

Quantitative Analysis of Interactive Behavior of Mitochondria and Lysosomes Using Structured Illumination Microscopy

Qixin Chen^{a,b,c†}, Xintian Shao^{a,b,c†}, Mingang Hao^{b†}, Zhiqi Tian^b, Chenran Wang^b, Fei Liu^{a,c}, Kai Zhang^d, Fengshan Wang^a, Peixue Ling^{a,c}, Jun-Lin Guan^b, Jiajie Diao^b

a. School of Pharmaceutical Sciences, Shandong University, Jinan 250101, China.

b. Department of Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA.

c. Shandong Academy of Pharmaceutical Science, Key Laboratory of Biopharmaceuticals, Engineering Laboratory of Polysaccharide Drugs, National–Local Joint Engineering Laboratory of Polysaccharide Drugs, Jinan 250101, China.

d. Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

†These authors contributed equally to this work.

Contact:

Peixue Ling, E-mail: lpxsdf@163.com, Address: 44 Wenhuxi Rd, School of Pharmaceutical Sciences, Shandong University, Jinan 250101, China.

Jun-Lin Guan, E-mail: guanjl@ucmail.uc.edu, Address: 3125 Eden Ave, Department of Cancer Biology,

20 University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA.

21 Jiajie Diao, E-mail: jiajie.diao@uc.edu, Address: 3125 Eden Ave, Department of Cancer Biology, University of
22 Cincinnati College of Medicine, Cincinnati, OH 45267, USA.

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24 **KEYWORDS** mitochondria; lysosome; mitophagy; membrane fusion, super-resolution imaging

25
26 **Abbreviations** CCCP: carbonyl cyanide m-chlorophenyl hydrazone; FWHM: full-width at half-maximum; LTR:
27 Lyso-Tracker Red; MLC: mitochondria and lysosome contact; MTG: Mito-Tracker Green; SIM: structured
28 illumination microscopy

29
30 **ABSTRACT**

31 Super-resolution optical microscopy has extended the spatial resolution of cell biology from the cellular level to
32 the nanoscale, enabling the observation of the interactive behavior of single mitochondria and lysosomes.
33 Quantitative parametrization of interaction between mitochondria and lysosomes under super-resolution optical
34 microscopy, however, is currently unavailable, which has severely limited our understanding of the molecular
35 machinery underlying mitochondrial functionality. Here, we introduce an M -value to quantitatively investigate
36 mitochondria and lysosome contact (MLC) and mitophagy under structured illumination microscopy. We found
37 that the M -value for an MLC is typically less than 0.4, whereas in mitophagy it ranges from 0.5 to 1.0. This
38 system permits further investigation of the detailed molecular mechanism governing the interactive behavior of
39 mitochondria and lysosomes.

41 Introduction

42 The crosstalk between mitochondria and lysosomes is involved in many cellular processes. For instance,
43 mitophagy, a process that selectively removes redundant or damaged mitochondria, plays an important role in
44 regulating the number of intracellular mitochondria and maintaining mitochondrial functions.¹ Dysregulated
45 mitophagy is implicated in many diseases, such as neurodegenerative diseases and cancer.²⁻⁴ To date, mitophagy
46 has been often studied at the cell level through methods such as flow cytometry,⁵ enzyme-linked
47 immunosorbent assay,⁶ western-blot,⁷ and confocal fluorescence microscopy.⁸⁻¹⁰ These methods only report the
48 cumulative level of mitophagy and ignore individual mitophagy events from the fusion between single
49 mitochondria and lysosome pair. Although confocal fluorescence microscopy can detect mitophagy using
50 mitophagy-specific dye, it is difficult to distinguish subcellular structures at a resolution beyond 200 nm.^{11, 12}
51 Moreover, confocal fluorescence microscopy does not provide details for the interactive behavior of individual
52 mitochondria and lysosome pairs. Thus, a novel strategy is needed to capture detailed information on the
53 crosstalk between individual mitochondria and lysosome pairs.

54
55 Recently developed super-resolution fluorescence microscopy techniques, such as stimulated emission depletion
56 (STED),^{11, 13, 14} structured illumination microscopy (SIM),^{12, 15, 16} and stochastic optical reconstruction
57 microscopy (STORM),¹⁷⁻¹⁹ as well as other single-molecule super-resolution imaging techniques,^{20, 21} have
58 provided new tools for investigating interactions between organelles at the subcellular level. And the interaction
59 between mitochondria and lysosomes have been brought into focus using these techniques. For example, SIM
60 super-resolution imaging has revealed a new type of interaction, mitochondria and lysosome contact (MLC).^{12,}
61 ²² Unfortunately, a parametrization system for the assessment of the interactive behaviors such as MLC and
62 mitophagy under a super-resolution microscope is currently unavailable.

63
64 To fill this gap, in this toolbox paper, we propose an analysis system, *M*-value, to quantitatively analyze the
65 crosstalk between mitochondria and lysosomes at the subcellular level. The *M*-value in MLC is less than 0.4,
66 whereas the *M*-value in mitophagy ranges from 0.5 to 1.0. Thus, this *M*-value system provides a robust platform
67 to quantitatively analyze the interactive behavior of subcellular organelles under super-resolution microscopy.

