Journal of Visualized Experiments

Toxicity Study of Zinc Oxide Nanoparticles in Cell Culture and in Drosophila melanogaster

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59510R4
Full Title:	Toxicity Study of Zinc Oxide Nanoparticles in Cell Culture and in Drosophila melanogaster
Keywords:	zinc oxide nanoparticles; toxicity; cell death; oxidative stress, MRC5 cells; Drosophila
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Additional Information:	
Question	**Response**
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TITLE:

Toxicity Study of Zinc Oxide Nanoparticles in Cell Culture and in *Drosophila melanogaster*

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KEYWORDS:

zinc oxide nanoparticles, toxicity, cell death, oxidative stress, MRC5 cells, *Drosophila*

SUMMARY:

We describe a detailed protocol for evaluating the toxicological profiles of zinc oxide nanoparticles (ZnO NPs) in particular, the type of cell death in human MRC5 lung fibroblasts and ROS formation in the fruit fly *Drosophila*.

ABSTRACT:

Zinc oxide nanoparticles (ZnO NPs) have a wide range of applications, but the number of reports on ZnO NP-associated toxicity has grown rapidly in recent years. However, studies that elucidate the underlying mechanisms for ZnO NP-induced toxicity are scanty. We determined the toxicity profiles of ZnO NPs using both in vitro and in vivo experimental models. A significant decrease in cell viability was observed in ZnO NP-exposed MRC5 lung fibroblasts, showing that ZnO NPs exert cytotoxic effects. Similarly, interestingly, gut exposed to ZnO NPs exhibited a dramatic increase in reactive oxygen species levels (ROS) in the fruit fly *Drosophila*. More in-depth studies are required to establish a risk assessment for the increased usage of ZnO NPs by consumers.

INTRODUCTION:

Nanotechnology refers to the application of nanosized materials that are used across all scientific fields, including medicine, materials science, and biochemistry. For instance, ZnO NPs which are known for their ultraviolet scattering, chemical sensing, and anti-microbial properties, as well as

high electrical conductivity, are utilized in the production of various consumer products such as food packaging, cosmetics, textiles, rubbers, batteries, catalyst for automobile tail gas treatment, and biomedical-related applications[1-3].

However, the burgeoning applications of ZnO NP-based products, leading to increased human exposure to ZnO NPs, have raised concerns on their potential adverse effects on human health. A number of in vitro cellular studies have demonstrated that ZnO NPs can induce oxidative stress, autophagy-related cytotoxicity, inflammation, and genotoxicity[4-8]. Notably, the toxicity of ZnO NPs is assumed to be caused by the dissolution of Zn to free Zn^{2+} ions, as well as the surface reactivity of ZnO, resulting in the cellular ionic and metabolic imbalances that are linked with impaired ionic homeostasis and an inhibition of ion transportation[4,7,9,10]. Importantly, studies have shown that the generation of reactive oxygen species (ROS) is one of the primary mechanisms underlying ZnO NPs-associated toxicity. Insufficient anti-oxidative activity following ROS insult has been shown to be responsible for eliciting the cytotoxicity and DNA damage [9]. The toxic effects of ZnO NPs have also been reported in animal models, including rodent[1], zebrafish[11,12], as well as the invertebrate *Drosophila*[13].

Drosophila serves as a well-established alternative animal model for toxicity screening of chemical entities and nanomaterials (NMs)[14,15]. Importantly, there are high levels of genetic and physiological similarity between human and *Drosophila* that justifies the use of *Drosophila* as an in vivo model for evaluating biological responses to environmental contaminants such as NMs[16]. Furthermore, there are many advantages of using *Drosophila* due to its small size, short lifespan, genetic amenability, and easy and cost-effective maintenance. Moreover, *Drosophila* has been widely adopted for the study of genetics, molecular and developmental biology, ever since its full genome was fully sequenced years ago back in 2000, therefore making it suitable for a variety of high-throughput screening and for tackling unresolved biological questions[17-21]. In recent years, a number of studies related to immunotoxicity using different types of NPs in *Drosophila* have been reported[15,22-24]. This fundamental new knowledge obtained from the studies using *Drosophila* has helped to provide more insights into our understanding of nanotoxicology.

ROS is a well-known culprit for cytotoxicity and genotoxicity caused by NPs, in particular, metal-based NPs[25]. ROS are oxygen-containing chemical species with higher reactive properties than molecular oxygen. Free radicals such as superoxide radical (O2−) and even, non-radical molecules such as hydrogen peroxide (H_2O_2) can act as ROS. Under normal physiological condition, they are required to maintain cellular homeostasis[26], however, excessive ROS due to overproduction or dysregulation of the antioxidant defense system can cause oxidative stress, leading to damage to proteins, lipids and deoxyribonucleic acid (DNA)[27]. For instance, as ROS levels increase and glutathione (GSH) level decreases concomitantly, disruption of adenosine triphosphate (ATP) synthesis takes place and lactate dehydrogenase (LDH) level increases in the medium, culminating in cell death[27].

Here, we provide protocols for performing cellular and genetic analyses using cultured mammalian cells and *Drosophila* to determine the potential adverse effects of ZnO NPs. An overview of the method used for the toxicity study of ZnO NPs is shown in **Figure 1**.

PROTOCOL:

1. Fluorescence activated cell sorting (FACS) analysis on lived/fixed cells

1.1 Sonicate ZnO NPs in suspension for 15 min.

1.2 Prepare ZnO NPs at various concentrations (e.g., 0, 10, 25, 50,100 and 200 µg/mL) using 1 mg/mL ZnO NP stock solution for the treatment of cultured cells.

1.3 Seed MRC5 human lung fibroblasts (1 x 10^5 cells/well) onto a 6-well culture plate a day in advance, and then treat the cells with 2 mL of ZnO NPs (in triplicates) for 8 h, 16 h, and 24 h.

1.4 At each time point, collect the cells by centrifuging at 300 x *g* for 5 min.

1.5 Wash the cell pellets twice with phosphate buffered saline (PBS).

1.6 Resuspend the cells with 1x binding buffer, which is composed of 0.1 M HEPES/sodium hydroxide (NaOH), 1.4 M sodium chloride (NaCl), and 25 mM calcium chloride ($CaCl_2$), at a concentration of 1 x 10^5 cells per 100 µL.

1.7 Add 5 µL of Fluorescein isothiocyanate (FITC) Annexin V stain and 5 µL of propidium iodide (PI) DNA stain, and incubate the cells for 15 min at room temperature (RT; 25 °C) in dark.

1.8 Top up the samples with an additional 400 µL of 1x binding buffer before sorting the cells by flow cytometry. A minimum of 10,000 cells is analyzed for each sample.

1.9 Tabulate the bar chart using the median intensity obtained.

2. Exposure of ZnO NPs to *Drosophila*

2.1 Add 1 mL of nanoparticles at different concentrations into vials, followed by 9 mL of fly food to make a final concentration of 0.1 mg/mL, 0.25 mg/mL or 0.5 mg/mL ZnO NPs.

2.2 Mix the nanoparticles with food thoroughly in the vials using the pipette.

2.3 Allow fly food containing ZnO NPs to cool for at least 2-3 h before use.

2.4 Introduce adult male and female flies into the vials for 5 days, and allow them to mate and lay eggs (which appear as white spots) on the surface of the food.

2.5 Remove the parental flies, and allow the eggs to undergo further development, which consists of 4 different developmental stages (embryonic, larval, pupal and adult stage).

3. Dissection of fly

3.1 Collect late 3rd instar larvae from the wall of the vials for analyses. Freshly laid eggs normally develop into late 3rd instar larvae after 72-120 h at RT.

3.2 Clean the dissection dish and fill up the well with dissection medium/PBS.

3.3 Dissect the larvae (late 3rd instar) under the stereomicroscope, using a pair of forceps.

3.4 Use the tip of the forceps to make a tiny hole and break open the cuticle layer of the larvae. Carefully pull out the gut and place it into a 1.5 mL microcentrifuge tube containing Schneider's *Drosophila* medium, prior to the fixing step using 1 mL of 4% paraformaldehyde (PF).

3.5 Fix the gut in PF for 10 min at RT, for subsequent experiments, such as immunostaining.

4. ROS detection using Dihydroethidium (DHE) staining

4.1 Treat larvae with various concentrations of ZnO NPs as described in step 2.1.

4.2 Following the dissection of the gut from 3rd instar larvae as described under section 3, incubate the gut in Schneider's *Drosophila* medium at RT before tissue staining is performed. Dissolve 1 µL of DHE dye (from the stock concentration of 30 mM) in 1 mL of Schneider's medium, making a final working concentration of 10- 30 µM DHE dye.

4.3 Incubate the gut for 5 min at RT in dark, and then wash three times using Schneider's medium for every 5 min.

4.4 Fix the gut with 4% PF (optional step) and mount the gut onto glass slides, with anti-fade mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). Capture images under a confocal microscope.

5. Measuring fluorescence using ImageJ software

5.1 Import the captured fluorescence images acquired using fluorescence microscopy or confocal laser scanning microscopy into the ImageJ software.

5.2 Click on the **Analyze** menu and select **Set measurements**.

5.3 Select the output measure such as area integrated intensity and mean grey value.

5.4 Click **Measure**.

5.5 Select a region without fluorescence to set the background.

5.6 Export the data into the Excel spreadsheet and determine the corrected total cell fluorescence (CTCF), using the calculation as shown below.

CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)

5.7 Construct a bar chart and perform statistical analysis.

REPRESENTATIVE RESULTS:

NP-exposed cells were processed with the cell staining reagent kit, followed by cell sorting using flow cytometry. ZnO NP-treated cells (bottom, right panel) exhibit a higher percentage of early (R3)/ late apoptotic cells (R6) than control cells (R5, bottom, left panel). Necrotic cell death is denoted by R4 (top, right panel) (**Figure 2**). The results of the FITC/Annexin V Assay on ZnO NP-treated MRC-5 fibroblasts are shown in **Figure 2**.

For the *Drosophila* experiments, sonicated ZnO NPs at various concentrations were added to fly food in 10 mL tubes and then mixed well using a pipette controller (**Figure 3**). Late third instar larvae collected from vials were dissected under the stereomicroscope. The larvae were first washed to remove remnants of remaining food (**Figure 4**). The outer cuticle layer was torn apart to expose internal organs. The gut was identified by the characteristic long and semitransparent appearance (whereas other organs appear opaque and light yellowish under the microscope) (**Figure 5**). The gut was carefully removed, without breaking, and transferred into a new microcentrifuge tube containing fixative on ice.

For the quantitation of fluorescence intensity such as the intensity of the DHE probe in the gut, the images were exported in JPEG or TIFF formats and opened with the ImageJ software. The part of the gut for analysis was selected and identified, for example, the midgut or hindgut region, and the fluorescence intensity of the region of interest (ROI) was measured. To compare the relative intensities of the different experimental groups, we employed the same quantitative confocal microscopy method described in the previous section. For comparison of fluorescence intensities, the parameter was set using the negative untreated control. Calculations of the signal intensity on the basis of calibration intensities of untreated control allowed a direct comparison between different experimental groups. **Figure 6** shows the average intensity of the DHE signal in the 3rd instar larval gut exposed to ZnO NPs at different concentrations. The gut of larvae treated with 0.5 mg/mL of ZnO NP treatment showed the highest fluorescence intensity.

The differences in relative intensities between all the experimental groups were further tabulated, and statistical analysis was performed, providing both qualitative and quantitative results (**Figure 7**).

FIGURE AND TABLE LEGNEDS:

Figure 1. Overview of the method used for toxicity study of ZnO NPs. For in vitro work, ZnO NP-treated cells were stained prior to flow cytometry analysis. For in vivo work, gut was dissected from 3rd instar larvae, followed by staining with DHE dye and image acquisition.

Figure 2. Dot plot of cells separated into different populations based on their FITC and PE staining. The pictograms show the results of FITC/Annexin V assay with 24 h treatment of ZnO-NPs on MRC-5. Statistical analysis of the cells at different stages can then be performed.

Figure 3. Preparation of ZnO NP-containing fly food medium. (**A**) Ingredients for fly food are added to water, allowed to swell, and boiled for 5 min. (**B**) After cooling down to 50 °C with stirring, Nipagin was added and mixed thoroughly. (**C**) Prepare a master mix for the nanoparticles (total volume not exceeding 10% of the final food volume). (**D**) Medium is then aliquoted, mixed with ZnO NPs at various concentrations and allowed to cool down completely before storage.

Figure 4. The whole gut dissection procedure. (**A**) Transfer 3rd instar larvae to a dissection disc. (**B**) Use forceps to gently hold a larva, and (**C**) wash away the remnant of food using saline. (**D**) Gently tear the cuticle apart without touching the gut and other internal organs. (**E**) Place the gut into the saline for subsequent procedure.

Figure 5. The anatomy of the digestive tract/gut. The gut extracted from the *Drosophila* larva is divided into three discrete domains of different developmental origin namely the foregut, midgut, and hindgut.

Figure 6. Staining of the gut tissue with DHE. (**A**) Prepare a master mix containing growth medium and DHE (a final concentration of 30 μM). (**B**) Add the master mix into the well of a dissection disc. (**C**) Transfer the dissected gut tissue into the well containing the DHE master mix. (**D**) Incubate at RT for 5 min and protect the tissue from light; wash 3x in PBS/saline for 5min. (**E**) Fix in 4% PF for 10 min; wash the tissue three times with PBS (optional). (**F**) Gently transfer the gut onto a glass slide, lay flat without having any tissue folded and mount with mounting medium before covering with cover glass.

Figure 7. Quantitation of fluorescent images using ImageJ. (**A**) Import the captured images. (**B**) Click on the **Analyze** menu and select **Set measurements**. (**C**) Select the area integrated intensity and mean grey value. Select a region without fluorescence to set the background. (**D**) Export the data into Excel and calculate the CTCF for subsequent statistical analysis.

DISCUSSION:

In order to assess if ZnO NP can induce apoptosis in MRC5 fibroblasts, we use flow cytometry to distinguish the cells from necrotic or apoptotic cell death. In normal live cells, phosphatidylserine (PS) is localized at the cell membrane. If apoptosis occurs, PS is translocated to the extracellular leaflet of the plasma membrane, allowing the binding of Annexin V labeled with fluorescein (FITC Annexin V) [29]. On the other hand, the red-fluorescent propidium iodide (PI), a nucleic acid binding dye, is impermeable to living cells and apoptotic cells but stains dead cells[30]. This allows us to identify the dead cells (red and green), apoptotic cells (green fluorescence) and live cells (little or no fluorescence), using a flow cytometer with the 488 nm line of an argon-ion laser for excitation.

For DHE staining of the *Drosophila* gut, it is important to reconstitute the dye with DMSO just

before you start the experiment, as prolonged storage may lead to an auto-oxidation of the dye that turns the dye into dark/purplish color[31,32]. In addition, the reconstituted stock solutions also tend to expire rather quickly, so one has to pay attention to the "expiry date". Alternatively, fluorogenic probes such as the green version of the photostable probe, which has the added ability to be multiplexed with stains, and produces much clearer signals than DHE, could be used. The dissected gut was transferred to Schneider's medium containing the dye at the desired concentration with no fixative added. This is to permit the incorporation of the dye in live cells, and hence staining is performed in the culture medium, to allow for better respiration of the cells.

With regard to the quantitation of DHE staining, for a start, avoid saturation of the pixels in control untreated cells. The imaging software program was used to determine exposure time by visually flagging saturated pixels. The untreated sample was used to set the exposure time and maintain the same parameters when capturing images for comparison of the intensity across different treatment groups which is important for quantification of the fluorescence image intensity later on. It is essential to acquire all images (control and treated samples) using the same system and acquisition settings/parameters, and the background should be standardized for all images (so that there is consistency for background subtraction)[33].

ACKNOWLEDGMENTS:

The study was supported by the grant number R706-000-043-490. The study does not represent the view of the grant sponsor.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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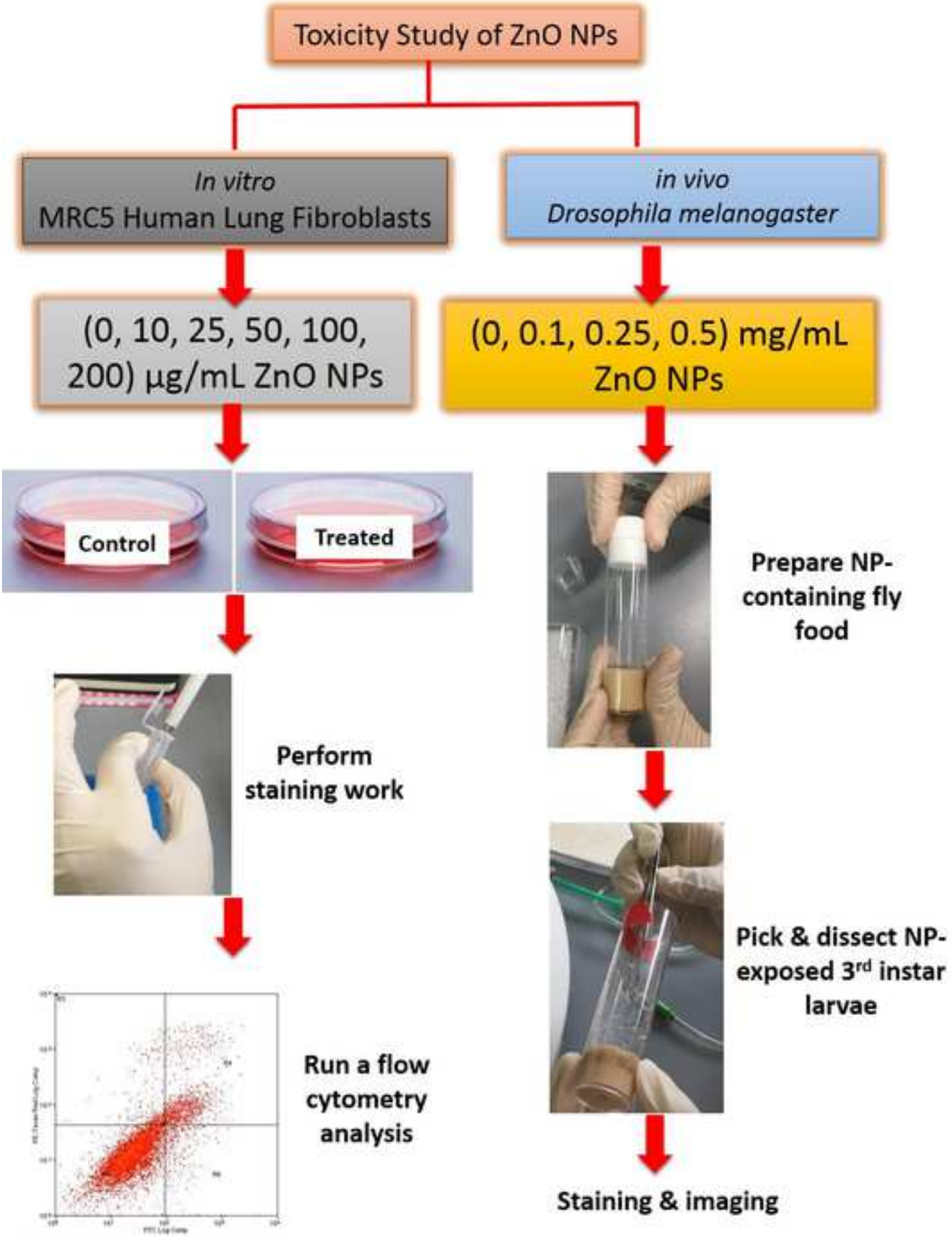

