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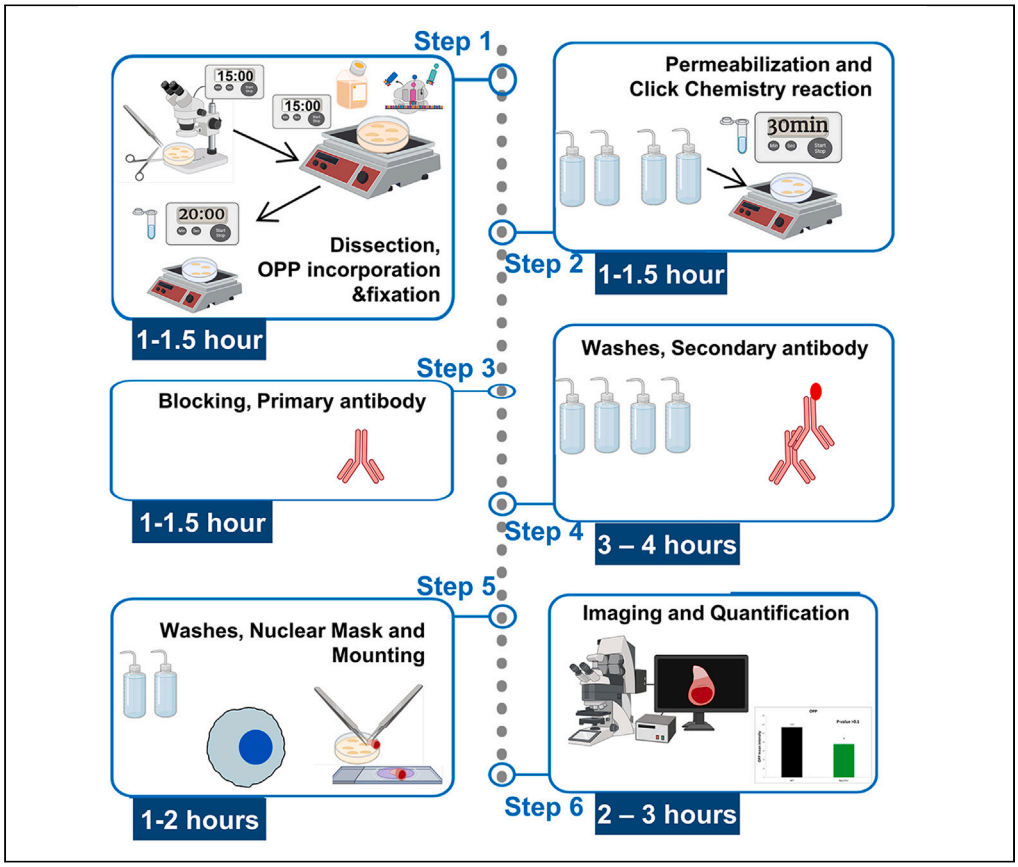
# Protocol for assessing translation in living *Drosophila* imaginal discs by O-propargyl-puromycin incorporation




Translation is a fundamental process of cellular behavior. Here, we present a protocol for measuring translation in *Drosophila* epithelial tissues using O-propargyl-puromycin (OPP), a puromycin derivative. We detail steps for larval dissection, OPP incorporation, fixation, OPP labeling, immunostaining, and imaging. We also provide details of quantification analysis. Significantly, OPP addition to methionine-containing media enables polypeptide labeling in living cells. Here, we study wing imaginal discs, an excellent model system for investigating growth, proliferation, pattern formation, differentiation, and cell death.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Visualizing protein synthesis in *Drosophila* tissues by click-based OPP labeling

Compare translation rates of different genotypes within mosaic *Drosophila* tissues

OPP incorporation can be combined with immunostaining

OPP incorporation into newly synthesized proteins in live epithelial cells

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# Protocol for assessing translation in living *Drosophila* imaginal discs by O-propargyl-puromycin incorporation

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## SUMMARY

**Translation is a fundamental process of cellular behavior. Here, we present a protocol for measuring translation in *Drosophila* epithelial tissues using O-propargyl-puromycin (OPP), a puromycin derivative. We detail steps for larval dissection, OPP incorporation, fixation, OPP labeling, immunostaining, and imaging. We also provide details of quantification analysis. Significantly, OPP addition to methionine-containing media enables polypeptide labeling in living cells. Here, we study wing imaginal discs, an excellent model system for investigating growth, proliferation, pattern formation, differentiation, and cell death.**

