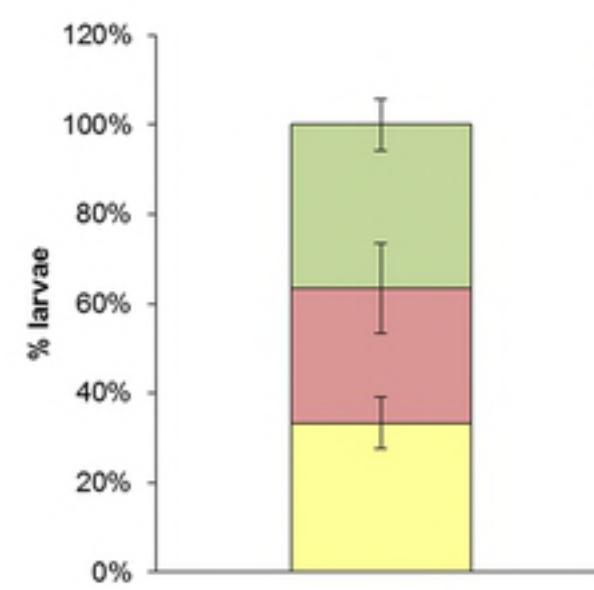
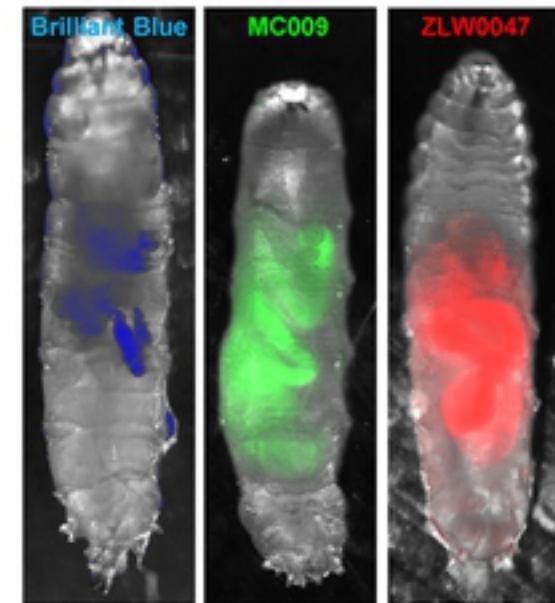
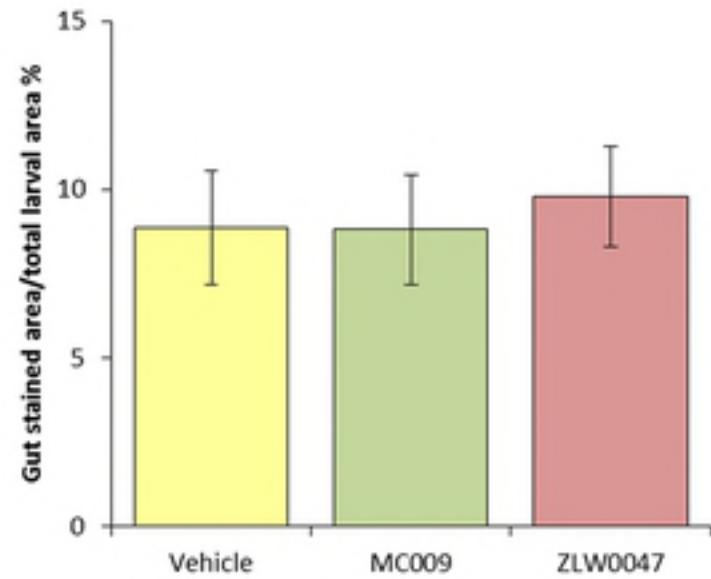
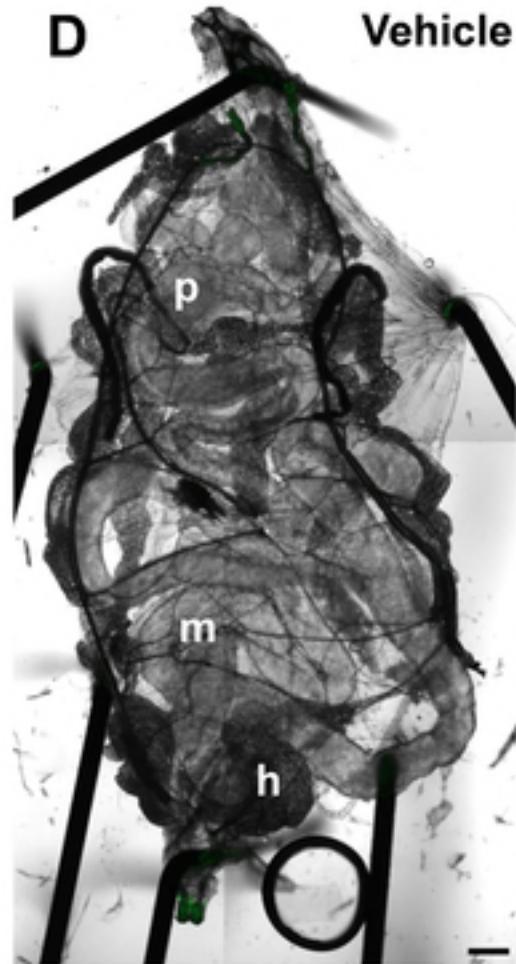