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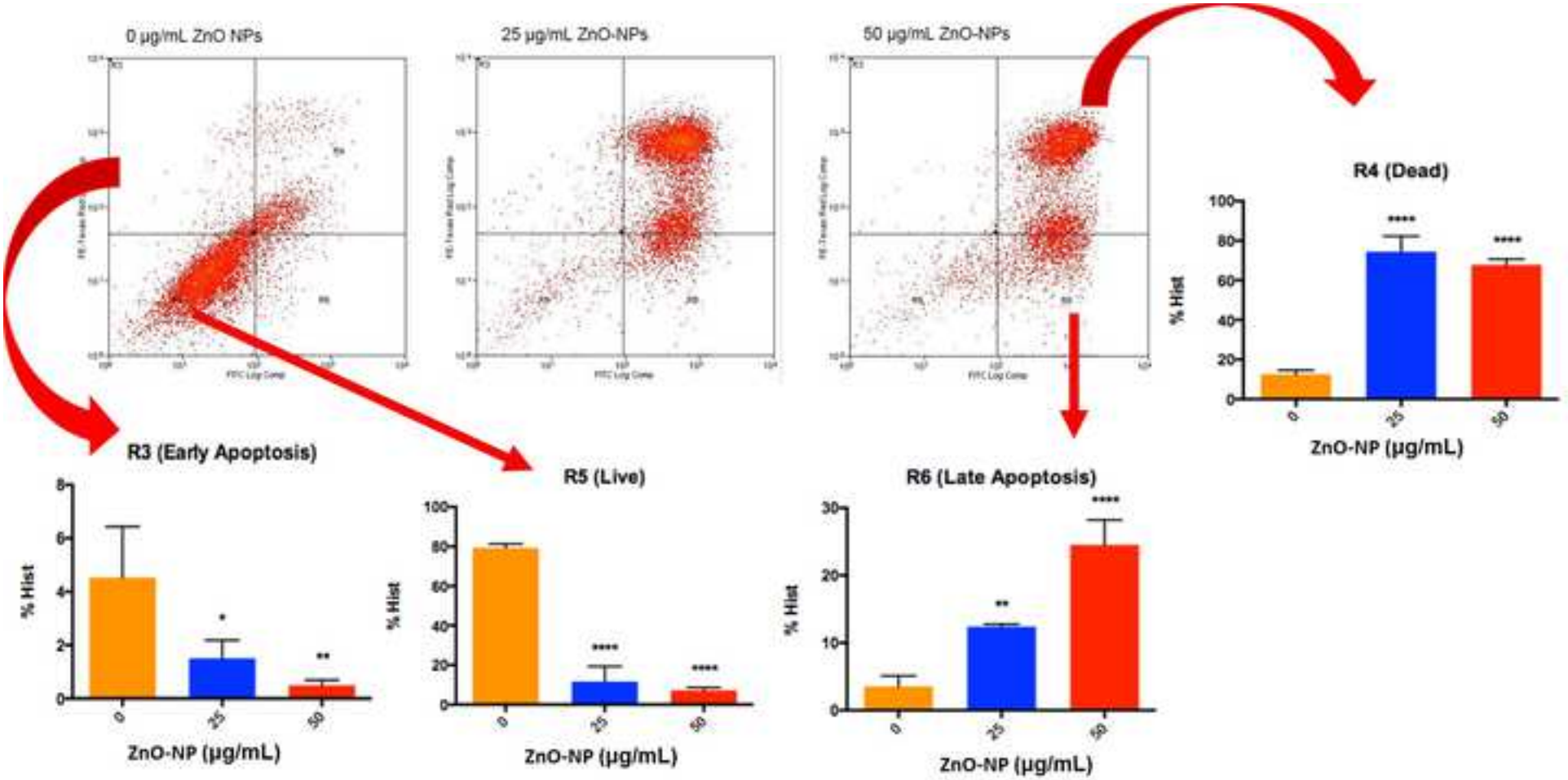

Figure 3

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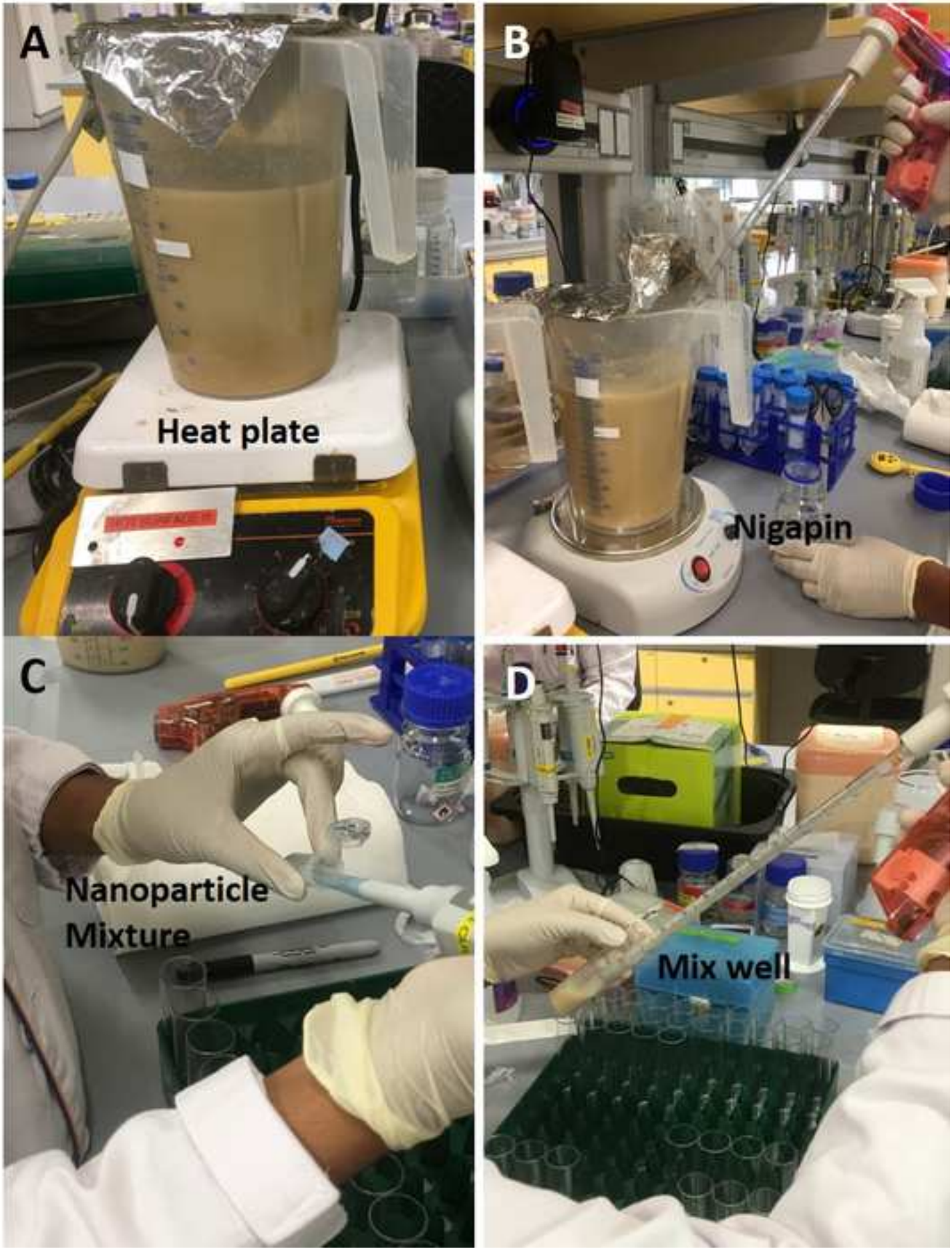