**For complete details on the use and execution of this protocol, please refer to Lee et al. (2018), Ji et al. (2019), and Kiparaki et al. (2022).[1–3]**

## BEFORE YOU BEGIN

1. Ensure that all the necessary buffers for the protocol are freshly prepared. These buffers include 1× PBS, Schneider medium supplemented with serum, fixatives, blocking agents, and antibody solutions.
2. It is recommended to conduct control experiments in parallel to the main experiments to ensure reproducibility of the OPP protocol. For example, in our original manuscript we used cycloheximide as negative control to confirm that OPP incorporation in cells of the imaginal discs depends on active translation.[1] In addition, we confirmed that cells with reduced levels of Tor, myc or eIF2a activity present reduced OPP incorporation. In that work, we showed that cells that have heterozygous mutations in ribosomal protein genes ($Rp^{+/-}$ cells) have reduced OPP incorporation compared to wild-type cells. Another negative control that we suggest is a sample without OPP, to distinguish the OPP signal from background cytoplasmic staining.

   *Note:* Although in this study we used the *Drosophila melanogaster* third instar larvae wing imaginal discs for OPP staining, this protocol can be used for other types of imaginal discs and tissue as well.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse anti-β-galactosidase (mAb40-1a) | DSHB (1:100) | RRID: AB_2314509 |
| Chemicals, peptides, and recombinant proteins | | |
| Sodium chloride | Sigma | S7653 |
| Fetal bovine serum, certified, heat inactivated, United States | Gibco | 10082139 |
| Schneider's *Drosophila* medium | Gibco | 21720024 |
| $Na_2HPO_4$ | Sigma | S5136 |
| $NaH_2PO_4$ | Sigma | S9638 |
| Pipes | Sigma | P1851 |
| EGTA | Sigma | E3889 |
| $MgCl_2$ | Sigma | M9272 |
| Triton X-100 | Merck | T8787 |
| BSA | Sigma | AB022 |
| Critical commercial assays | | |
| Click-iT Plus OPP Alexa Fluor 594 Protein Synthesis Assay Kit | Thermo Fisher Scientific | C10457 |
| Click-iT Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit | Thermo Fisher Scientific | C10456 |
| Experimental models: Organisms/strains | | |
| *D. melanogaster* mutant for *RpL27A* Df(2L)M24F11 | Bloomington Drosophila Stock Center | Flybase: FBab0001492 |
| Software and algorithms | | |
| ImageJ | Schneider et al.[4] | https://imagej.nih.gov/ij/ |
| BioRender | | https://www.biorender.com |
| Other | | |
| PYREX 9 depression glass spot plate | Corning | 7220-85 |
| VECTASHIELD | Vector Labs | H-1000 |
| Dumont #5: fine forceps | Fine Science Tools | 11254-20 |
| Student Vannas spring scissors | Fine Science Tools | 91500-09-(1) |
| PYREX Spot Plates | Fisher Scientific | 13-748B |

***Note:*** For our experiments, we have used the Click-iT Plus OPP Alexa Fluor 594 (or 488) Protein Synthesis kit from Thermo Fisher (https://www.fishersci.com/shop/products/moleular-probes-click-it-plus-opp-alexa-fluor-594-protein-synthesis-assay-kit/C10457).

## MATERIALS AND EQUIPMENT

| **Click reaction** | | |
|---|---|---|
| Reagent | Dilution | Amount |
| Click-iT OPP Reaction Buffer (Component C)* | 1:11 | 88 μL |
| Copper Protectant (Component D)** | 1:50 | 20 μL |
| Click-iT Reaction Buffer Additive (Component E)*** | 1:100 | 10 μL |
| Alexa Fluor picolyl azide (Component B) (Alexa) | 1:40–1:50 | 2–2.5 μL |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Dilution | Amount |
| $ddH_2O$ | N/A | ~880 μL |
| | Total | 1 mL |