68 **Results**

69 *MLC in living HeLa cells under SIM*

70 To investigate MLC events in living HeLa cells using super-resolution imaging, we first incubated cells with a
71 commercially available mitochondrial probe (Mito-Tracker Green, MTG, 100 nM) and a lysosomal probe
72 (Lyso-Tracker Red, LTR, 200 nM) for 30 min, and then observed mitochondria (green) and lysosomes (red)
73 under a SIM (Fig. 1A). The intracellular mitochondria showed spherical, rod-shaped or filamentous
74 arrangement in an irregular manner at a resolution of approximately 150 ± 24.67 nm ($n=10$) (Fig. S1).
75 Mitochondria are approximately 0.3 μm in width and 0.6 - 10.0 μm in length (Fig. S2), which is consistent with
76 our previous results.¹² In particular, there were more filamentous mitochondria than spherical and rod-shaped
77 ones, a benchmark of healthy cells. In contrast, the diameter of spherical lysosomes (red color) was 0.6 ± 0.16
78 μm ($n=200$) (Fig. S3). Both MTG and LTR allow for high-resolution staining under SIM, which enabled us to
79 capture and understand the dynamic process of MLC (Fig. 1A, B). MLC events occur frequently in living cells
80 (Fig. 1B) with a dynamic process from contact to separation (Fig. 1B, white arrows). In addition, we observed
81 that mitochondria were surrounded by multiple lysosomes (Fig. 1C). Various types of MLC events,^{12, 22} such as
82 point contact (Fig. 1E-7), extended contact (Fig. 1E-8), and surrounding contact (Fig. 1E-9) were also observed.

84 *Quantitative analysis of whole-cell MLC*

85 Recently, it has been reported that dysregulated contact sites formed by mitochondria and lysosomes are linked
86 to Parkinson's disease.²³ We reason that proteins involved in MLC will provide a new perspective and drug
87 targets for the treatment of this disease. A quantitative analysis platform to analyze MLC at the subcellular level
88 would therefore allow us to understand the biological functions and evaluate the therapeutic effects of different
89 drugs. Here, we introduce a quantitative analysis system, *M*-value that is derived from the full-width at
90 half-maximum (FWHM) of organelle images, for understanding MLC events at the subcellular level. FWHM
91 refers to the full width of the image at half-maximum value and can directly reflect the resolution of image.
92 MTG-stained Mitochondria and LTR-stained lysosomes result in yellow spots when they merge (Fig. 1H).
93 Herein, we use the following calculation formula to define the *M*-value of the merge.

$$M = \frac{2Y}{R_{\text{FWHM}} + G_{\text{FWHM}}}$$

R_{FWHM} indicates the FWHM of red (lysosome); G_{FWHM} indicates the FWHM of green (mitochondria); Y indicates the merging distance between R_{FWHM} and G_{FWHM} .

We will demonstrate that the M -value is a simple and well-defined number that can be used to quantitatively assess MLC events in live cells. We first observed that a typical MLC event (Fig. 1B, D) generates an ensemble of M -values below 0.4. We hypothesize that an M -value 0.4 can be the upper bound to characterize dynamic MLC events. To support this idea, we calculated the M -values of a large number of MLC events ($n=100$) (Fig. 1C, E, F, G and Fig. S4-8) and found that the M -values of all MLC events were indeed below 0.4 (Fig. 1J).

Quantitative analysis of whole-cell mitophagy

Mitophagy has been commonly studied by co-localizing fluorescently stained mitochondria and autophagosomes with confocal microscopy in cells.²⁴ However, this strategy is not suitable for the quantitative analysis of mitochondria-lysosome interactions at the subcellular level. To address this issue, we set out to use the M -value to quantitatively analyze mitophagy.

Mitophagy was triggered by the incubation of HeLa cells with 10.0 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a common mitochondria damage inducer, for 12 h.^{12, 24, 25} Cells were then stained with MTG and LTR and observed under SIM (Fig. 2A). Treatment with CCCP broke mitochondria into spherical shapes with varying sizes compared to untreated cells, which primarily showed filamentous mitochondria (Fig. 1A). In addition, CCCP treatment induced more frequent mitophagy (Frame 1-3 in Fig. 2A), as evidenced by the appearance of more yellow spots resulting from fusion between mitochondria (green) and lysosomes (red) (Fig. 2A inset 1-3 and Fig. 2B). This result indicates that more mitophagy occurs under pathological conditions. Because dysfunctional mitophagy is closely associated with many diseases,²⁻⁴ a quantitative analysis of mitophagy is of great significance for the drug development.

119

120 With this goal in mind, we calculated the M -value of the mitophagy after the cell was exposed to CCCP for 12 h
121 (Fig. 2A blue enlargement, and Fig. 2D). The M -value of the mitophagy event is greater than 0.5, and some
122 even reaches close to 1.0 (Fig. 2C, E). To test whether the M -value can be applied to all mitophagy events, we
123 performed a quantitative analysis to more mitophagy events ($n=100$) (Fig. S9-12). The M -values in all
124 mitophagy events consistently range from 0.5 to 1.0 (Figure 3A). Thus, the M -value platform provides a robust
125 system to differentiate MLC (0-0.4) and mitophagy (0.5-1.0) in physiological and pathological conditions.

126

127 To verify the applicability of using the M -value for MLC and mitophagy, we next applied the M -value to
128 quantitative analysis of an MLC (Fig. 3B, C) and a mitophagy event (Fig. 3D, E) at different rotation angles
129 (0° - 180°). We found that the M -value for MLC and mitophagy is consistent at different observation angles.
130 Finally, CCCP-induced mitophagy was confirmed with a commercially available mitophagy detection dye,²⁴
131 which generates higher red fluorescence compared with untreated cells (Fig. 3F).

