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Click-iT® Plus OPP Alexa Fluor® Protein Synthesis Assay in Embryonic Cells

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Abstract

This protocol describes a method to assess relative changes in the level of global protein synthesis in the preimplantation embryo using the Click-iT® Plus OPP Protein Synthesis Assays. In this assay, O-propargyl-puromycin (OPP), an analog of puromycin, is efficiently incorporated into the nascent polypeptide of newly translated proteins in embryonic cells. OPP is fluorescently labeled with a photostable Alexa Fluor™ dye and detected with fluorescence microscopy. The intensity of the fluorescence is quantitatively analyzed. This is a fast, sensitive, and non-radioactive method for the detection of protein synthesis in early embryo development. It provides a tool for analyzing the temporal regulation of protein synthesis, as well as the effects of changes in the embryonic microenvironment, and pharmacological and genetic modulations of embryo development.

Keywords: Embryo development, Maternal-embryonic transition, O-propargyl-puromycin, Protein synthesis

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Graphical abstract:

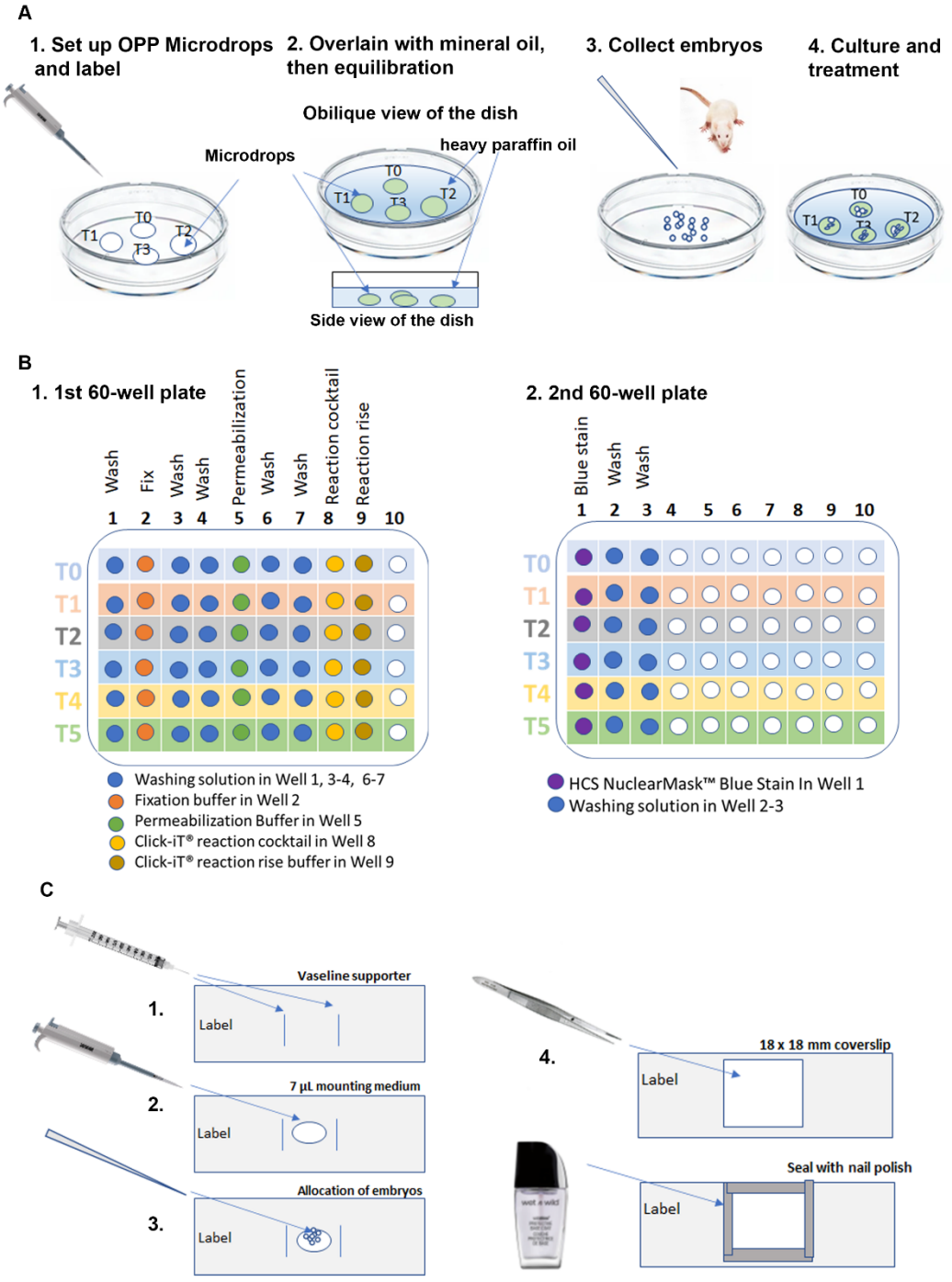

Figure 1. Brief overview of the procedures of the Click-iT® Plus OPP Alexa Fluor® protein synthesis assay in embryonic cells.

(**A**) Set up OPP treatments: (1) Set up microdrops containing 50 µL of OPP working solution and label different treatments on the back of culture dishes (*e.g.*, T0, T1, T2, and T3); (2) The drops are overlain with 2–3 mm heavy paraffin oil and then equilibrated in incubator for 2 h; (3) Collect the embryos from female reproductive tracts or following *in vitro* culture in desired treatments; (4) Culture embryos in the equilibrated OPP working solution for

2–6 h. (**B**) Example of OPP detection procedures working with 60-well plates labeled as T0, T1, T2, T3, T4, and T5 for different treatments: (1) The first 60-well plate is used for the procedures of washing, fixation, permeabilization, and Click-iT® OPP detection. (2) The second 60-well plate is for DNA staining and washing. **(C)** Slide preparation: (1) Label the required number of slides and set up vaseline coverslip supports; (2) Add mounting medium; (3) Transfer embryos into mounting medium; (4) Set coverslip; (5) Seal the coverslip with nail polish.