Figure 4

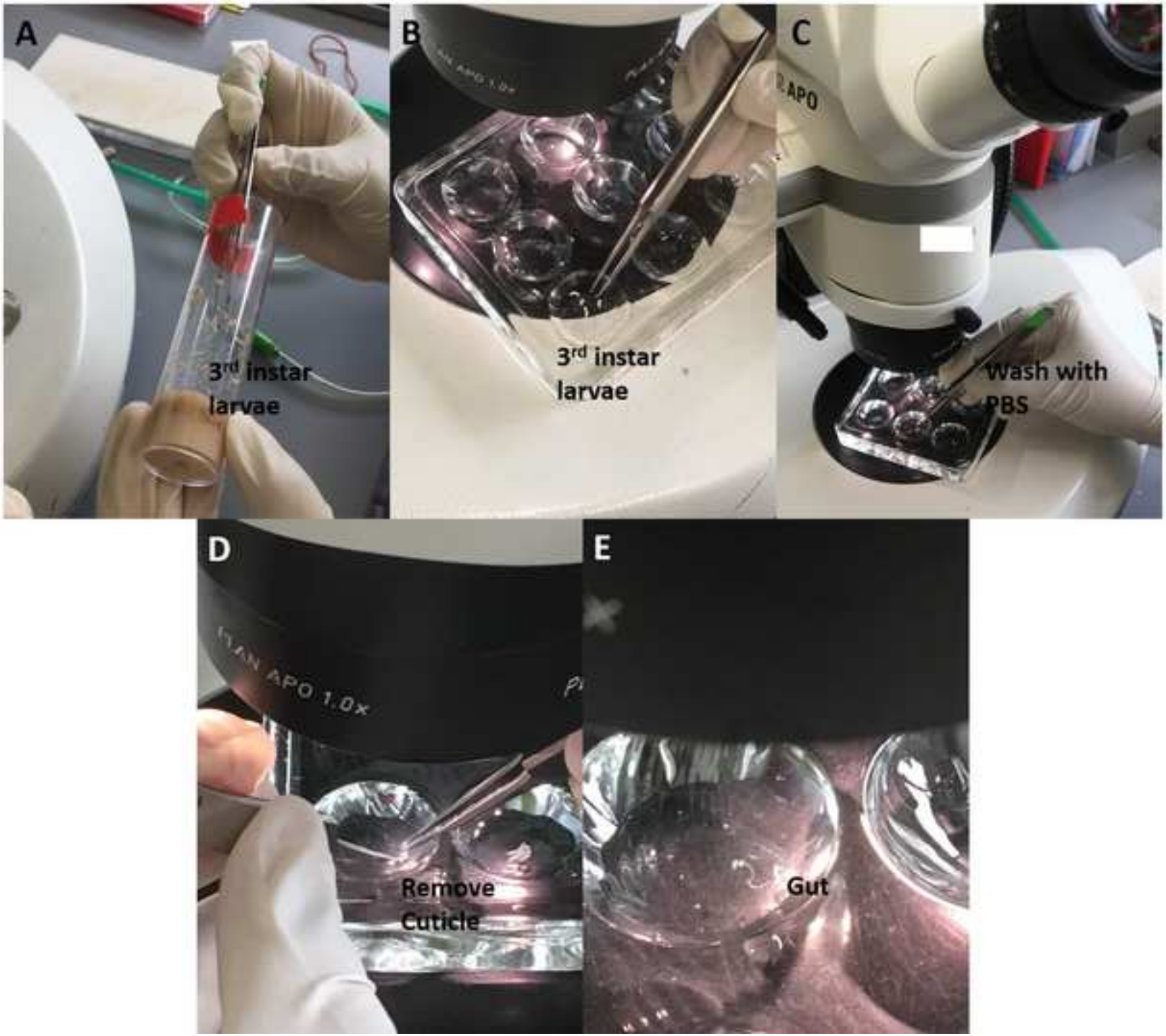

Figure 5

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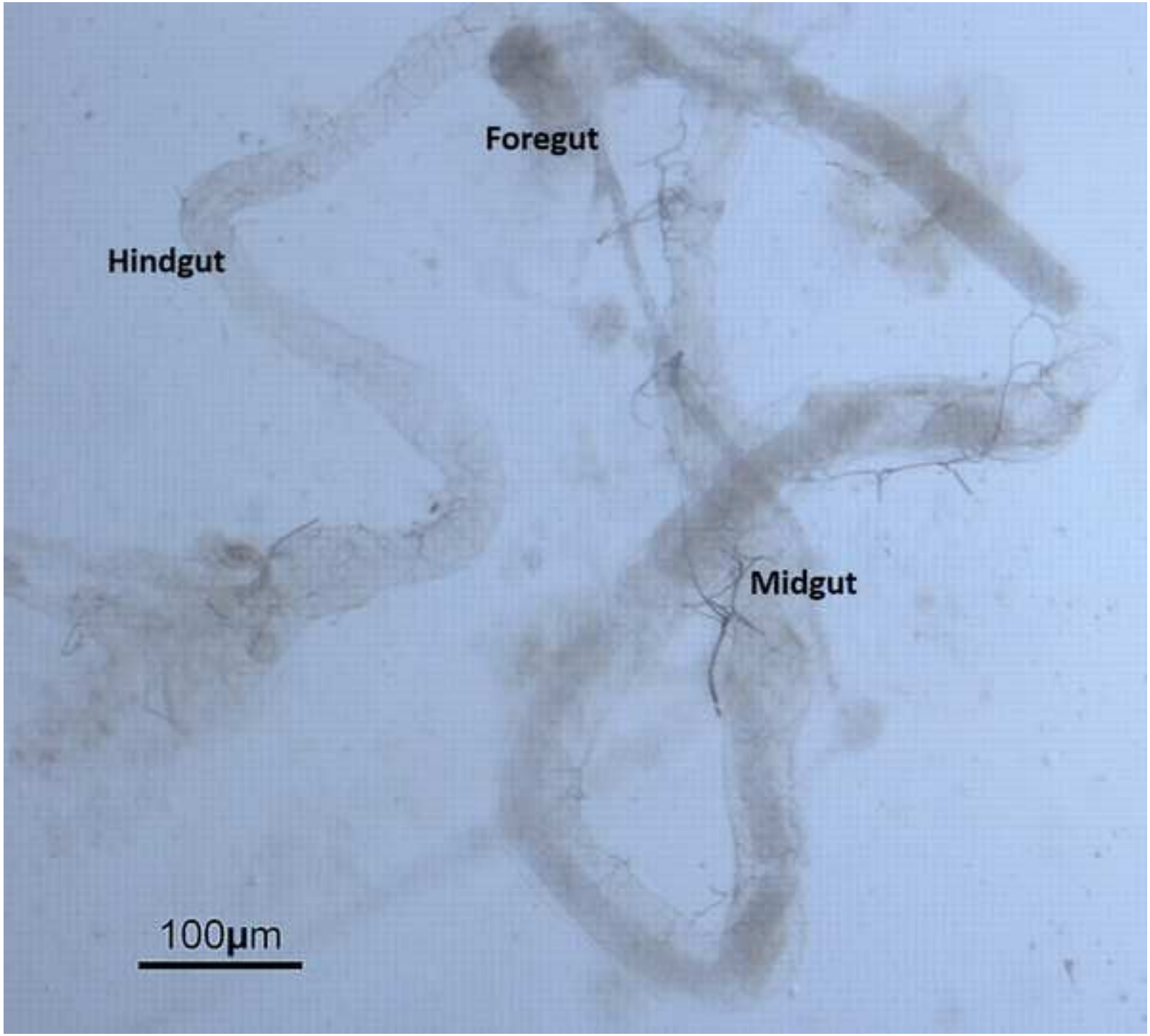

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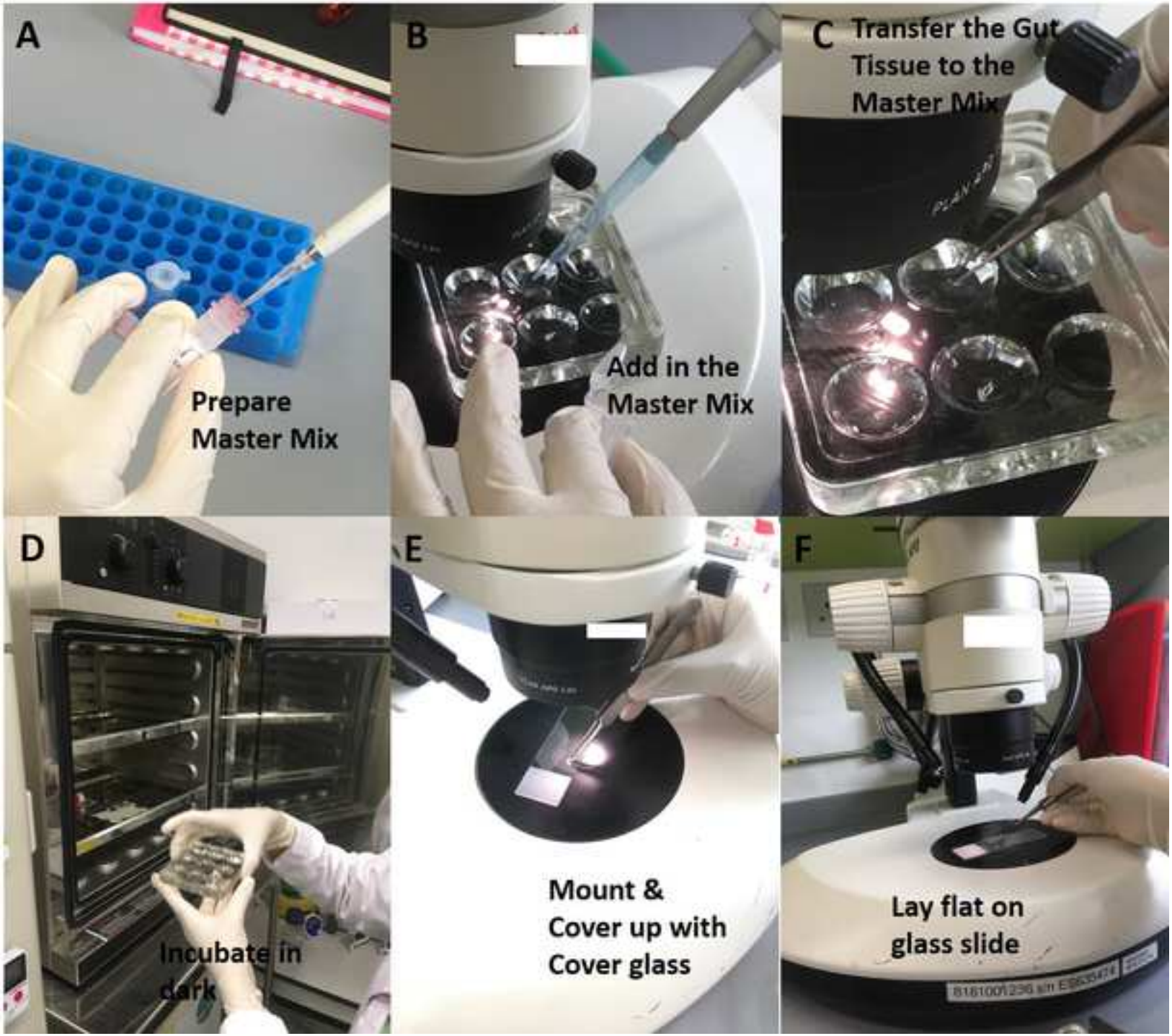