Note on storage conditions:
*According to manufacturer guidelines the 1× Click-iT OPP Reaction Buffer is prepared by adding the 4 mL of Component C to 36 mL deionized water (1:10 dilution). This 1× Click-iT OPP Reaction Buffer is then recommended to be stored at 4°C and remains stable for up to 6 months. However, we opted to simplify the process by directly dissolving Component C into the final Click reaction mix. By adopting this approach, Component C can be stored in an undiluted form at 4°C, extending its usability.
**We have noticed that Component D is the first component of the kit to be exhausted.
***Upon dissolving Component E in deionized water (total volume of 2 mL), dispense the solution into 40 aliquots of 50 μL each (this is the 10× solution of Component E). Store the aliquots at −20°C and thaw one aliquot just prior to use. The thawed aliquot of Component E should be stored in 4°C and used up to 3–4 days, until the color of the solution turns yellowish. Alternative, you may choose to aliquot the solution into even smaller volumes. Notice that: In the provided instructions, the manufacturer suggests preparing the 1X Click-iT OPP Reaction Buffer Additive by diluting the 10X solution of Component E at a ratio 1:10 in deionized water. It is advised to prepare the 1× solution fresh and use it on the same day. However, we have omitted this intermediate dilution by directly dissolving the 10× solution of Component E to prepare the Click-iT Plus reaction cocktail. As a result, the dilution of Component E in our final Click reaction stands at 1:100, differing from the recommended 1:10 ratio outlined in the manufacturer's instructions final reaction mix.

| **10× PBS solution** | |
|---|---|
| Reagent | Final concentration |
| NaCl | 1.3 M |
| $Na_2HPO_4$ | 70 mM |
| $NaH_2PO_4$ | 30 mM |
| Note: pH 7.4, store at room temperature (RT) for up to 1 year.<br>Diluted 1× PBS should be stored at 4°C | |

| **2× PEM buffer** | |
|---|---|
| Reagent | Final concentration |
| Pipes | 200 mM |
| EGTA | 2 mM |
| $MgCl_2$ | 2 mM |
| Note: pH 6.9 by KOH. Sterilize by filtering. Storage at 4°C for up to 6 months. | |

| **1× PBT buffer** | |
|---|---|
| Reagent | Final concentration |
| PBS solution | 1× (dilute the 10× PBS solution 1:10) |
| Triton X-100 | 0.2% |
| BSA | 0.5% |
| Note: Sterilize by filtering. Storage at 4°C for up to 6 months. | |

Fixative solutions should be prepared fresh prior to fixation.

- **Option A:** 4% Formaldehyde Solution (FA, Pierce, Cat #28908) diluted in 1× PEM buffer.
- **Option B:** 4% Formaldehyde Solution (FA, Pierce, Cat #28908) diluted in 1× PBS.

## STEP-BY-STEP METHOD DETAILS

### Dissection, OPP incorporation, and fixation—Day 1

Timing: 1–1.5 h

In this step, the larvae are inverted to make the imaginal discs accessible to the labeling medium, which is supplemented with O-propargyl-puromycin (OPP). The labeling time is short (only 15 min), but sufficient to allow OPP incorporation into newly synthesized proteins. Following the OPP labeling, the *Drosophila* tissues are fixed.

1. To begin, transfer the flies to a fresh vial and allow them to lay eggs for one day. Depending on the genotype, proceed with the dissection of third instar larvae after 4–5 days which marks Day 1 of this Protocol.

   ***Note:*** The dissection time depends on the developmental growth of the flies. For example, Minute flies require more days (1–2) to reach the same developmental stage compared to wild-type files.

   ***Note:*** During this period, it is possible to generate clones by utilizing heat shock inducible flippase and FRT recombination.

2. Dissection.
   a. Collect wandering third instar larvae of the desired genotype from the fly food and wash them twice with 1× PBS in a 9-well glass plate.

   ***Note:*** This is important to remove any excess of food particles that may be adhered to their cuticles and may interfere with the subsequent reactions.

   ***Note:*** 1× PBS is freshly prepared using a 10× PBS solution.

   b. Transfer the clean larvae (usually 20 in number) to a new well containing Schneider medium (~500 μL) which is supplemented with 10% FBS serum (Schneider/FBS medium).

   ***Note:*** FBS is important to ensure that the larvae have the appropriate nutrients and growth factors to support survival and growth of the tissues during the protocol.

   c. Using a pair of forceps dissect the larvae.
      i. Grab the mouth hooks of the larvae and with a microdissection spring scissors cut and remove the posterior one-third of the larvae (usually around 20 larvae).
      ii. Invert dissected larvae using forceps.
      iii. Remove quickly the largest portion of unrelated tissues (i.e., fat body, salivary glands, gut).

   ***Note:*** Complete this step within 10–15 min of initial dissection, at room temperature (RT, temperature range between 22°C and 25°C).