Background

Fertilization triggers embryonic genome activation, whereby most maternal transcripts and proteins are degraded, followed by the generation of a new transcriptome and resulting proteome (Gao *et al.*, 2017; Svoboda, 2018). These processes are required for the transition from maternal to embryonic control of development and create a cellular environment conducive of the totipotent state of the early embryo. There has been extensive analysis and development of tools for monitoring the changes to transcription at this time, but changes in translation have received less attention. New protein synthesis during the process of maternal to embryonic transition is tightly regulated and can be quantitatively evaluated with several methods, such as ^{35}S-Methionine (Crosby *et al.*, 1988), two-dimensional gel electrophoresis (Latham *et al.*, 1991), and mass spectrometry (Gao *et al.*, 2017). Here we report the development and use of a rapid alternative method for the analysis of global changes in the level of protein synthesis in the early embryo. The Click-iT® Plus OPP Protein Synthesis Assay is a non-toxic and non-radioactive method for the detection of nascent protein synthesis utilizing fluorescence microscopy and high-throughput imaging. O-propargyl-puromycin (OPP) is a membrane-permeable alkyne puromycin analog that forms covalent bonds with nascent polypeptide chains with cells. The addition of the Alexa Fluor® picolyl azide and the Click reaction reagents leads to a chemoselective ligation or “click” reaction between the picolyl azide dye and the alkyne OPP, and the modified proteins are detected by imaged-based analysis. The assay has been demonstrated to preserve cell morphology and has been used in NIH3T3, HeLa, C2C12 cells, BPAE, U-2 OS, CHO-M1, and A549 (Mateu-Regue *et al.*, 2019; Enam *et al.*, 2020; Liu *et al.*, 2012), and the embryonic cells of preimplantation development (Li *et al.*, 2021).

This protocol is designed to semi-quantitatively assess the relative changes in protein synthesis during the period of mouse maternal to embryonic transition. The first stage is to test and optimize the concentration of the OPP, then confirm the specificity of the assay by use of the known protein synthesis inhibitor Cycloheximide and inhibitor of translation elongation, 4EGI-1 (eIF4E inhibitor) (Li *et al.*, 2021).