Figure 7

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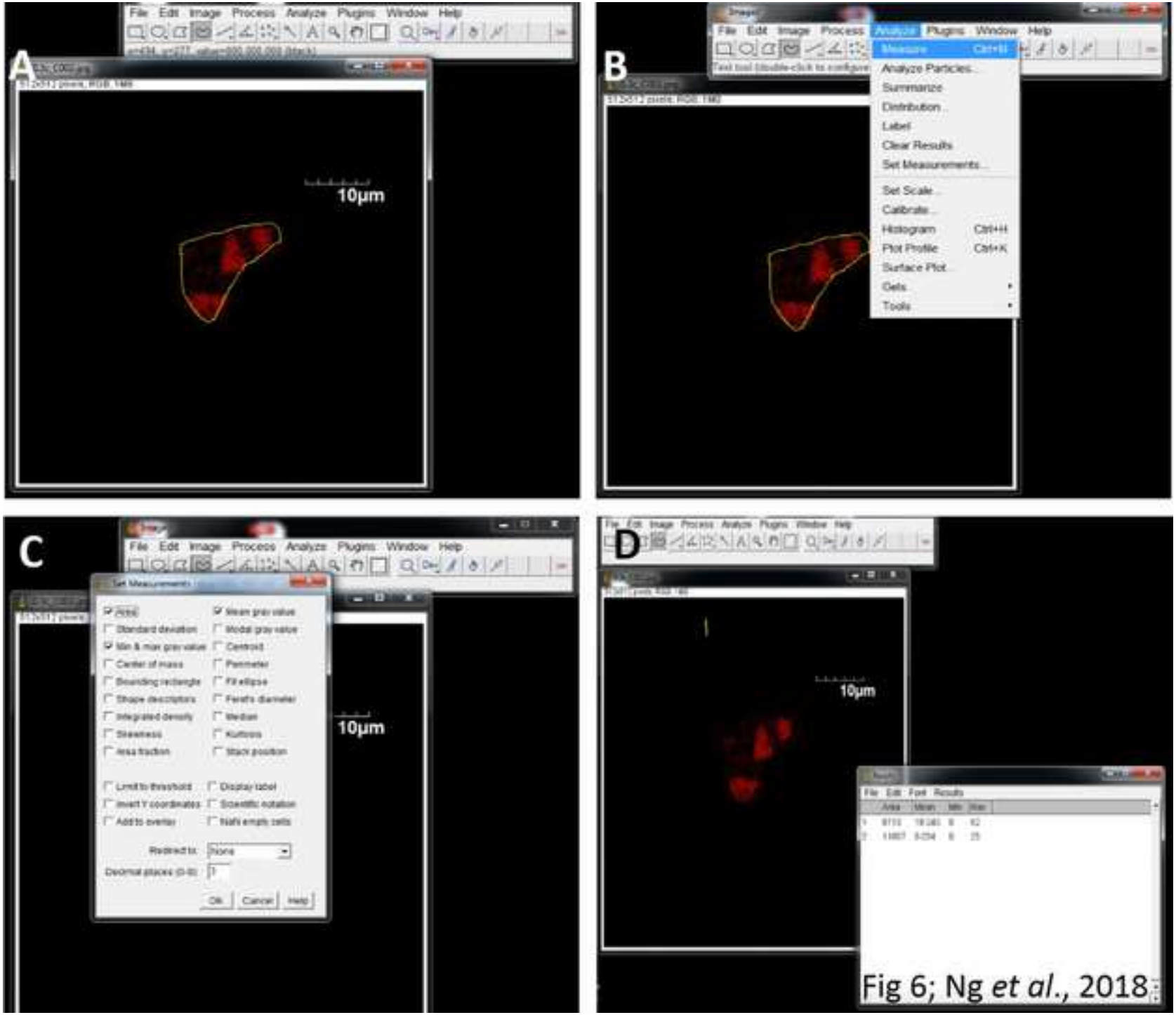

Name of Material/ Equipment	Company	Catalog Number
15% Methyl 4-Hydroxybenzoate	Sigma Aldrich	
4% Paraformaldehyde	Sigma Aldrich	P6148
Bacto Agar	BD biosciences	
cncCK6/TM3, Sb		
cornmeal, glucose, yeast brewer	Sigma Aldrich	
CyAn ADP with Summit Software	DAKO	
Dihydroethidium (Hydroethidine)	Thermo Fisher Scientific	D11347
FITC Annexin V Apoptosis Detection Kit I	BD biosciences	556547
Fluorescent microscope	Olympus	
Glucolin	Supermarket	
Image J software	NIH	
MRC5 human lung fibroblast	ATCC	CCL-171
Schneider's *Drosophila* medium	Thermo Fisher Scientific	21720-024
vectashield antifade mounting medi	Vector Laboratories	H-1200
wild- type *Canton-S; Sod2N308/CyO*	NIG-FLY	
Zinc Oxide Nanoparticles	Sigma Aldrich	721077

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Product Specification

Product Name:
Zinc oxide, dispersion – nanoparticles, <100 nm particle size (TEM), ≤40 nm avg. part. size (APS), 20 wt. % in H_2O

Product Number: **721077**
CAS Number: 1314-13-2

ZnO

TEST	Specification
Appearance (Color)	Off-White to Tan
Appearance (Form)	Conforms
Dispersion	
ICP Major Analysis	Confirmed
Confirms Zinc Component	
Concentration	18 - 22 % wt
% ZnO	
Size	< 100 nm
pH	6.0 - 9.0

Specification: PRD.2.ZQ5.10000005883

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Signature:	
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June 26, 2019

Bing Wu
Science Editor
JoVE

Re: JoVE59510R1 - [EMID:fb7538a54fbcab42]

Dear Dr. Bing Wu,

Please find our revised manuscript entitled "Toxicity study of Zinc Oxide Nanoparticles in cell culture and in *Drosophila* melanogaster" for publication in JoVE.

Your decision letter requested us to revise our manuscript in accordance with the comments made by the editorial team and to submit a revised version of the manuscript. We thank you and the editorial team for the insightful comments, which greatly improved our manuscript. In our response to that, we have made significant changes to the manuscript by rephrasing sentences to avoid the textual overlap from our previous publication. We hope that our revised manuscript addresses his/her concerns and that it is now suitable for publication.

Thank you very much for your consideration.

Sincerely,
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Yong Loo Lin School of Medicine
National University of Singapore
Singapore
Tel: +65 65167973
E-mail: antbgh@nus.edu.sg

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The manuscript has been modified and the updated manuscript, 59510_R3.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: The manuscript has already been sent twice for rigorous editing service to improve the readability.

2. Please add a short description of the figure in Figure Legend.
Response: As pointed out by the editorial team, we have included a short description of the figure in Figure Legend.

3. Figure 5: Please use μm instead of um.
We have used μm instead of um in Figure 5.

4. Step 3.1: Please write this step in the imperative tense.
Response: As suggested, we have revised step 3.1 and changed it to imperative tense.