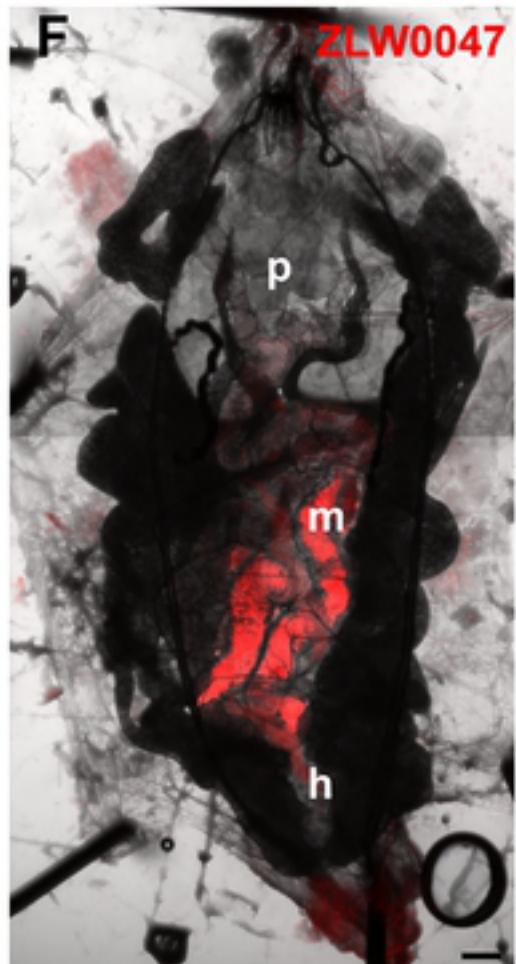
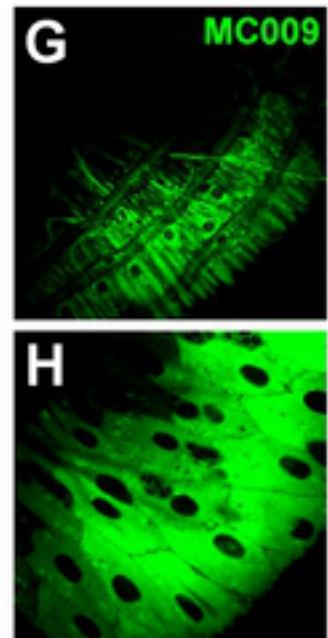
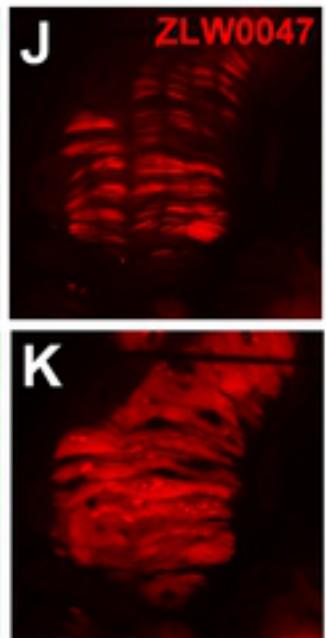