Materials and Reagents

A. Plastic and glass ware

1. 60-well culture plates (Nunc, Naperville, IL, USA, LUX 5260)
2. 35 cm × 10 mm Petri dishes (Thermo Scientific, catalog number: 150318)
3. Cover glasses, 18 × 18 mm (Merck, BR470045)
4. Microscope slides, 25 mm × 75 mm (Merck, S8902)

B. Animals

Six-eight-week-old female mice

C. Click-iT® Plus OPP Alexa Fluor® protein synthesis assay (Thermo Scientific, catalog number: C10456), including

1. Component A: Click-iT® OPP Reagent, 20 mM in DMSO

2. Component B: Alexa Fluor® 488 picolyl azide in DMSO solution
3. Component C: Click-iT® OPP Reaction Buffer (10× solution containing Tris-buffered saline)
4. Component D: Copper Protectant
5. Component E: Click-iT® Reaction Buffer Additive
6. Component F: Click-iT® Reaction Rise Buffer, containing 2 mM sodium azide.
7. Component G: NuclearMask™ Blue Stain, 2,000× concentrate in water

D. Chemicals and reagents

1. Dulbecco′s Phosphate Buffered Saline (PBS) (Sigma, catalog number: D5773)
2. Triton X-100 (Sigma, catalog number: T8787)
3. Bovine serum albumin (BSA) (Sigma, catalog number: B2064)
4. Paraformaldehyde (PFA) (Sigma, catalog number: P6148)
5. DMSO (Sigma, catalog number: D8418)
6. Click-iT® Plus OPP Alexa Fluor® protein synthesis assay kit (see Recipes)
7. Fixation buffer (10 mL) (see Recipes)
8. 1× PBS (50 mL) (see Recipes)
9. Permeabilization buffer (1 mL) (see Recipes)
10. Washing solution (10 mL) (see Recipes)
11. KSOM medium (see Recipes)

Equipment

1. Dissection microscope (Olympus, SZx7)
2. Nikon ECLIPSE 80i microscope, Plan Apo 40×/1.0 oil objective (Nikon, Tokyo, Japan)
3. pH meter SevenDirect SD20 (Mettler Toledo)
4. Incubator, Cytomat 2 C470-LiN (Themo Fisher Scientific)

Software

1. ImageJ (http://imagej.net/Fiji)
2. SPSS for Windows (Version 22.0, SPSS Inc., Chicago, IL, USA)

Procedure

ANIMALS AND TREATMENTS

A. Animals and embryo collection

Six-eight-week-old female mice were superovulated by intraperitoneal injection of 5 IU equine chorionic gonadotropin (eCG) (Ningbo Second Hormone Factory, China) and 44–48 h later, 5 IU human chorionic gonadotrophin (hCG) (Livzon, Zhuhai, China), and then placed with fertile males overnight. Pregnancy was confirmed by the presence of a copulation plug in mated females the following morning. Zygotes or two-cell embryos were recovered 20 h and 40 h post-hCG from mated females, respectively. The embryos were collected in HEPES-buffered modified human tubal fluid medium (HEPES-HTF) (O'Neill, 1997), and all components of the media were tissue culture grade (Sigma Chemical Company, St Louis, MO, USA) and contained 3 mg bovine serum albumin/mL (Sigma).

B. Medium preparation and treatments

1. Calibrate the concentration of OPP for embryo treatment
   To minimize the embryonic toxicity of OPP during the period of embryo treatment, several doses of OPP should be tested. For example, dilute Component A (20 mM) with KSOM medium (Lawitts and Biggers, 1993) to produce a range of OPP concentrations (*e.g.*, 50, 37.5, and 25 μM) to 400 μL of final working solutions (Table 1).

   Table 1. Prepare a series of working solutions of Click-iT® OPP (Component A)

OPP (μM)	KSOM (μL)	Component A (μL)	DMSO (μL)	Total volume (μL)
0	399	0	1	400
25	399	0.5	0.5	400
37.5	399	0.75	0.25	400
50	399	1	0	400

