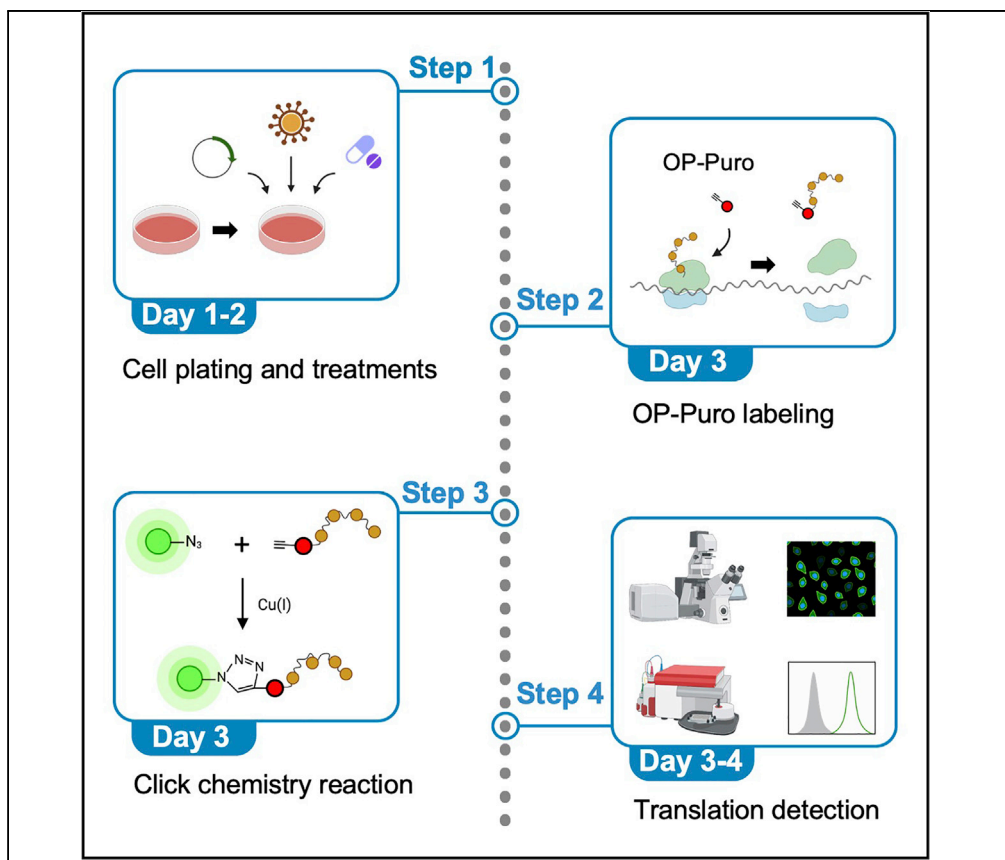


Protocol

Protocol for assessing translational regulation in mammalian cell lines by OP-Puro labeling



Translational regulation is a fundamental step in gene expression with critical roles in biological processes within a cell. Here, we describe a protocol to assess translation activity in mammalian cells by incorporation of O-propargyl-puromycin (OP-Puro). OP-Puro is a puromycin analog that is incorporated into newly synthesized proteins and is detected by click chemistry reaction. We use OP-Puro labeling to assess translation activity between different cell types or cells under different growth conditions by confocal microscopy and flow cytometry.

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

Jack Chun-Chieh Hsu, Joanna B. Pawlak, Maudry Laurent-Rolle, Peter Cresswell

chunchieh.hsu@yale.edu (J.C.-C.H.)
peter.cresswell@yale.edu (P.C.)