132

133 *Comparison of interactive behavior of mitochondrial and lysosome using epi-illumination fluorescence* 134 *microscopy, confocal microscopy, and SIM*

135 To demonstrate the advantages of SIM, we compared the performance of epi-illumination fluorescent
136 microscopy, confocal microscopy, and SIM in resolving mitochondria-lysosome interaction using the same
137 staining process with MTG and LTR (Fig. 4). Mitochondria and lysosomes appeared as green and red plaques in
138 untreated cells under an epi-illumination fluorescence microscope, and a large area of yellow plaque was
139 generated in the overlaid image (Fig. 4A). One cannot capture any information about MLC events at this spatial
140 resolution, which may even cause a misinterpretation of mitophagy in untreated cells. Figure 4B showed the
141 morphology of mitochondria and lysosomes under a confocal microscope. Although confocal microscopy
142 successfully located mitochondria and lysosomes at the subcellular level, its spatial resolution (~ 500 nm, Fig.
143 S13) is not enough for the quantitative investigation of the detailed mitochondria-lysosome interaction below
144 100 nm, compared with SIM (Fig. 4C).

145

146 In order to further investigate the mitochondria-lysosome interaction under pathological conditions using
147 different optical microscopy techniques, we incubated 10.0 μ M CCCP with HeLa cells for 12 h before staining
148 with MTG and LTR. As expected, neither epi-illumination fluorescence microscopy (Fig. 4D) nor confocal
149 microscopy (Fig. 4E) was able to clearly capture mitophagy at the subcellular level. In contrast, SIM provides
150 excellent imaging quality that can resolve the mitochondria-lysosome interaction (Fig. 4F).

151

152 *Application of M-value to ATG13 and FIP200 knockout cell*

153 Since the *M*-value system is feasible to quantitatively separate MLC (<0.4) and mitophagy (0.5-1.0), we then
154 applied *M*-value to analyze the crosstalk of mitochondria and lysosomes in ATG13 knockout (ATG13 KO) and
155 FIP200 knockout (FIP200 KO) HeLa cells. Both ATG13 and FIP200 play important roles in the process of
156 autophagy and mitophagy (Fig 5A).^{26, 27 28, 29} The endogenous ATG13 and FIP200 in wild-type (WT) cells were
157 knocked out with a CRISPR/Cas9 gene editing assay (Fig 5B). The ATG13 KO and FIP200 KO cells were then
158 exposed to media with or without 10.0 μ M CCCP for 12 h prior to staining with MTG and LTR. In untreated
159 ATG13 KO cells (Fig 5C), mitochondria showed a continuous rod shape, and interacted with lysosomes as
160 MLC (*M*-value, 0.245) (Fig 5E-1). After CCCP treatment, damaged mitochondria appeared as granules with
161 various sizes (Fig 5C), which is similar to those in CCCP-treated WT cells (Fig 2A). MLC (*M*-value, 0.186)
162 also occurred under CCCP treatment (Fig 5E-2). In contrast, compared to CCCP-treated WT cells (Fig 2A),
163 mitophagy events in CCCP-treated ATG13 KO cells significantly decreased. In FIP200 KO cells, no mitophagy
164 was observed before and after CCCP-treatment (Fig 5D, E-3, E-4), whereas MLC still occurred. These results
165 demonstrated that ATG13 and FIP200 are required for mitophagy. The *M*-value system is applicable to
166 distinguish the two events in autophagy-defective cells.

167

168 **Discussion**

169 Mitochondria are important organelles for energy conversion in eukaryotic cells and are involved in a variety of
170 biological processes such as intracellular homeostasis, proliferation, senescence, and death.^{30, 31} At the same
171 time, functions of mitochondria are regulated by their interactions with lysosomes through processes such as
172 MLC and mitophagy.^{12, 22, 32, 33} MLC is widely found in cells, and it was recently discovered that RAB7 GTP

173 hydrolysis plays a key role in the MLC process.²² Mitophagy is an autophagy process that selectively removes
174 redundant or damaged mitochondria, which regulates the number of mitochondria in cells and maintains normal
175 mitochondrial function.^{2, 4, 34} Herein, the interactive behaviors of individual mitochondria and lysosome pairs
176 were observed under SIM. Compared to conventional tools such as epi-illumination and confocal fluorescence
177 microscopy, SIM offers an improved spatial resolution to capture detailed information of the dynamic
178 mitochondria-lysosome interactions in live cells.

180 Another bottleneck that limits our understanding of mitochondria-lysosome interaction is a lack of quantitative
181 analysis platform for data generated from SIM. To address this challenge, we developed an *M*-value platform
182 based on the FWHM of organelle images. This platform allows us to successfully differentiate MLC from
183 mitophagy because their *M*-values are bounded within different range: an *M*-value below 0.4 implies MLC and
184 an *M*-value ranging from 0.5 to 1.0 implies mitophagy. Thus, the *M*-value quantitative analysis system based on
185 the super-resolution microscopy technique opens a new avenue to adopt automatic image analyzing software for
186 high-throughput study of subcellular organelle interactions.

188 We envision that the *M*-value platform will specifically benefit high-throughput drug screening as an evaluation
189 index of pharmacodynamics. Currently, high-throughput drug screening mainly relies on reporter gene systems,
190 fluorescent labeling detection, micro-chemical technology, and fluorescence imaging technology.³⁵ Among
191 them, the combination of fluorescence imaging and automatic analysis is an excellent screening strategy for
192 micro-, sub-cellular image analysis in living cells.³⁶ However, the confocal microscopy systems commonly
193 used in these high-throughput screening systems cannot provide sufficient spatial resolution to capture organelle
194 interactions in live cells.^{37, 38} Combining artificial intelligence and SIM imaging with *M*-value system will
195 significantly retrench the process of early drug discovery and shorten the drug screening cycle to promote the
196 success rate of novel drug discovery.