   ***Note:*** Practice may be required to invert the larvae fast enough. Untrained users can refer to published videos demonstrating dissections of wing and eye imaginal discs.[5,6]

3. OPP incorporation.
   a. Remove the medium used during dissection and add fresh Schneider/FBS medium supplemented with Component A.

   ***Note:*** The concentration of Component A is in the range of 10–20 μM, which corresponds to a dilution of 1:1,000–1:2,000.

   b. Gently shake the samples for 15 min at RT, at speed 100 rpm.

   ⚠ CRITICAL: OPP becomes covalently attached to the nascent amino acid chains and blocks further elongation. Longer incubation time should be avoided due to potential toxicity,

and additionally since it will increase the dependence of the OPP signal on turnover rate of the truncated polypeptides. Differences in proteasome function between samples could then affect the signal as much as differences in translation rate.[4,7]

4. Fixation.
   a. Wash the samples once with 1× PBS and immediately proceed to sample fixation.

   *Note:* For sample fixation we use freshly prepared 4% Formaldehyde Solution (FA, Pierce, Cat #28908) diluted either in PEM buffer(100 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$) or 1× PBS for 20 min at RT.

   ⚠ CRITICAL: Follow safety measures when handling formaldehyde, since it is highly toxic. Wearing gloves and working in a chemical fume hood are good safety practices to prevent accidents.

### Permeabilization and click-chemistry reaction—Day 1

Timing: 1–1.5 h

5. Permeabilization and Click chemistry reaction.
   a. Wash the samples twice with 1× PT (1× PBS, 0.2% Triton X-100).
   b. Wash with 0.5% Triton X-100 in 1×PBS for 10 min.

   *Note:* At this point, thaw the Click Components.

   c. Block with 3% BSA in 1× PBS for 10 min.

   *Note:* While blocking with 3% BSA, prepare the Click reaction mix according to the proportions in the Materials section.

   *Note:* The mix should be used within 10 min.

   d. Remove the blocking solution and add 300–400 μL of Click reaction mix to each sample in the 9-well glass plate.
   e. Incubate the samples in the dark at RT, on a shaker, for 30 min.
   f. Remove reaction mix and wash once with the Rinse buffer (component F).
   g. Wash with 3% BSA in PBS for 2 min.

   *Note:* If there are no primary antibodies, proceed to NuclearMask staining (step 8).

### Blocking and primary antibody incubation—Day 1

Timing: 1–1.5 h

6. Blocking and Primary Antibody Incubation.
   a. Block 1 h with PBT (1× PBS, 0.2% Triton X-100, 0.5% BSA).

   *Note:* Prepare primary antibody at proper dilution (e.g., mouse anti-b-gal mAb40-1a dilution used 1:100) in PBT

   b. Incubate in primary antibody overnight (12–16 h) on shaker (4°C–8°C).

### Secondary antibody incubation—Day 2

Timing: 3–4 h

7. Secondary Antibody Incubation.
   a. Wash the samples thrice with PT (1× PBS + 0.2% Triton X-100), 5–10 min for each wash.
   b. Add secondary antibody.

   *Note:* Depending on the Alexa Fluor dye of the Click reactions use the appropriate secondary antibody to avoid overlap with the OPP signal in the same channel. For example, if using 594 picolyl azide, the secondary antibody could be Alexa 488 conjugated. For 488 picolyl azide use 555 or 647 conjugated secondary antibody diluted in PBT.

   c. Incubate the samples on shaker for 2–3 h in the dark at RT.
   d. Wash the samples thrice with PT (1× PBS + 0.2% Triton X-100), 5–10 min for each wash.

### NuclearMask staining and mounting—Day 2

Timing: 1–2 h

At this step we label nuclei using the NuclearMask component of the kit, before we proceed to sample mounting. It is important to include NuclearMask staining since OPP labeled proteins are released from ribosomes and localize to various subcellular compartments. Translational rates are determined by the signal intensity in the ring around or within the nucleus identified by NuclearMask.

8. NuclearMask Staining.
   a. Wash once with 1× PBS.

Add NuclearMask solution into the samples and incubate them for 30 min at RT in the dark on a shaker.

*Note:* Dissolve NuclearMask solution (Component G) in 1× PBS (1:2,000)

⚠ CRITICAL: NuclearMask Blue Stain is a known mutagen and therefore appropriate precaution is required.