Highlights

Protocol for the measurement of global translation activity at the single-cell level

Incorporation of OP-Puro into newly synthesized proteins in living cells

Fluorescent labeling of incorporated OP-Puro using click chemistry reaction

Confocal microscopy and flow cytometry to define translation activity

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Protocol

Protocol for assessing translational regulation in mammalian cell lines by OP-Puro labeling

Jack Chun-Chieh Hsu,^{1,6,*} Joanna B. Pawlak,^{1,2} Maudry Laurent-Rolle,^{2,3,4} and Peter Cresswell^{1,5,7,*}¹Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA²Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, USA³Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT 06520, USA⁴Center for Infection and Immunity, Yale University School of Medicine, New Haven, CT 06520, USA⁵Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520, USA⁶Technical contact⁷Lead contact*Correspondence: chunchieh.hsu@yale.edu (J.C.-C.H.), peter.cresswell@yale.edu (P.C.)
<https://doi.org/10.1016/j.xpro.2022.101654>

SUMMARY

Translational regulation is a fundamental step in gene expression with critical roles in biological processes within a cell. Here, we describe a protocol to assess translation activity in mammalian cells by incorporation of O-propargyl-puromycin (OP-Puro). OP-Puro is a puromycin analog that is incorporated into newly synthesized proteins and is detected by click chemistry reaction. We use OP-Puro labeling to assess translation activity between different cell types or cells under different growth conditions by confocal microscopy and flow cytometry. For complete details on the use and execution of this protocol, please refer to Hsu et al. (2021) and Hsu et al. (2022).

BEFORE YOU BEGIN

The protocol is divided into four steps: 1) cell plating and treatment(s); 2) OP-Puro labeling; 3) fixation, permeabilization, and click chemistry reaction; and 4) translation detection. OP-Puro is incorporated onto the C-termini of newly synthesized proteins thereby prematurely terminating protein synthesis. During OP-Puro incorporation, a “clickable” alkyne is introduced into newly synthesized proteins that can be labeled using an azide-conjugated fluorophore and detected through click chemistry reaction.

Two different readouts can be used to detect the OP-Puro labeled fluorescence signal: confocal microscopy and flow cytometry. This protocol is set up to study translational regulation in various mammalian cell lines and is applicable to a variety of purposes, including comparing translation efficiency in cells with different genotypes or under different growth conditions, cells treated with metabolic inhibitors, including translation inhibitors, and virally infected cells (Hsu et al., 2021, 2022). We have used this protocol to determine the correlation between translation efficiency and expression of the antiviral protein viperin at the single cell level (Hsu et al., 2022) as well as to screen SARS-CoV-2 viral proteins for translation inhibitory activity (Hsu et al., 2021).

Cell plating

In this protocol, cell plating is a critical step. Adherent cells of interest should be seeded at a density reaching 70%–90% confluence on the day of OP-Puro labeling. This step is crucial to ensure active cell growth and translation. After cell plating, the cells should be left to adhere for at least 12 h before being challenged with cell stress inducers, transfection reagent or viral infection.



Preparation of cell culture

⌚ Timing: 1–2 days

This protocol is set up for adherent mammalian cell lines. We have optimized it for use on human cell lines (HEK293T, HeLa, Huh-7 cells), African green monkey kidney epithelial cell lines (Vero, Vero E6 cells), as well as immortalized mouse bone marrow-derived macrophage cell lines (Hsu et al., 2021, 2022). In this description, we use HEK293T cells as a model. Minor optimizations might be required for other cell lines, which will be highlighted in the text. Although we have not applied the protocol to non-mammalian and suspension cell lines, similar protocols based on OP-Puro incorporation have been widely used to study protein synthesis in mouse small intestine (Liu et al., 2012), Chinese hamster ovary (CHO) cells (Nagelreiter et al., 2018), hematopoietic stem cells (Signer et al., 2014), muscle stem cells (Zismanov et al., 2016), skin stem cells (Blanco et al., 2016), colorectal cancer organoids (Morrall et al., 2020a, 2020b), and Calu-6 cells upon SARS-CoV-2 infection (Fisher et al., 2022).