198 **Materials and methods**

199 *Materials*

Mito-Tracker Green (#M7514, MTG) and Lyso-Tracker Red (#L12492, LTR) were obtained from Invitrogen (Eugene, Oregon, USA). Mitophagy dye was obtained from Dojindo Laboratories (#MD01-10, Kumamoto, Japan), Carbonyl cyanide 3-chlorophenylhydrazone (#045200, CCCP) was obtained from Thermo Fisherscientific (Grand Island, NY, USA). Penicillin-streptomycin (#15140163, 10,000 units/ml), Fetal bovine serum (#26140079, FBS), Dulbecco's modified Eagle's medium (#11965118, DMEM) and other cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). Primary and secondary antibodies used in this study were GAPDH (#5174, Cell Signaling Technology, Beverly, MA, USA), FIP200 (#12436, Cell Signaling Technology, Beverly, MA, USA) and ATG13 (#13273, Cell Signaling Technology, Beverly, MA, USA) and HRP-linked anti-rabbit IgG (#7074, Cell Signaling Technology, Beverly, MA, USA). HeLa cells were a generous gift from Dr. Carolyn M. Price lab (University of Cincinnati).

Cell culture

Cells were cultured in DMEM supplemented with 10% FBS, penicillin-streptomycin (100 units/ml) in a 5% CO₂ humidified incubator at 37 °C.

Live cell labeling

Cells were incubated with 100 nM MTG for 30 min and further co-incubated with 200 nM LTR at 37 °C for another 30 min in free DMEM, and washed with free DMEM 3 times and observed using a fluorescence microscopy, confocal laser scanning microscopy or OMX 3D-SIM super-resolution microscope.

Confocal laser scanning microscopy

The images were obtained using a LSM-710 confocal laser scanning microscope (Carl Zeiss, Inc.) equipped with a 63×/1.49 numerical aperture oil-immersion objective. I 1. and were analyzed with ZEN 2012 (Carl Zeiss, Inc.) and ImageJ software (National Institutes of Health). All fluorescence images were analyzed with ImageJ software (<https://imagej.nih.gov/ij/>).

226 ***OMX 3D-SIM super-resolution microscope imaging and analysis***

227 A total of 2×10^5 cells were seeded on a glass bottom microwell dish and incubated with 2 ml of DMEM
228 medium supplemented with 10% FBS for 24 h, followed by CCCP or vehicle treatment for 12h. After treatment,
229 the cells were washed 3 times with pre-warmed free DMEM medium, stained with 100 nM MTG for 30 min,
230 co-incubated with 200 nM LTR at 37 °C for another 30 min, and washed with free DMEM for 3 times. Finally,
231 cells were cultured in phenol-free medium (#1894117, Gibco, Grand Island, NY, USA) and observed under an
232 OMX 3D-SIM super-resolution microscope (Bioptechs, Inc) that is equipped with an Olympus 100 \times /1.49
233 numerical aperture oil-immersion objective lens and solid-state lasers.

234

235 Firstly, the lasers (488 nm and 561 nm) were turned on and waited for 20 min to make the instrument thermally
236 stable. After cleaning the lens, an immersion oil droplet (refractive index 1.516) was placed on the lens. Finally,
237 the sample was placed on a tray for imaging. In order to reduce photobleaching, a 10 ms exposure time and 1%
238 transmission excitation power was used to focus the sample and find the cells. Images were captured with an
239 electron-multiplying charge coupled device (EMCCD) camera (Photometrics Cascade II) with a gain value of
240 3000 at 10 MHz. The exposure time was set to 50 ms per frame. Whole-frame images (512 \times 512 pixels) were
241 acquired using Z-stacks with a step size of 0.2 μ m.

242

243 SIM images were analyzed with ImageJ software equipped a fluorescence intensity measurement tool. The
244 intensity profile along the perpendicular (white solid lines, Fig. S14) of mitochondria-lysosome contact (white
245 dash lines, Fig. S14) was used for the *M*-value calculation.

246

247 ***CRISPR/Cas9-mediated knockout of FIP200 and ATG13 in HeLa cells***

248 The pX458 plasmid (pSpCas9(BB)-2A-GFP; Addgene) was used as the cloning backbone for expressing
249 sgFIP200 and sgATG13. Two complementary oligos for each sgRNA were denatured, annealed and ligated into
250 linearized pX458 vector digested by BbsI (New England Biolabs). Empty constructs and pooled pX458-sgRNA
251 were transiently transfected into HeLa cells respectively using Lipofectamine 3000 (Invitrogen). After 48 hr, the
252 transfected cells were sorted based on the fluorescence of GFP (reporter) using a FACSAria cytometer (BD

253 Biosciences). Sorted individual cells were cultured in a 96-well plate and were subjected to Western blot
254 analyses. At least three different clones were pooled for functional experiments. The sgRNA sequences targeting
255 FIP200 and ATG13 were based on the published literature using CRISPR/Cas9 library.^{39, 40} sgRNA sequences of
256 FIP200 and ATG13 are listed below:

257 sgFIP200: CAGGTGCTGGTGGTCAATGG

258 sgATG13-1: TCACCCTAGTTATAGCAAGA

259 sgATG13-2: CAGTCTGTTGTACACCGTGT

260 sgATG13-3: GACTGTCCAAGTGATTGTCC

261

262 ***Western-blot assay***

263 Cells were cultured in 3.5 cm diameter plates (80-90% confluence), washed by PBS buffer, and lysed for 15
264 min on ice using RIPA buffer (#C2978, Sigma, St. Louis, MO, USA) containing an anti-protease mix (#PI78415,
265 Thermo Scientific, Waltham, MA, USA). Protein concentration was measured by BCA assay (#23225, Thermo
266 Scientific, Waltham, MA, USA). Equal amounts of proteins were subjected to SDS-PAGE and immunoblotting
267 as described previously.⁴¹

268

269 **Competing financial interests**

270 The authors declare no competing financial interests.

271

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279

280 **Author contributions**

281 Q.C., X.S. and Z.T. collected all OMX 3D-SIM super-resolution microscope data. Q.C. and X.S. analyzed and
282 processed the OMX 3D-SIM data. Q.C. cultured cell. M.H performed confocal laser scanning microscopy. K.Z.,
283 F.W., P.L., J.G., and J.D. conceived the project, designed the experiments, and wrote the manuscript with the
284 help of all authors.