9. Mounting imaginal discs.
   a. Dissect imaginal discs of interest (e.g., wings, eye) from the inverted larva body and mount them on slides by using VECTASHIELD mounting medium. Seal the edges of the coverslip with clear nail polish.

   *Note:* 14 µL is sufficient for 24 × 24 mm coverslips.

10. Proceed to imaging or store slides at 4°C or at −20°C (for longer periods) prior to imaging.

### Imaging and analysis—Day 3

Timing: 2–3 h

11. Image the samples under a confocal microscope, using the appropriate channels.

    *Note:* Images were taken using 40× oil immersion objective.

*Note:* The intensities of all the lasers and detectors were set to avoid excessive or weak signal. The Quick LUT feature in the Leica software was used to assess the intensity saturation and optimizing the settings for the image capturing.

12. Save all images in the original format as well as any other format appropriate for subsequent analysis. For Image J quantification, .tif images of a single section were used. For details, check the "quantification and statistical analysis" section below.

## EXPECTED OUTCOMES

OPP was originally used as a click chemistry reagent for fluorescent labeling of nascent protein synthesis in tissue culture cells (Liu et al. 2012).[8] OPP labeling is detected both in the cytoplasm and in the nucleus. If we aim to compare nascent protein synthesis between different cells in mosaic tissues or between different genotypes, it is important to determine the signal intensity at the level where nuclei are present in both cell types. Comparison of cytoplasmic labeling in one cell type with nuclei labeling in another cell type can lead to inaccurate conclusions. Importantly, control experiments should be done in parallel. For example, in the original paper (Lee et al., 2018) where we first used OPP to measure bulk translation in *Drosophila* mosaic wing imaginal discs, we showed that OPP incorporation is reduced in cells that have mutations in genes that affect translation, including Tor, myc and Gadd34 (Figure 4 and S4 in[1]). By using this protocol we confirmed that cells that have heterozygous mutations in ribosomal protein genes ($Rp^{+/-}$ cells) have reduced bulk translation rates compared to wild-type cells. Unexpectedly, we showed that the transcription factor Xrp1, and not a reduced number of ribosomal subunits, was responsible for the reduced translation in the $Rp^{+/-}$ cells. This finding then led to the discovery that Xrp1 reduced global translation through PERK- dependent phosphorylation of eIF2α, a key mechanism of global regulation of CAP-dependent translation.[3] Figure 1 shows a mosaic wing disc containing cells with heterozygous mutations in the ribosomal protein gene *RpL27A* ($RpL27A^{+/-}$ cells) and wild-type cells. $RpL27A^{+/-}$ cells (labeled green) present reduced OPP incorporation compared to wild-type cells. Control clones that differ only in the presence of the Ubi-GFP transgene do not present differences in OPP incorporation (Figures 1K–1Q). Notice that OPP labeling in imaginal discs from wild-type larvae is intrinsically patchy, likely as a consequence of the patchy activation of the Tor pathway (Figure 1M).[1,9]

## QUANTIFICATION AND STATISTICAL ANALYSIS

We recommend using the ImageJ analysis tool for quantification purposes of microscopic images. For Image J quantification, .tif images of a single section were used. For consistent and reliable comparison between different tissues, imaging should be performed with the same confocal settings, such as laser power, gain, and objective. For quantification of OPP signal, the .tif image of a single confocal section showing the overlap staining for both NuclearMask and β-gal was opened in the ImageJ software and multiple regions of interest (ROI) were drawn in each disc for each genotype (Figure 1E). We selected regions that contained nuclei for both genotypes and we avoided cytoplasmic regions. In our case we focus our analysis in the pouch region of the wing discs, but similar analysis can be performed for the other areas of the wing, such as the notum. For example, in the disc shown in Figures 1A–1D, by selecting the "Freehand selection" drawing tool we draw 4 ROIs for regions containing $RpL27A^{+/-}$ cells (labeled green) and 4 different ROIs for regions containing wild-type cells (unlabeled) (Figure 1E). By pressing Ctrl+T, we added each ROI in the ROI Manager tool. We opened the .tif image showing NuclearMask staining and transferred all saved ROIs from the ROI manager to the NuclearMask image (Figure 1F). By selecting each ROI separately and pressing Ctrl+M we calculated the mean fluorescence intensity of each ROI for the NuclearMask staining (Figure 1I). We verified that our areas contain similar percentage of nuclei by checking the mean intensity value of the NuclearMask fluorescence (Figure 1I). We perform similar analysis for the .tif image of the b-gal channel, to confirm the genotype of the areas. For example, in our case b-gal labels the $RpL27A^{+/-}$ cells. Therefore we confirm that the areas #1, #2, #3 and #4 contain $RpL27A^{+/-}$ cells, while areas #5, #6, #7 and #8 wild type cells (Figures 1G and 1I). Finally, we opened the .tif image