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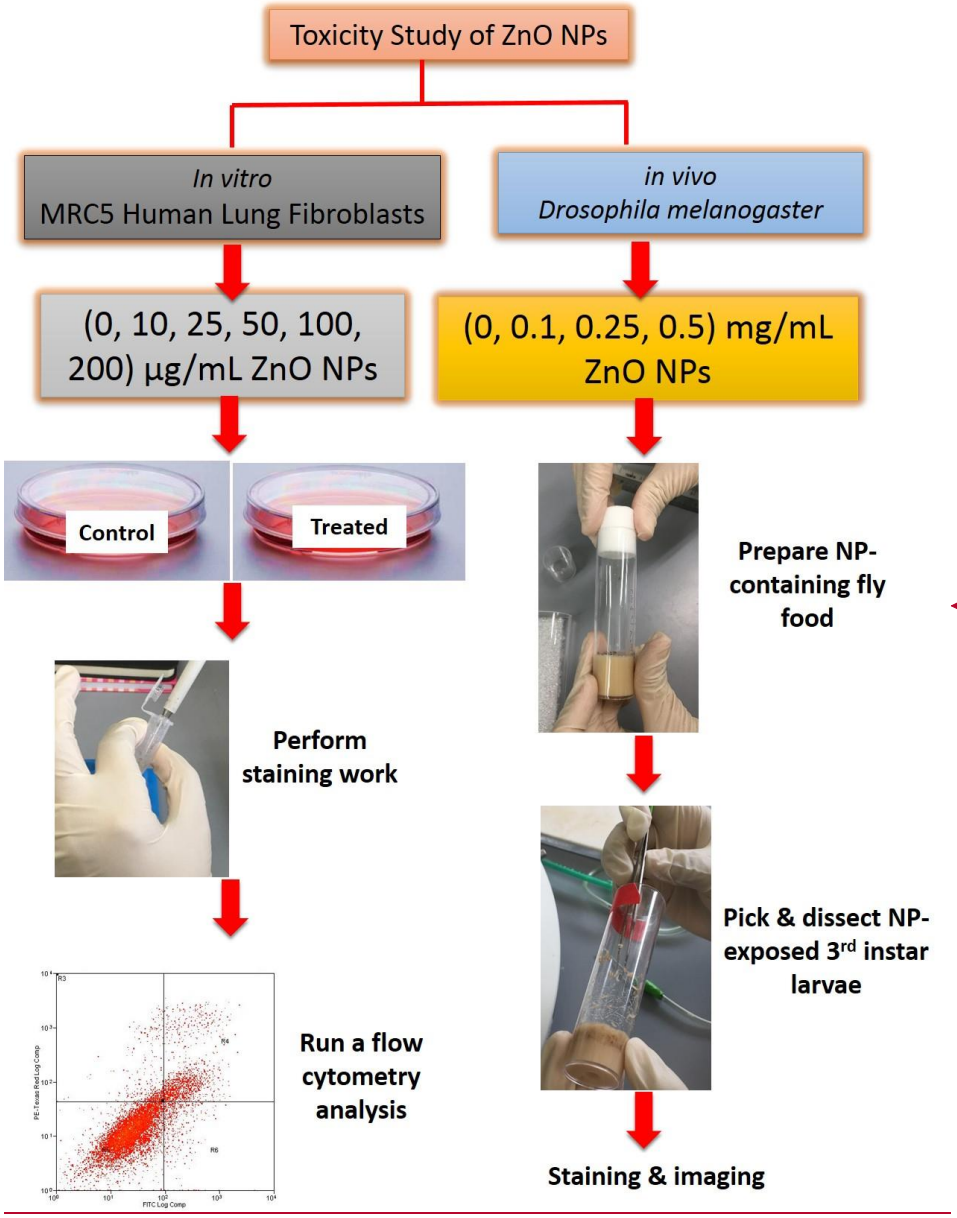

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Figure 1 **Overview of the method used for toxicity study of ZnO NPs.** For *in vitro* work, ZnO NP-treated cells were stained prior to flow cytometry analysis. For *in vivo* work, gut was dissected from 3rd instar larvae, followed by staining with DHE dye and image acquisition.

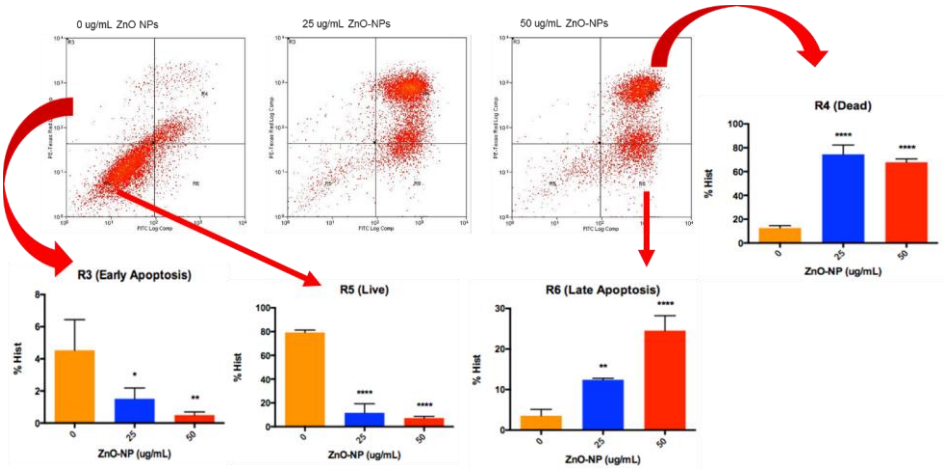

Figure ~~1~~ 2 **Dot plot of cells separated into different populations based on their FITC and PE staining.** The pictograms show~~s~~ the results of FITC/Annexin V assay on 24 h treatment of ZnO-NPs on MRC-5. ~~Dot plot of cells separated into different populations based on their FITC and PE staining.~~ Statistical analysis of the cells at different stages can then be performed.

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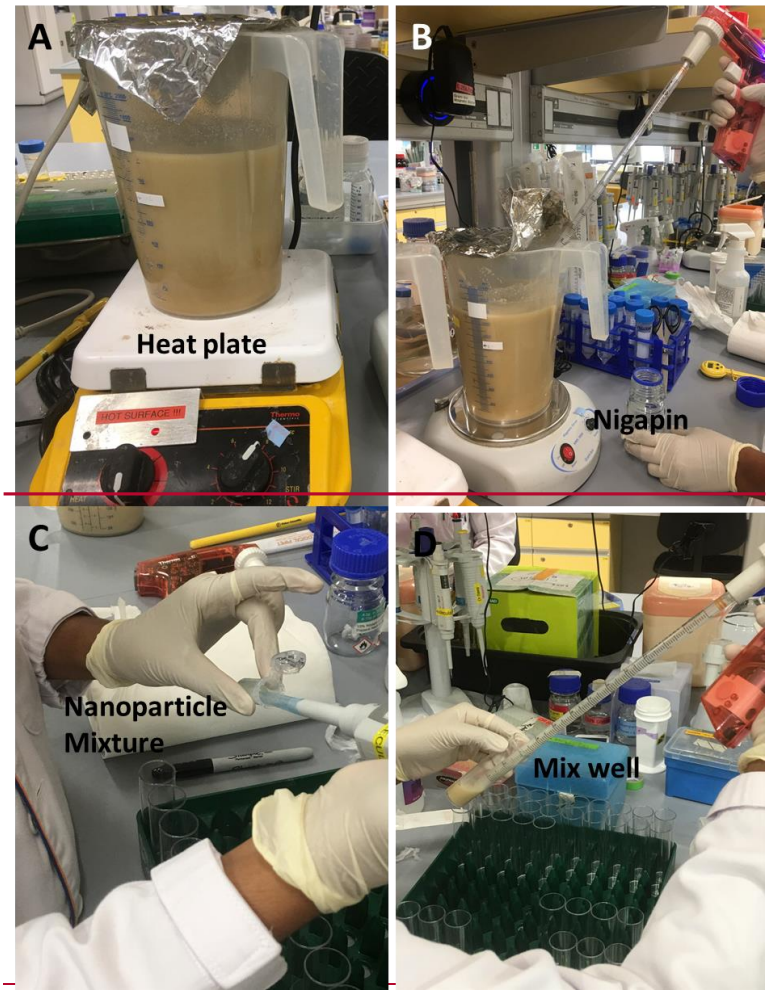
A
Heat plate
B
Nigapin
C
Nanoparticle
Mixture
D
Mix well