   The example is to prepare three doses of OPP. The control is 0 μM OPP in KSOM medium. Adjust the concentration of DMSO to 0.25% (v/v) in all working solutions.
   Note: Total volume can be proportionally adjusted depending on the number of treatments; The concentration of OPP can be readjusted or optimized for the different experimental designs. A range of culture medium types are suitable for use depending upon the experimental design (e.g., hTF medium and KSOM, supplemented with or without amino acids).
2. OPP treatment (Figures 1 A1-4)
   a. Set up microdrops on culture dishes. Each drop contains 50 μL of OPP working solutions overlaid with 2 mm of heavy paraffin oil (Sigma).
      Optional: use 60-well Terasaki plates (LUX 5260, Nunc, Naperville, IL, USA), each containing 10 μL of OPP working solutions.
   b. Equilibrate in 5% CO_2 incubator at 37°C for 2 h.
   c. Transfer the embryos into OPP working solutions.
      Optional: embryo density is optional, e.g., 1–10 embryos in 10 μL of working solutions. Keep the same density in all treatments.
   d. Culture and treat the embryos for 2–6 h at 37°C, with 5% CO_2 in air tension.
      CRITICAL: (1) It was suggested that the incubation periods with OPP be between 30 min and 1 h to maximize detection and minimize toxicity (Liu et al., 2012; Signer et al., 2014). However, translation in preimplantation embryos may be different from the somatic cells. In simple formulated medium, 2-cell embryo expressed a peak signal after 6 h treatment with 37.5 μM OPP (Li et al., 2021). Thus, we recommend testing different incubation periods (Figure 2B) with different concentrations of OPP (Figure 2 A) and doses of drugs (Figure 2C) for each stage of embryo development used before the final experimental design is determined (as in the example in Table 1). (2) Similar calibrations are suggested if the experiments are performed in different oxygen concentrations, as this protocol was designed for the OPP treatment in air tension before final experimental design is determined.

STEP-BY-STEP METHOD DETAILS

Note: To facilitate systematically processing embryos, we use 60-well Terasaki plates (Figure 1 B1–2). Each well can contain 10 μL of solution and 1–10 embryos. Transfer embryos with minimal solution into the well of the next step and use clean pipettes for each transfer stage.

A. Proceed to embryo fixation and permeabilization (Figure 1 B1–2)

1. Work at room temperature. Transfer embryos treated with OPP into washing solution and rinse once to remove the media.
2. Transfer embryos into 10 µL fixation buffer/well. Incubate for 15 min at room temperature.
3. Wash embryos twice with washing solution to remove fixative.
4. Transfer embryos into 10 µL of permeabilization buffer and incubate for 20 min at room temperature.

B. Click-iT® OPP Detection (Figure 1 B1–2)

1. Prepare Click-iT® reaction cocktail according to Table 2.

Table 2. Click-iT® reaction cocktail

Reaction components (as supplied in kit)	Number of wells		
	10 wells	50 wells	100 wells
Click-iT® OPP Reaction Buffer (1× concentrate)	88 µL	0.44 mL	0.88 mL
Copper Protectant (Component D)	2 µL	10 µL	20 µL
Alexa Fluor® picolyl azide (Component B)	0.25 µL	1.25 µL	2.5 µL
Click-iT® Reaction Buffer Additive (10× solution)	10 µL	50 µL	0.1 mL
Total reaction volume	0.1 mL	0.5 mL	1 mL

Note: Use the Click-iT® reaction cocktail within 15 min of preparation. The number of wells can vary depending on the experiment. It is important to calculate the number of wells you will plate before you start the experiment.

2. Wash embryos twice with 10 µL of washing solution
3. Transfer embryos into 10 µL of Click-iT® reaction cocktail.
4. Incubate for 30 min at room temperature. Protected from light.
5. Wash once with 10 µL per well of Click-iT® reaction rise buffer (Component F).

C. DNA Staining (Figure 1 B1-2)

Note: The following protocol is based upon 10 µL of HCS NuclearMask™ Blue Stain working solution per well.

1. Dilute HCS NuclearMask™ Blue Stain (Component G) solution (1:2,000, v/v) in 1× PBS to obtain a 1× HCS NuclearMask™ Blue Stain working solution.
2. Transfer embryos into 10 µL of 1× HCS NuclearMask™ Blue Stain working solution. Incubate for 30 min at room temperature, protected from light.
3. Wash twice with washing solution and proceed to Imaging and Analysis (below).