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- 367

368 **Figure legends**

369 **Figure 1.** Whole cell quantitative analysis of mitochondrial-lysosome contact (MLC) in living cells. (A) SIM
370 image of mitochondria (Green) and lysosomes (Red). 1-11 represent MLC events. (B) A representative dynamic
371 process of MLC in a living HeLa cell. White solid lines indicate the fluorescence intensity shown in (D). (C)
372 Partial enlargement of (A). White solid lines indicate the fluorescence intensity shown in (G). (E) and (H)
373 Representative MLC events. White solid lines indicate the fluorescence intensity shown in (F). (I) Schematic
374 diagram of calculation formula for the *M*-value of MLC. (J) *M*-value range of MLC dynamic events. Scale bars:
375 A 5.0 μm , B, C, E, and H 0.5 μm , insets of A 0.5 μm .

376

377 **Figure 2.** Quantitative analysis of fusion between mitochondria and lysosome in CCCP-treated cells. (A) Frame
378 1-3 of mitochondrial and lysosome fusion events. White solid lines indicate fluorescence intensity shown in (C).
379 (B) 3D-SIM surface plot of representative fusion between mitochondria and lysosome. (D) Representative
380 fusion events in (A). White solid lines indicate fluorescence intensity shown in (E). Scale bars: A 5.0 μm , B and
381 D 0.5 μm , inset of A 0.5 μm .

382

383 **Figure 3.** *M*-value of WT cells with and without CCCP treatment. (A) *M*-value distribution of 100 MLC events
384 in normal cells and 100 mitophagy fusions in CCCP-treated cells. (B) A MLC event at different angles. The
385 solid white line indicates the fluorescence intensity shown in (C). (D) A mitophagy at different angles. The
386 white solid line indicates the fluorescence intensity shown in (E). (F) Cells incubated with mitophagy dye with
387 or without CCCP treatment. Scale bars: B 0.5 μm , F 5.0 μm .

388

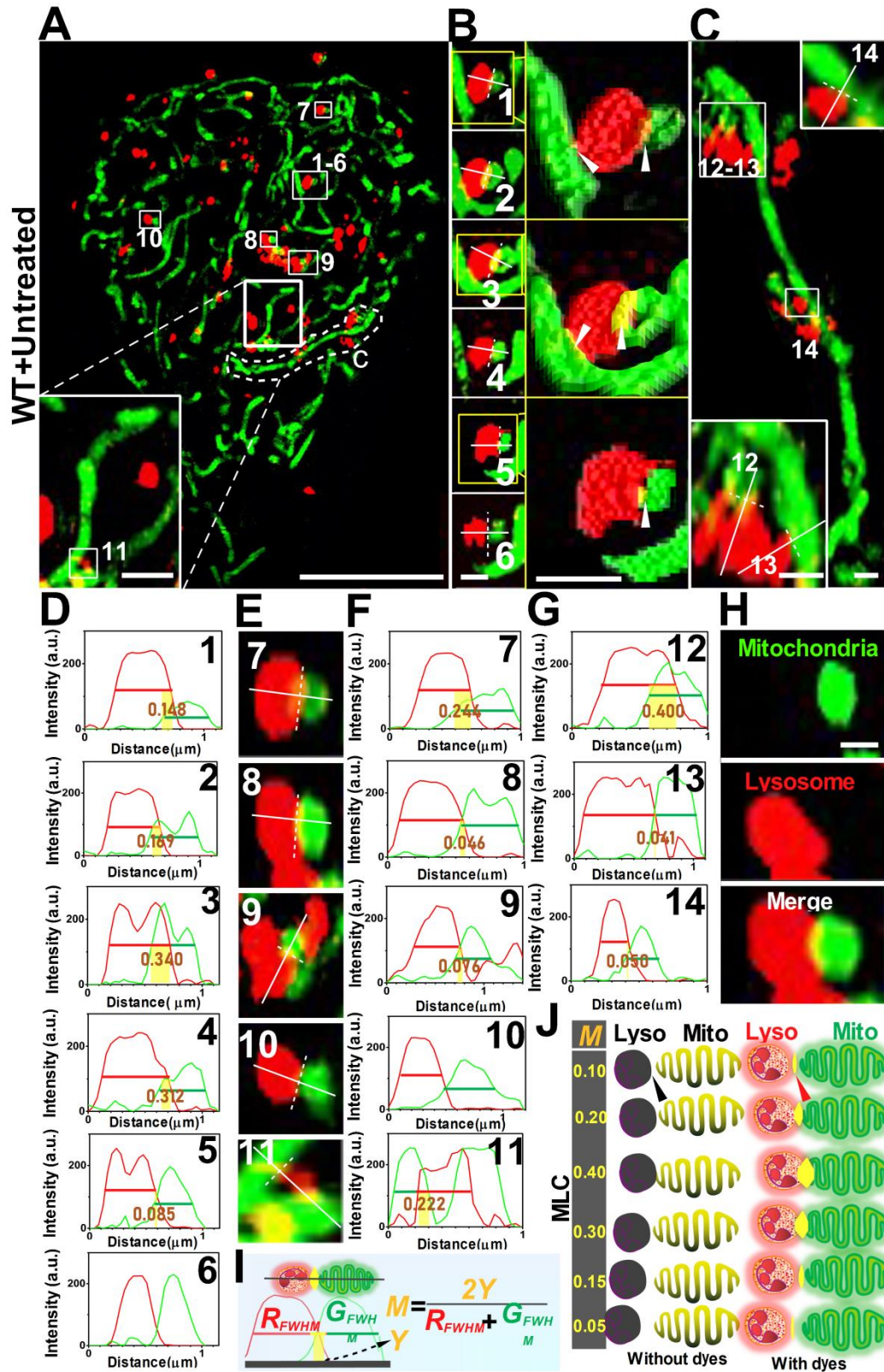
389 **Figure 4.** Comparison of interactive behavior of mitochondrial and lysosome using fluorescence microscopy,
390 confocal microscopy and SIM. Mitochondrial and lysosome were stained with MTG and LTR. Images of
391 untreated cells under fluorescence microscopy (A), confocal microscopy (B), and SIM (C). Images of
392 CCCP-treated cells under fluorescence microscopy (D), confocal microscopy (E), and SIM (F). Scale bars: 1.0
393 μm .