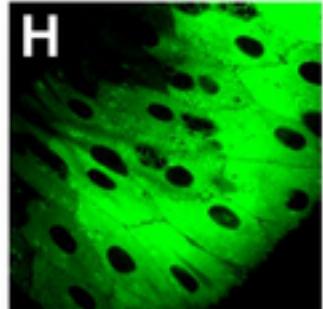
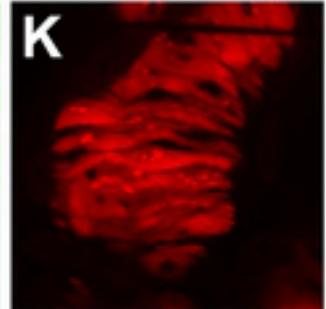
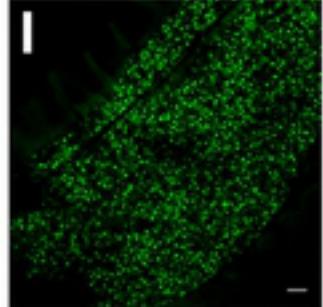
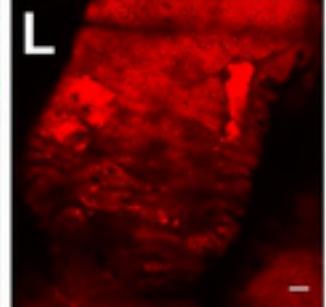