D. Imaging

1. Make slides (Figure 1 C1–5)
   a. Clean the glass slides and the coverslips with ethanol.
   b. Use an insulin syringe with 18–22 G needle filled with vaseline. Make two parallel lanes of vaseline on the slide to support the coverslip. The vaseline lane should 1–2 mm thick.
   c. Add a drop of 7 µL 1× PBS onto a slide. (Optional: use standard anti-fade mounting media).
   d. Transfer 10 embryos/drop and gently put an 18 × 18 mm coverslip on with forceps; remove any excess fluid from edges of the coverslip with a lint-free absorbent tissue.
   e. Seal with nail polish (optional: PathTech, https://www.pathtech.com.au). Take care not to move the coverslip to avoid squashing the embryos).
2. Scan the slide under fluorescence microscope with filters appropriate for DAPI/Hoechst and FITC for Alexa Fluor® 488 (Figures 2 A–C).
3. Whole section image is captured with mercury lamp UV illumination on a Nikon ECLIPSE 80i microscope

with a Nikon Plan Apo 40×/1.0 oil objective. (Any similar fluorescent microscope systems are suitable).

E. Fluorescence measurement and Data analysis

1. Measure the intensity of the fluorescent OPP stain
   The fluorescent intensity of nascent protein synthesis in the fluorescent channel in the nucleus or whole embryo can be measured by suitable analysis software, *e.g.*, ImageJ and ImagePro Plus (Media Cybernetics, Inc.).
   a. With ImageJ, open the images of the embryo with dual staining of OPP and NuclearMask blue stain.
   b. In the Analyze menu, open Set Measurements and tick “Area”, “Integrated density”, “Mean gray value”, and Standard deviation”.
   c. In the image of NuclearMask blue stain, outline the single nucleus using freehand selection of region of interest (ROI). From Analyze > Tool, open ROI Manager. Add this ROI to ROI Manager and rename it.
   d. Allocate the ROI for the outlined nuclei from ROI Manager to the image of OPP stain of same embryo. This is the exact localization of the nucleus for the dual staining images of same embryo.
   e. Select “Measure” from the analyze menu. The results are shown as a popup box with a stack of values for that nucleus of the embryo cell. The same way to measure the fluorescence of OPP stain of another nucleus.
   f. Directly measure the fluorescent stain of the outlined whole embryo using freehand selection of ROI.
   g. Record and copy the integrated density (stand for the fluorescent intensity of OPP stain) from the popup box for ROI of each nucleus or whole embryo. Set up an Excel spreadsheet for data analysis from all treatments and controls.
   h. Subtract the values in the control treatment of 0 μM OPP from the values from all treatments and controls in the same experiments. The resulting values are used for analysis.
2. Statistical analysis
   Statistical analysis was performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA) (Optional: other Statistic software). Fluorescence intensity (AU, arbitrary units of optical density of staining) was quantitatively analyzed by univariate analysis of variance. This parameter was set as the dependent variable, while the test treatments and drug doses were the independent variables. Experimental replicates were incorporated into the model as covariates. Differences between individual independent variables were analyzed by the least significance difference test. Less than a 5% probability ($P < 0.05$) was considered significant.
   Critical: The results from quantitative analysis of fluorescent intensity can vary between experiments due to a range of uncontrollable variables. It is therefore important that embryos from each treatment were processed at the same time and in parallel. All treatments were exposed to the same preparations and dilutions of all reagents. Similarly, all preparations from an experiment were examined microscopically within the same session, and identical microscope and camera settings were used. All image analysis was carried out in an identical manner for each embryo within an experiment. All preparations were carried out by the same experienced operator throughout the study.