1. Thaw cells of interest. Keep cells actively growing and prevent over confluency. Culture cells for at least one passage before plating the cells for OP-Puro labeling experiment.

⚠ **CRITICAL:** Cell proliferation and translation are sensitive to cell culture conditions. Keep cell culture conditions consistent, including temperature, CO₂ level, serum-free medium composition and the lot of fetal bovine serum (FBS).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
10× PBS	AmericanBio	Cat#AB11072
DMEM, high glucose	Thermo Fisher Scientific	Cat#11965092
Fetal Bovine Serum (FBS)	GeminiBio	Cat#100-106
Bovine Serum Albumin (BSA)	American Bioanalytical	Cat#AB01088
Saponin	Sigma-Aldrich	Cat#S7900
Tris-HCl	AmericanBio	Cat#AB02005
16% formaldehyde	Electron Microscopy Sciences	Cat#15710
ProLong Gold Anti-Fade Reagent	Thermo Fisher Scientific	Cat#P36930
Hoechst 33342	Thermo Fisher Scientific	Cat#H3570
O-Propargyl-puromycin	Abcam	Cat#ab146664
L-Ascorbic acid	Sigma-Aldrich	Cat#A4544
Copper sulfate	Sigma-Aldrich	Cat#C1297
Azide-fluor 488	Sigma-Aldrich	Cat#760765
AZDye 647 Azide	Click Chemistry Tools	Cat#1299
Cycloheximide	Sigma-Aldrich	Cat#C4859
Experimental models: Cell lines		
Human: HEK293T	ATCC	Cat#CRL-3216; RRID: CVCL_0063
African green monkey: Vero E6	ATCC	Cat#CRL-1586; RRID: CVCL_0574
Software and algorithms		
Leica Application Suite	Leica Camera	NA
ImageJ	National Institutes of Health	RRID:SCR_003070
FlowJo	FlowJo LLC	RRID:SCR_008520
Other		
Costar 12-well clear TC-treated multiple well plates	Corning Inc.	Cat#354108
SUPERFROST microscope slides (3" × 1" × 1 mm)	Thermo Scientific	Cat#4951F-001

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Coverslips	Bellco Glass Inc.	Cat#1943-00012
STELLARIS 8 confocal microscope	Leica Camera	NA
Accuri C6 Flow Cytometer	Becton, Dickinson and Company (BD)	NA

MATERIALS AND EQUIPMENT

Reconstitute special reagents

O-propargyl-puromycin (OP-Puro) from Abcam is shipped as a solid powder (50 mg per tube) and should be stored at -20°C . Once reconstituted in 50 μL of DMSO to make a 20 mM stock solution, OP-Puro stock solution can be stored at -20°C for up to 24 months.

Cycloheximide (CHX) is reconstituted to 50 mg/mL in DMSO. CHX stock solution should be stored at -20°C for up to 12 months. **Attention**, CHX is an extremely hazardous substance. It is toxic to the environment. Avoid release to the environment. Follow your institutional guidelines or contact your Environmental Health and Safety department for proper waste disposal.

Azide-fluor 488 from Sigma-Aldrich is shipped as a solid orange powder. Once reconstituted at 2 mM in DMSO, Azide-fluor 488 should be stored at -20°C in the dark for up to 24 months. We also used AZDye 647 Azide (Click Chemistry Tools), which provides a strong far-red fluorescence, and it is compatible with GFP-tagged protein expression or additional immunofluorescence staining. We recommend using lower AZDye 647 Azide concentration ($< 1 \mu\text{M}$) for FACS analysis. These azide-conjugated fluorophores are light sensitive, therefore, perform the staining procedure without intense direct light exposure. We have tested and used these two azide-conjugated fluorophores, but more azide-conjugated fluorophores can be used for click chemistry reaction.