394

395 **Figure 5.** ATG13/FIP200 knockout cells under SIM. **(A)** Illustration of the role of ATG13 and FIP200 in
396 mitophagy. **(B)** Western blot for detecting the protein expression of ATG13 and FIP200. Images of untreated
397 and CCCP-treated ATG13 KO cells **(C)** and FIP200 KO cells **(D)**. **(E)** Representative *M*-value calculations
398 indicate MLC events in ATG13KO cells **(1,2)** and FIP200 KO cells **(3,4)** with or without CCCP treatment.
399 Scale bars: **C** 5.0 μm , **E** 0.5 μm .

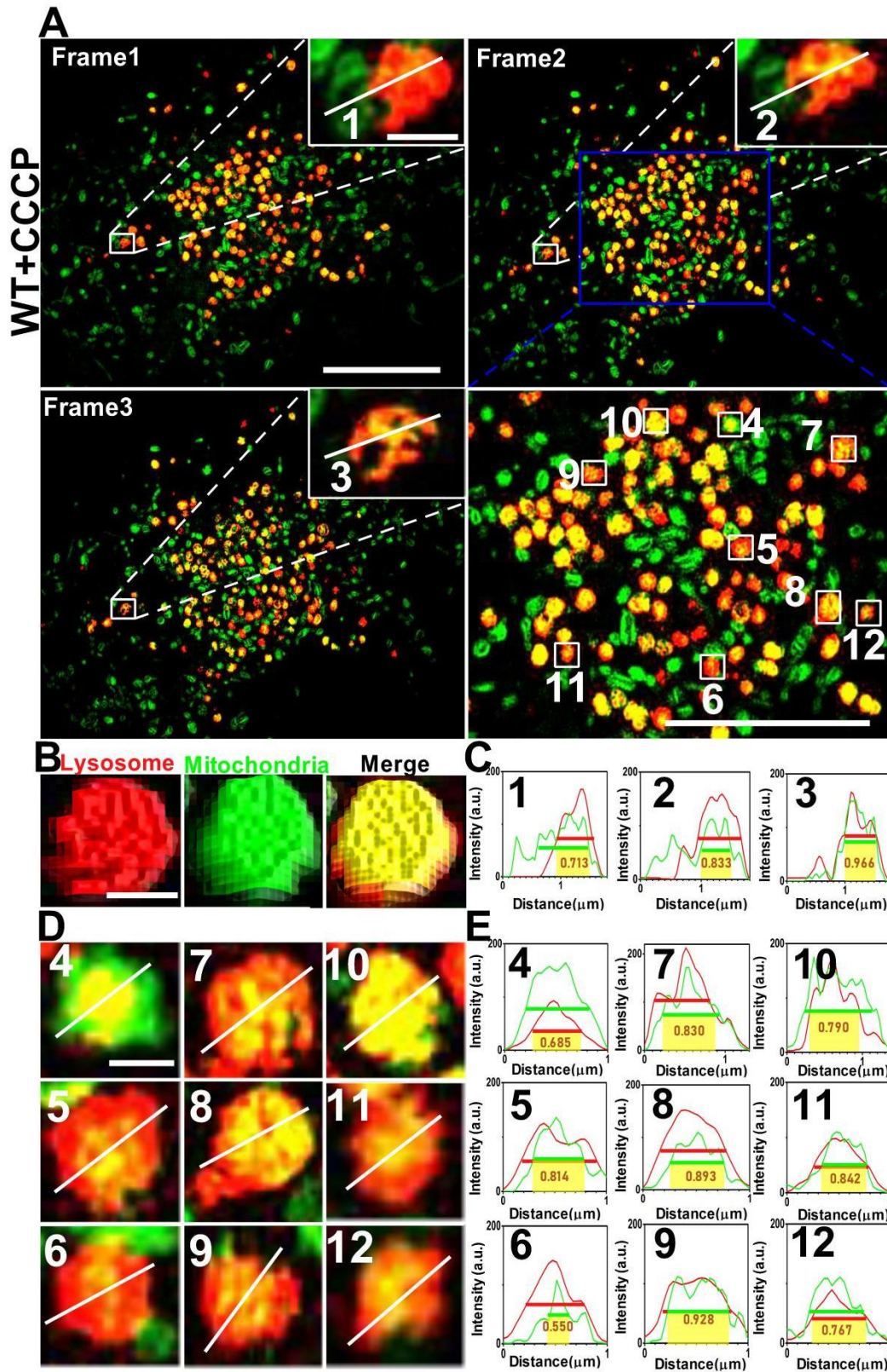
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401 **Fig. 1**