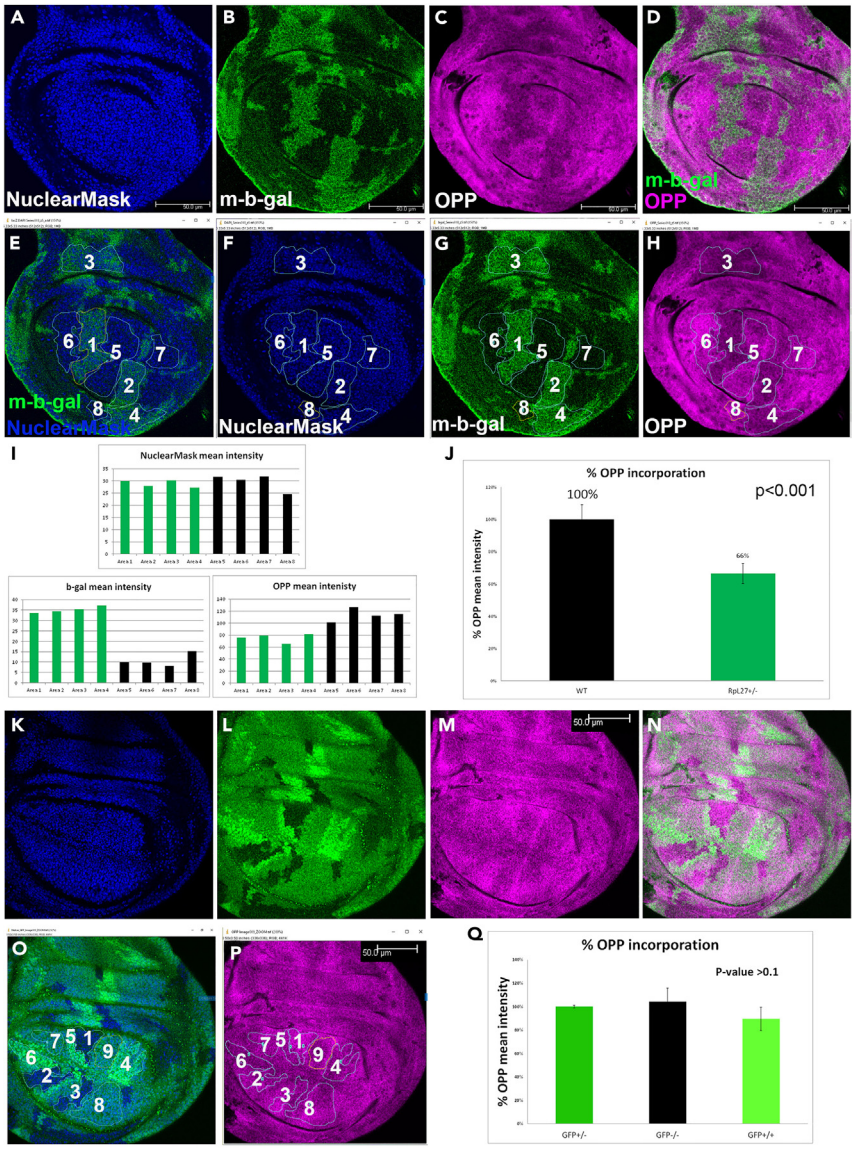


**Figure 1. Protein synthesis in mosaic wing imaginal discs**

(A–D) Protein synthesis in mosaic wing imaginal discs containing wild-type cells and *RpL27A*$^{+/-}$ mutant cells. *RpL27A*$^{+/-}$ mutant cells (labeled with m-b-gal, panel B) present reduced OPP incorporation compared to wild-type cells (unlabeled). Genotype: hsFLP; alz *RpL27A*$^{-}$ FRT40/FRT40.

(E–J) Regions of Interests were drawn to quantify the differences of OPP incorporation between wild-type cells and *RpL27A*$^{+/-}$ mutant cells. ($p < 0.001$ by Student's t test) Figure reprinted with permission from Lee et al., 2018.