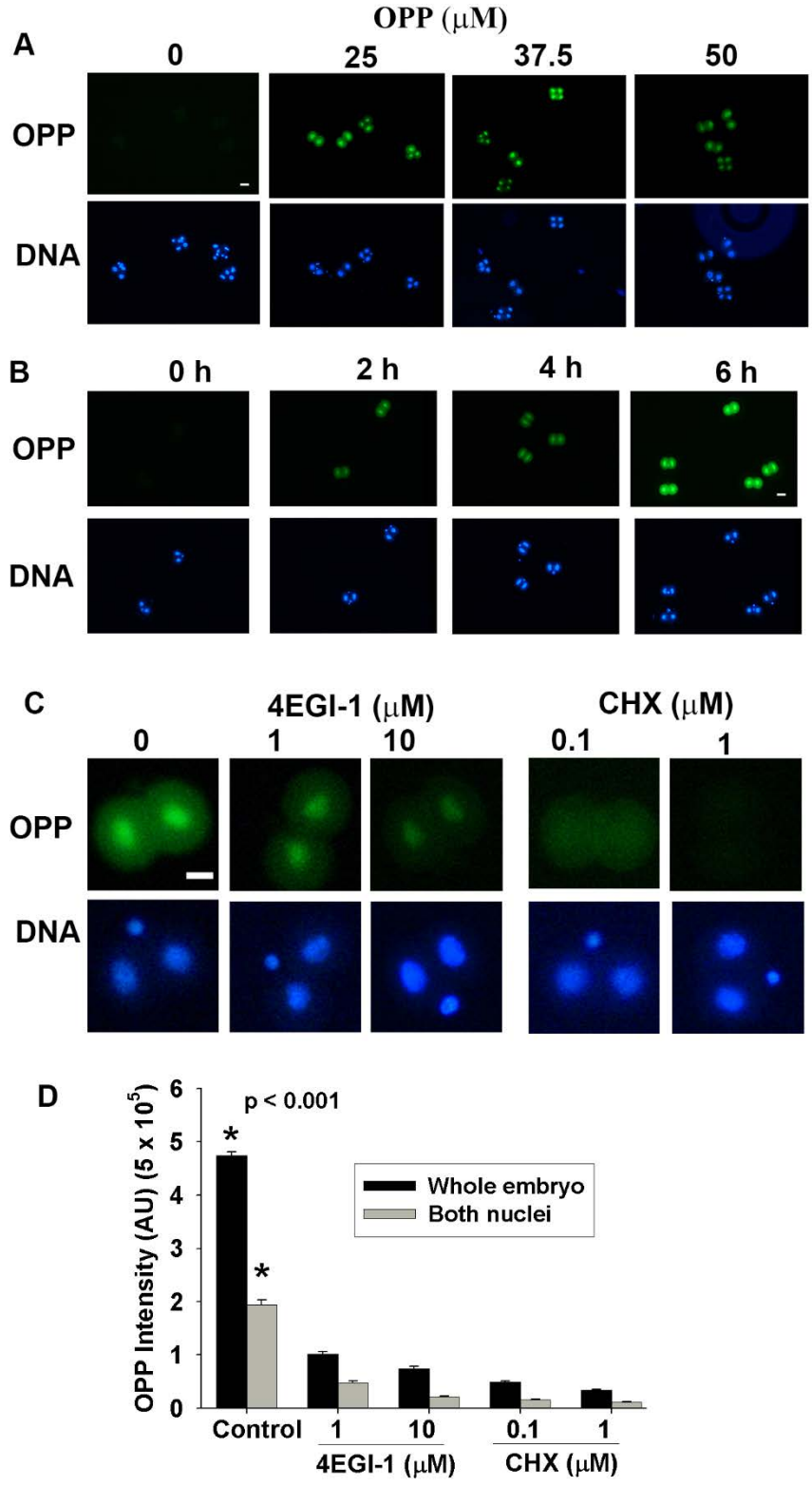

Figure 2. Analysis of OPP staining in the embryonic cells.

(A) Whole-section images representative of fluorescence changes in wild-type two-cell embryos treated either without or with a range of OPP concentrations for 24 h. There were at least ten embryos in each treatment group. All embryos were arrested at 2-cell stage in the 50 μM OPP group. **(B)** Two-cell embryos were collected from

female reproductive tracts and treated with 37.5 µM OPP for 0, 2, 4, and 6 h. The images are representative of a total of 30 embryos from three independent replicates. **(C)** Two-cell embryos were treated with either the 4EGI-1 (EIF4E inhibitor) (Li *et al.*, 2021) cycloheximide (CHX) (Schneider-Poetsch *et al.*, 2010) for 6 h. Images are representative of three independent replicates for ten embryos for each treatment. **(D)** OPP staining intensity in embryos treated with 4EGI-1 or CHX) in **(C)**, compared with the control (no inhibitor). Data are Mean ± S.E.M. (univariate analysis of variance). * Statistically different ($P < 0.001$) from all other treatments. Scale bars: 10 µm.