A**B****C****D****E****F****G****J****H****K****I****L**

Fig 7

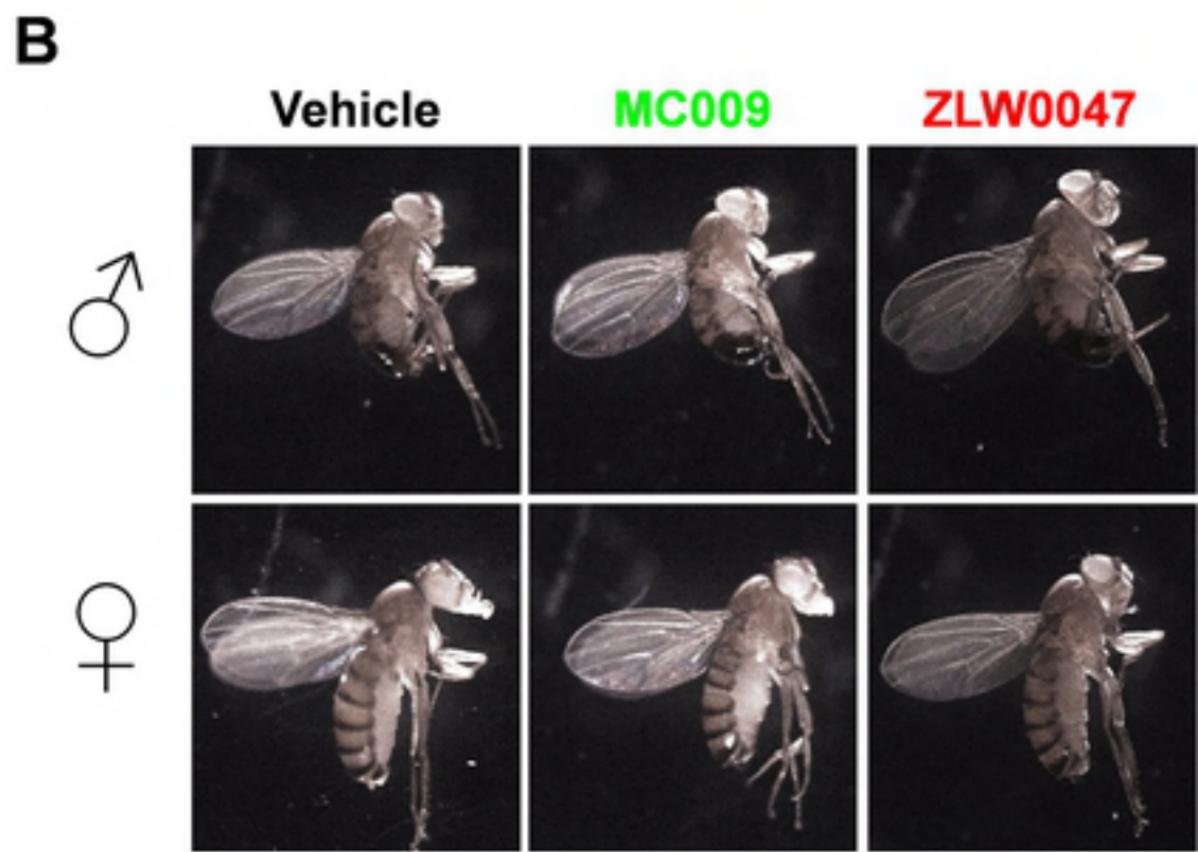
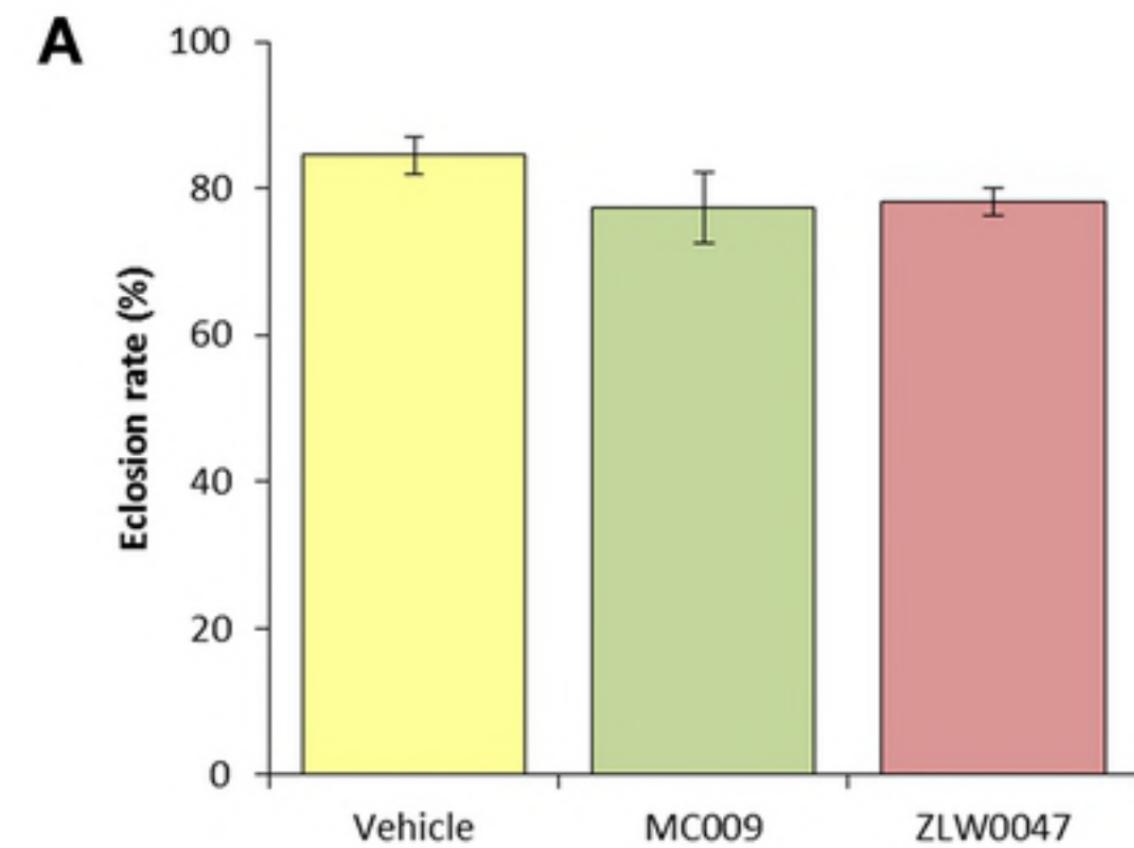


Fig 8