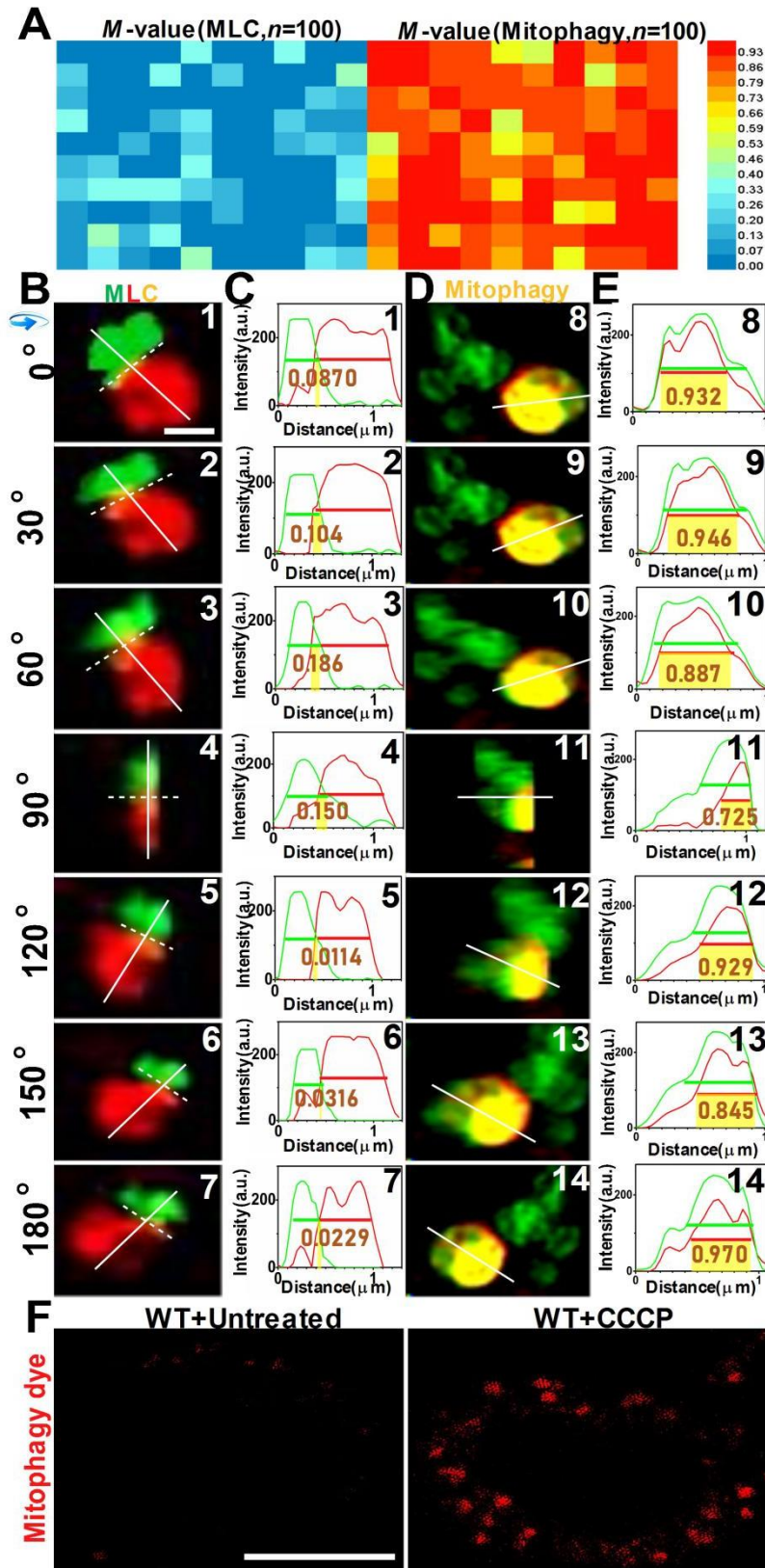


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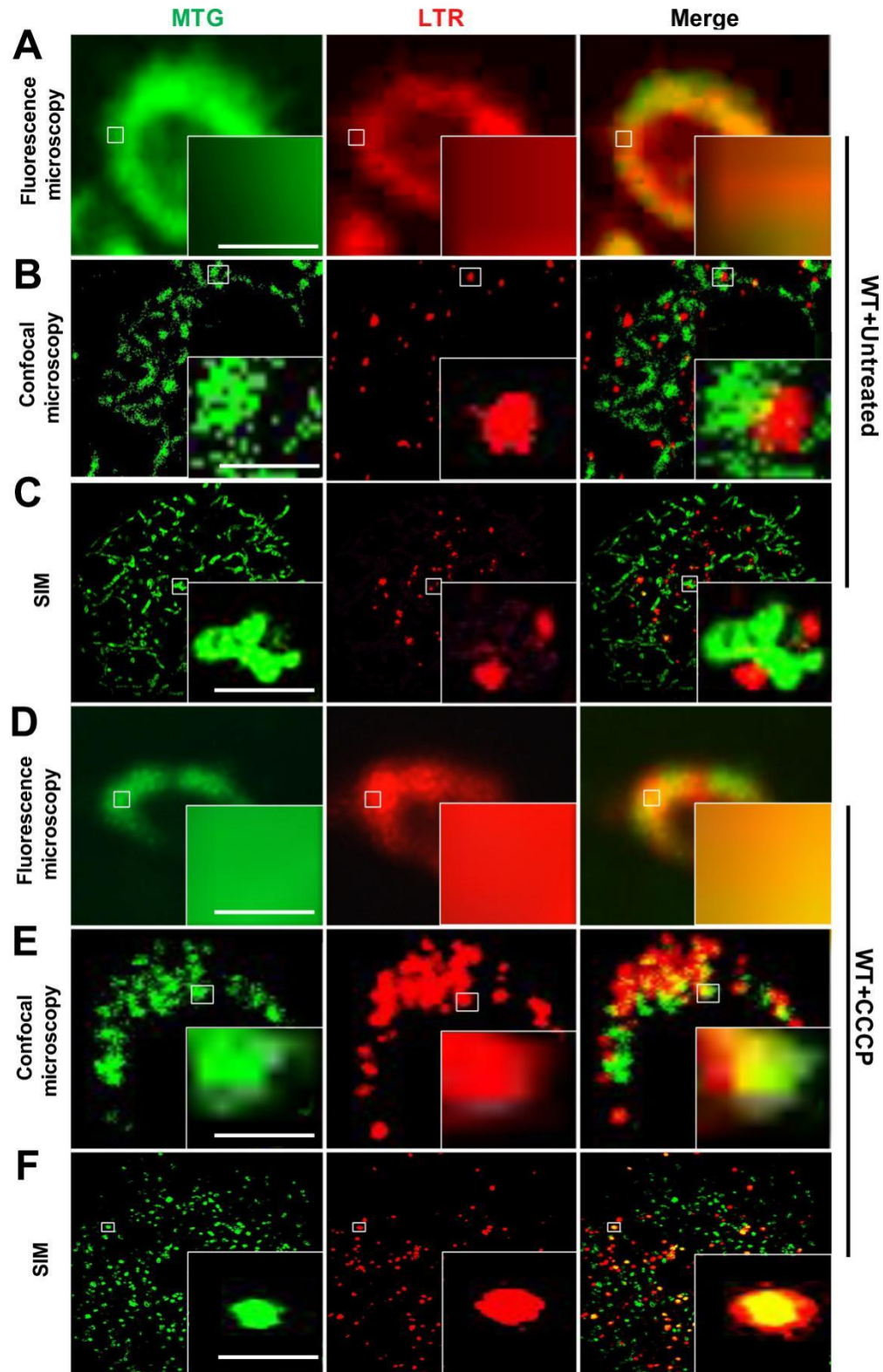
406 **Fig. 3**