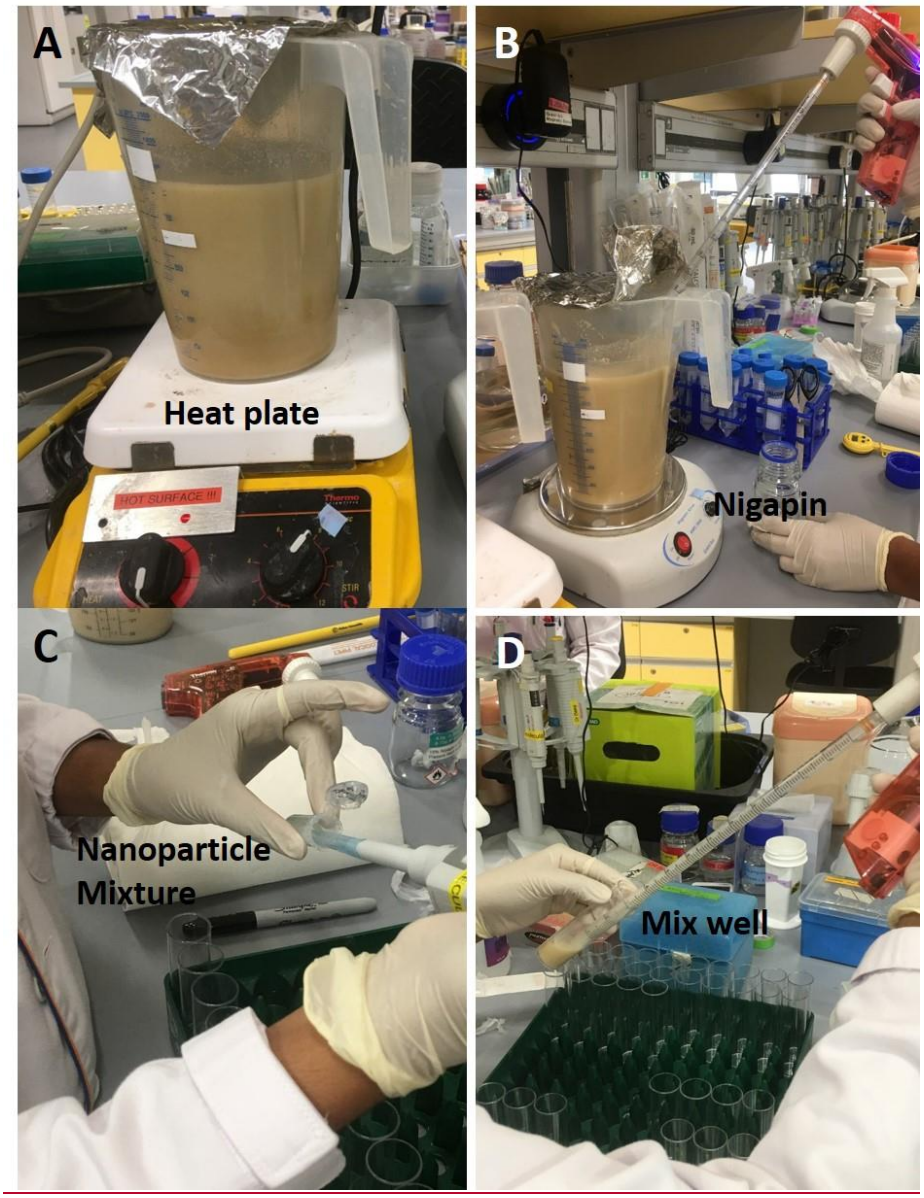

***Figure ~~2~~ 3* Preparation of ZnO NP-containing fly food medium.** A) Ingredients for fly food were added to water and allowed to swell, and boiled for 5 min. B) After cooling down to 50 °C with

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stirring from time to time, Nipagin was added and mixed thoroughly. C) Prepare a master mix for the nanoparticles (total volume not exceeding 10% of the final food volume). D) Medium is then aliquoted, mixed with ZnO NPs at various concentrations and allowed to cool down completely before storage.

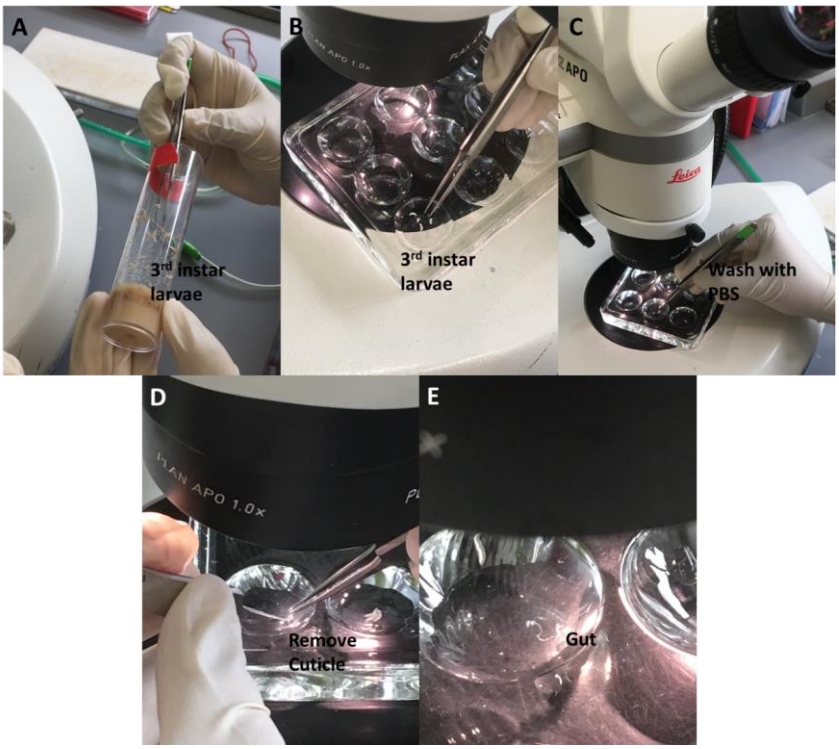

***Figure ~~3~~ 4* The whole gut dissection procedure.** A) Transfer 3rd instar larvae to a dissection disc. B) Use forceps to gently hold a larva, and C) wash away the remnant of food using saline. ~~C~~D) Gently tear the cuticle apart without touching the gut and other internal organs. E~~D~~) Place the gut into the saline for subsequent procedure.

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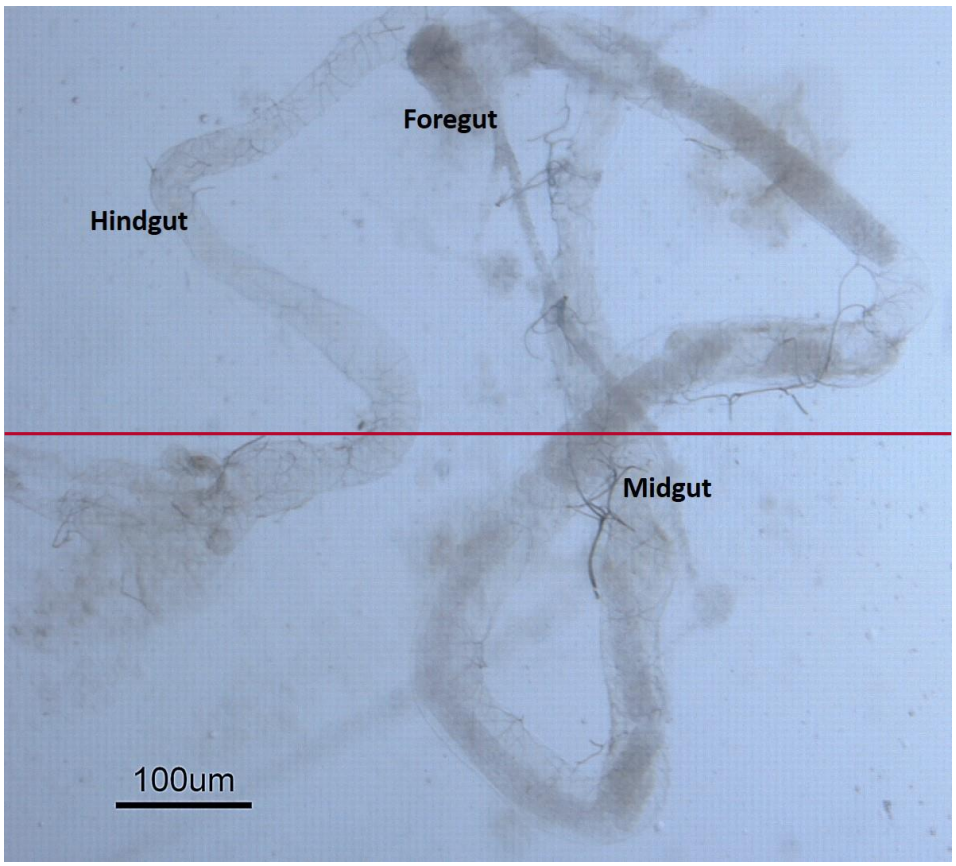
Foregut
Hindgut
Midgut
100um

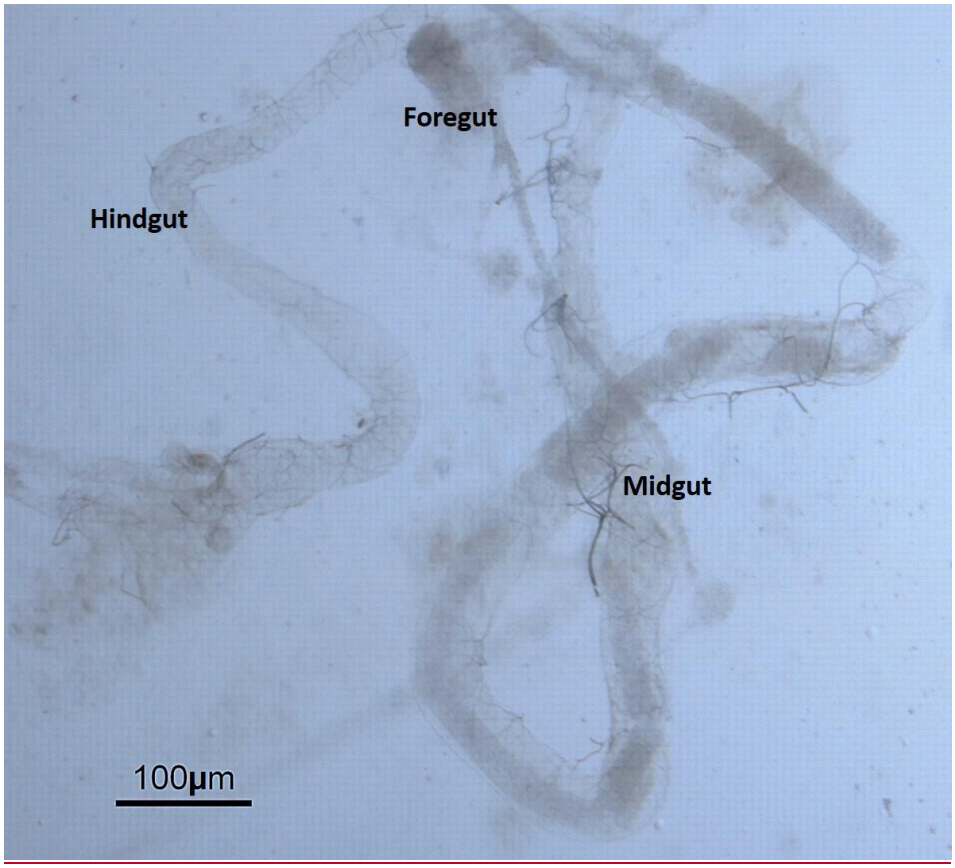

***Figure 4-5* The anatomy of the digestive tract/gut.** The gut extracted from the *Drosophila* larva is divided into three discrete domains of different developmental origin namely the foregut, midgut, and hindgut.