(K–Q) Protein synthesis in mosaic wing imaginal discs containing control clones differing only in levels of the Ubi-GFP transgene. Cells do not present different levels of OPP incorporation ($p > 0.1$, one-way ANOVA) Genotype: hsFLP; UbiGPP FRT40/FRT40.

showing the OPP staining and by the same way we measured the MEAN value of OPP fluorescence for each ROI (Figures 1H and 1J). Using Microsoft Excel, we calculated the AVERAGE and the standard deviation (SD) of the genotypes. We performed statistical comparisons between *RpL27A* $^{+/-}$ mutant areas and wild type areas by Student's t-test. Similar analysis of neutral clones differing only in the presence of the Ubi-GFP transgene do not present different levels of OPP incorporation. We performed statistical comparison between double GFP positive clones (GFP$^{+/+}$), GFP negative clones (GFP$^{-/-}$) and the background unrecombined cells (GFP$^{+/-}$) by one way ANOVA test. Notice

that due to the inherent patchiness of the OPP signal in wing imaginal disc, in addition to the quantification, we find the visual comparison important.

## LIMITATIONS

OPP as a puromycin analog, is known to be cytotoxic, since it inhibits protein synthesis by disrupting peptide transfer and causing premature chain termination during translation. Therefore OPP labeling can lead to cell death and stress responses, which can interfere with the interpretation of the results. Minimize the labeling time in order to not affect cell viability or secondary effects.

In addition, we favor comparing translational cellular rates of distinct genotypes within the same tissue, using mosaic analysis generating tools, as opposed to comparing the translational rates of distinct genotypes from different imaginal discs. If it is necessary to compare cells from different imaginal discs, we recommend co-labeling control samples together with experimental samples. We use the OPP labeling of these control discs as a way to normalize OPP signaling between genotypes and ensure that the treatment of different samples is consistent and comparable.

We have observed that the translational rates vary depending on the developmental stage of the larva (unpublished observations).Therefore, researcher should be cautious to compare the translational rates between discs that may differ in their developmental stage. Our suggestion is co-staining with developmental markers (such as Senseless for the wing disc) to ensure that we are comparing the translational rates of distinct genotypes at the same developmental stages.

Importantly OPP incorporation in polypeptides leads to their release from the ribosome and diffusion over long distances from the site of synthesis, even after short OPP incubations.[10] Therefore, OPP labeling cannot faithfully detect the site of polypeptide synthesis.[10]

## TROUBLESHOOTING

### Problem 1

Black areas, indicating the absence of OPP signaling in those regions (step 2).

### Potential solution

The reason of the appearance of black areas in part of the tissues could be the mechanical damage of the tissue during dissection. Cells that are damaged during dissection do not incorporate OPP, resulting in the absence of signal in those areas. Additionally, we have noticed that dead cells (e.g., apoptotic), do not incorporate OPP.

### Problem 2

Uneven signal is detected in some areas of the imaginal discs (step 3).

### Potential solution

This could be true due to unequal access of Component A throughout the tissues. To avoid this, remove any larval tissues that are attached to the imaginal discs (such as the fat body and gut) and ensure that there is adequate shaking during the incubation period with Component A and the subsequent incubation procedures.

### Problem 3

Positive control experiment does not present differences in OPP incorporation.

### Potential solution

Ensure that all buffers are prepared according to the provided instructions.

Use multiple positive control experiments.

### Problem 4
NuclearMask staining is weak (step 8).

### Potential solution
Increase the concentration of the NuclearMask buffer. Use dilution 1:1,000, instead of 1:2,000.

### Problem 5
The quantification of OPP incorporation among samples presents significant variability (step 11).

### Potential solution
Discs may differ in their developmental stages. Co-stain with a developmental marker to compare discs at similar stages.

## RESOURCE AVAILABILITY

### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marianthi Kiparaki (kiparaki@fleming.gr).

### Materials availability
This study did not generate new unique reagents. All reagents are available as specified in the key resources table.

### Data and code availability
This study did not generate or analyze datasets or codes.

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## AUTHOR CONTRIBUTIONS
M.K. established and optimized the protocol, performed research, created images, and wrote and revised the manuscript. N.E.B. revised the final manuscript. N.E.B. and M.K. supervised the study.

## DECLARATION OF INTERESTS
The authors declare no competing interests.

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