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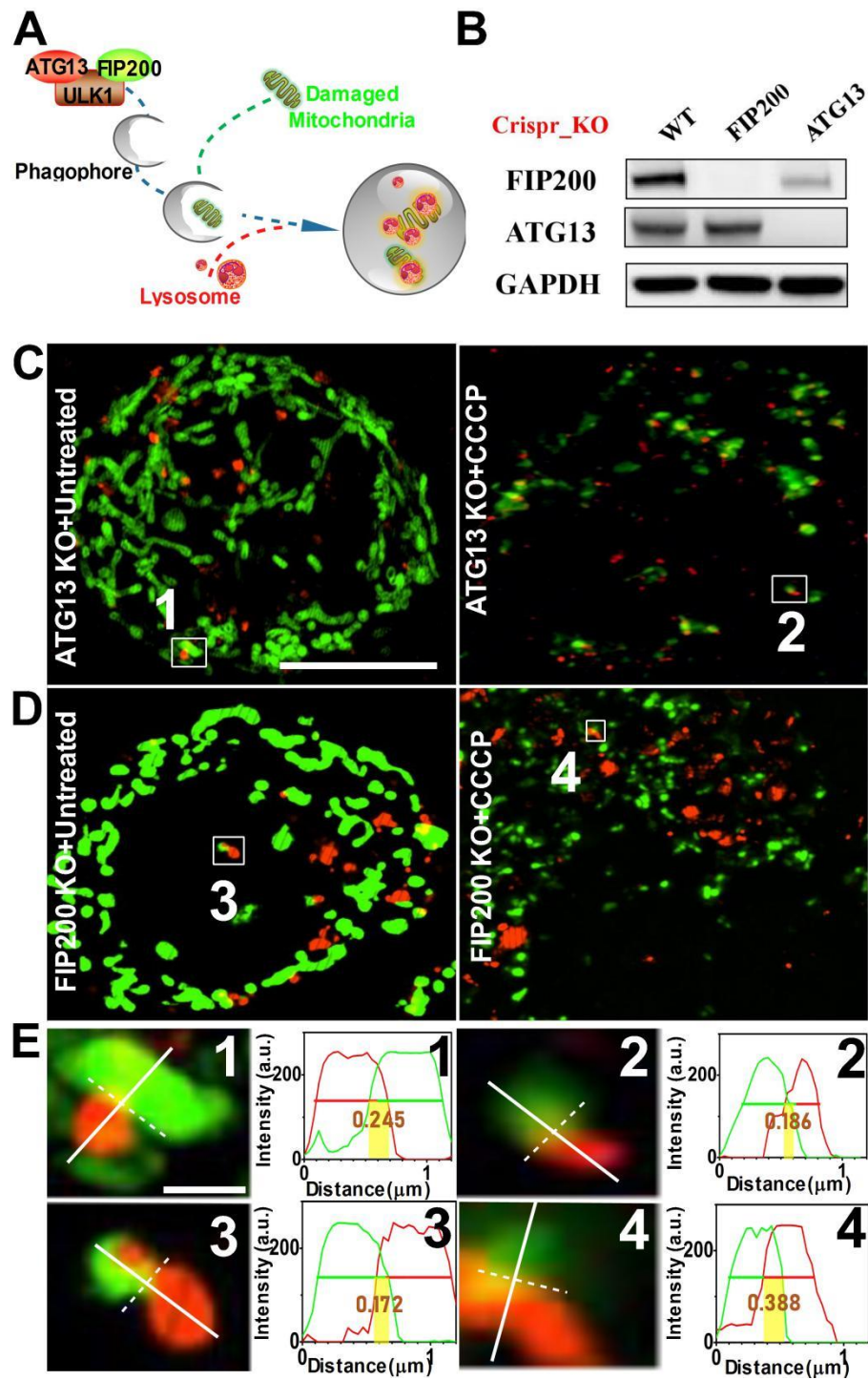
409 **Fig. 4**



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411

412 **Fig. 5**



413

414