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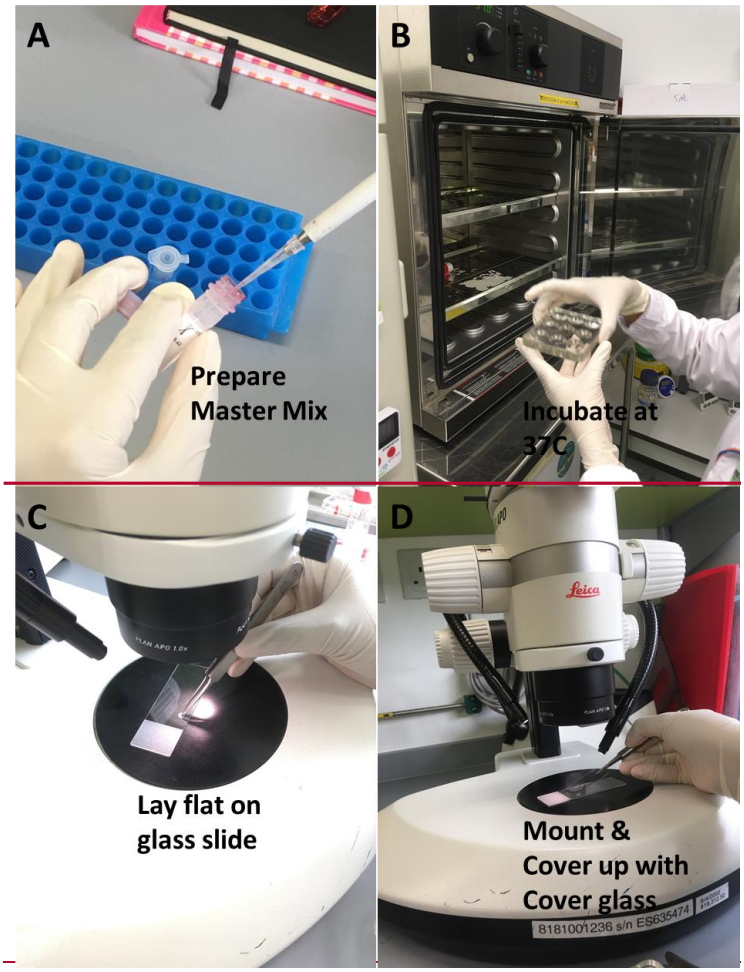
A
Prepare
Master Mix
B
Incubate at
37C
C
Lay flat on
glass slide
D
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Cover glass
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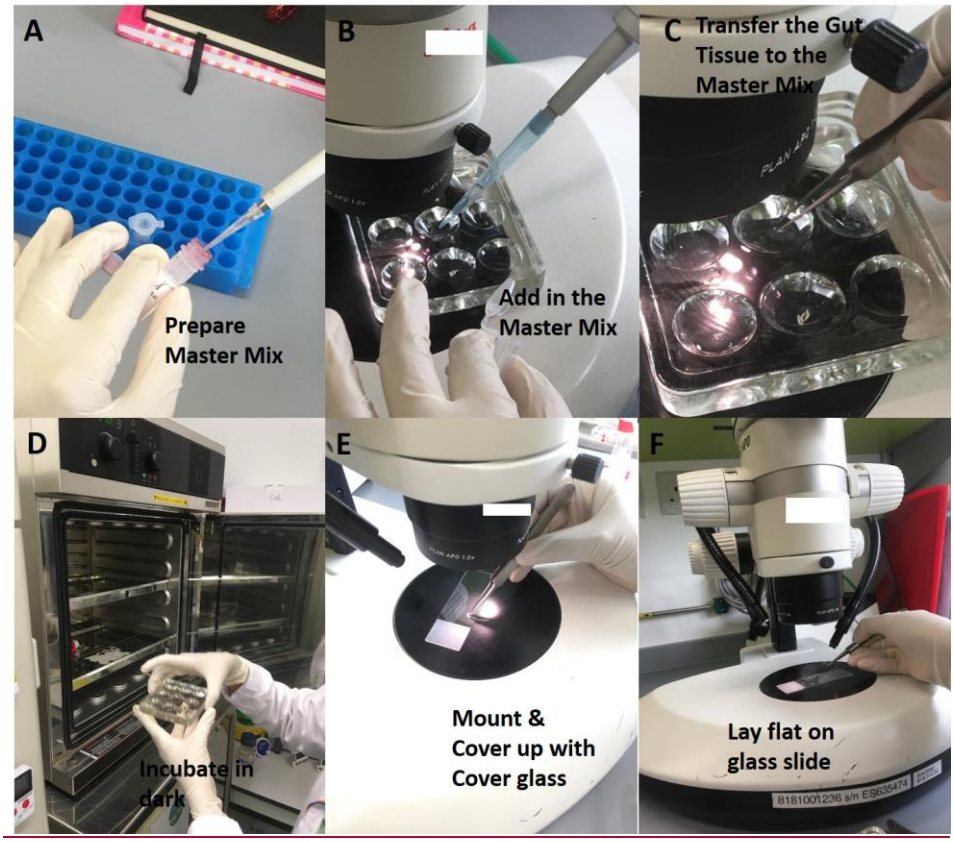

***Figure ~~5~~ 6* Staining of the gut tissue with DHE.** A) Prepare a master mix containing growth medium and DHE (to a final concentration of 30 µ~~u~~M). B) Add the master mix into the well of a dissection disc. C) Transfer the dissected gut tissue into the well containing the DHE master mix. D) Incubate at ~~37°C~~ RT for ~~30~~ 5 min and protect the tissue from light; wash 3x 5mins interval, in PBS/saline. ~~C~~E) Fix in 4% PF for 10 min; wash the tissue thrice with PBS (optional). ~~D~~F) Gently transfer the gut onto a glass slide, lay flat without having any tissue folded and mount with mounting medium before covering with cover glass.

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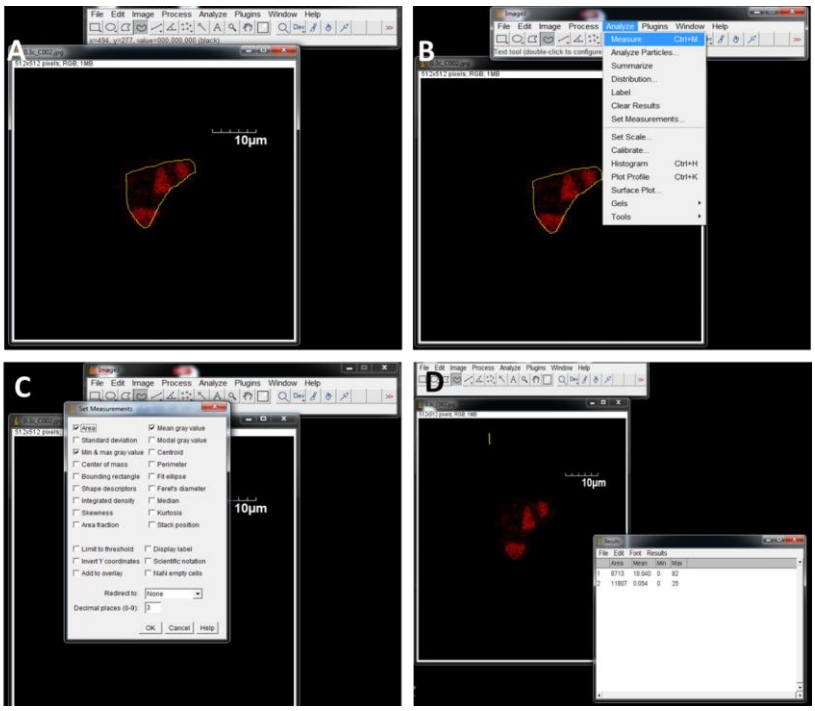

***Figure 6 7* Quantitation of fluorescent images using ImageJ.** A) Import the captured images. B) Click on the Analyze menu and select "set measurements". C) Select the area integrated intensity and mean grey value. Select a region without fluorescence to set the background. D) Export the data into Excel and calculate the CTCF for subsequent statistical analysis.

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