Recipes

1. Click-iT® Plus OPP Alexa Fluor® protein synthesis assay kit

a. Allow reagent vials to completely thaw and warm to room temperature before opening.
b. Prior to use, briefly centrifuge Click-iT® OPP Reagent (Component A) and NuclearMask™ Blue Stain (Component G) to maximize reagent recovery.
c. Prepare a 10× stock solution of the Click-iT® Reaction Buffer Additive (Component E). Add 2 mL of deionized water to the vial (containing 400 mg) and mix until completely dissolved. After use, aliquot 50 µL/each, store any remaining stock solution at ≤ –20°C.
Note: When stored as directed, this stock solution is stable for up to 1 year.
d. Prepare 1× Click-iT® OPP Reaction Buffer.
Transfer all the solution in the Component C bottle (4 mL) to 36 mL of deionized water. Rinse the Component C bottle with some of the diluted Click-iT® OPP Reaction Buffer to ensure the transfer of all the 10× concentrate.
Note: Use the Click-iT® reaction cocktail within 15 min of preparation. To prepare smaller amounts of 1× Click-iT® OPP Reaction Buffer, dilute 1 volume from the Component C bottle with nine volumes of deionized water. After use, store any remaining 1× solution at 2°C–8°C. When stored as directed, 1× Click-iT® OPP Reaction Buffer is stable for 6 months.
e. Prepare working solution of NuclearMask™ Blue Stain (Component G).
Prepare it just prior to use. Dilute HCS NuclearMask™ Blue Stain (Component G) solution 1:2,000 in PBS to obtain a 1× HCS NuclearMask™ Blue Stain working solution.

2. Fixation buffer [3.6% (w/v) Paraformaldehyde in PBS] (10 mL)

a. Weigh Paraformaldehyde 0.37 g.
b. Add to MilliQ H_2O 10 mL in a glass container.
c. Add 1.0 M NaOH 14 µL.
d. Warm and stir on a 50–60°C hotplate until completely dissolved. It takes about 20 min.
e. Add PBS 0.096 g and stir until completely dissolved.
f. Cover the container and leave until it reaches room temperature.
g. Adjust pH at 7.4 with 1 N HCl at room temperature.

3. 1× PBS (50 mL)

a. Weigh 0.4798 g PBS powder.
b. Add into 50 mL MilliQ H_2O and mix well.

4. Permeabilization buffer (1 mL)

a. Prepare prior to use.
b. 5 µL of Triton X-100
c. Add into 995 µL of 1× PBS and mix well.

5. Washing solution (10 mL)

a. Weigh 0.3 g BSA

b. Add into 10 mL of 1× PBS. Make sure it is completely dissolved before use.
Note: Always use the fume hood and follow safety measures when preparing and handling paraformaldehyde. Personal protection equipment should be used during use.

6. Composition of KSOM medium (100 mL)

555 mg of NaCl
18.5 mg of KCl
4.75 mg of KH_2PO_4
4.95 mg of $MgSO_4•7H_2O$
25 mg of $CaCl_2•2H_2O$
210 mg of $NaHCO_3$
3.6 mg of Glucose
2.2 mL of Na-Pyruvate
0.174 mL of DL-Lactic Acid
4 mg of EDTA
14.6 mg of GL-Glutamine and 100 mg Bovine serum albumin
Note: All reagents were purchased from Sigma.
Add MilliQ purified water to 100 mL, mix well, and sterilize by filtration with 22 mm Millex-GV Filter, 0.22 μm (MERCK).

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Competing interests

The authors declare no competing interests
AUTHOR CONTRIBUTIONS: X. Jin. and C.O. supervised the study. X. Jin. C.O. and X.H. designed and wrote the manuscript. Y.L., X.J. L.C., J.T., M.H., and J.L. performed the experiments.

Ethics

Animal experiments were approved by and conducted according to ethics guidelines from relevant research institutes and universities. Hybrid (C57BL/6 X CBA/He) mice were housed and bred at the Wenzhou Medical University.

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