Ascorbic acid from Sigma-Aldrich is shipped as a solid white powder that should be stored at 4°C . Ascorbic acid stock solution (500 mM) should be freshly prepared in ddH₂O. The ascorbic acid solution should be added last to click chemistry reaction solution which should then be applied immediately. Ascorbic acid solution is prone to oxidization by air and becomes yellow. Do not use it once it turns yellow.

Tris-HCl and CuSO₄ stock solutions can be stored at 4°C for up to 12 months.

Solutions

Fixation solution (8 mL)

Reagent	Final concentration	Amount
Formaldehyde (16%)	1%	0.5 mL
PBS (10 \times)	1 \times	0.8 mL
ddH ₂ O	N/A	6.7 mL
Total	N/A	8 mL

△ CRITICAL: Formaldehyde is a highly toxic, human carcinogen and a suspected reproductive hazard.

Always work in a chemical fume hood with proper personal protective equipment and follow safety measures when handling formaldehyde.

Note: Fixation solution should be freshly made.

Permeabilization buffer (20 mL)		
Reagent	Final concentration	Amount
Saponin (10%)	0.1%	0.2 mL
FBS (100%)	3%	0.6 mL
PBS (10×)	1×	2 mL
ddH ₂ O	N/A	17.2 mL
Total	N/A	20 mL

Note: Permeabilization buffer should be freshly made.

Click chemistry reaction solution (5 mL)		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.5; 1.5 M)	150 mM	0.5 mL
CuSO ₄ (100 mM)	1 mM	50 μL
Azide-fluor 488 (2 mM)	30 μM	75 μL
ddH ₂ O	N/A	3.375 mL
Ascorbic acid (500 mM; added before use)	100 mM	1 mL
Total	N/A	5 mL

Note: Immediately before application, add ascorbic acid to the final concentration as described above.

⚠ **CRITICAL:** Click chemistry reaction solution should be freshly prepared.

Note: The final concentration of Azide-fluor 488 should be optimized for the cell line of interest empirically. 5–50 μM is a good starting range. Other Azide-conjugated fluorophores can be used for click chemistry reaction. We have used AZDye 647 Azide (Click Chemistry Tools) for the reaction, which provides a strong far-red fluorescence detection and is compatible with GFP-tagged protein expression and conventional immunofluorescence staining. We recommend using lower AZDye 647 Azide concentration (< 1 μM) for flow cytometry analysis.

Alternatives: OP-Puro-based protein synthesis detection kits are available from Click Chemistry Tools and ThermoFisher. However, we did not test these kits.

FACS buffer (5 mL)		
Reagent	Final concentration	Amount
FBS (100%)	3%	150 μL
PBS (10×)	1×	0.5 mL
ddH ₂ O	N/A	4.35 mL
Total	N/A	5 mL

Note: FACS buffer should be freshly made.

STEP-BY-STEP METHOD DETAILS

Cell plating

⌚ **Timing:** 2 h

Translation is sensitive to cell confluence. Overconfluent cells may have less active cellular translation. Plate cells at least one day before OP-Puro labeling at a density that will reach 80% confluence

on the day of OP-Puro labeling. We recommend that you test and determine the optimal plating density for your cell line of interest.

1. For cell cytometry experiments, plate the cells in a 12-well plate at optimal density that will reach 80% confluence on the day of OP-Puro labeling. We recommend a plating density of $2\text{--}4 \times 10^5$ cells per well for HEK293T cells.

△ **CRITICAL:** Avoid over confluence on the day of labeling, which may inhibit translation.

△ **CRITICAL:** Supply sufficient culture medium. We usually use 2 mL medium supplemented with 10% FBS for a 12-well format. More medium might be required for prolonged culture times or different cell lines for active cell proliferation.

Note: For confocal microscopy, we plate cells on coverslips in a 12-well plate. Polylysine-coated coverslips can improve the growth and adhesion of some cell lines (e.g., HEK293T).

Note: Plate three extra wells for controls: 1) no OP-Puro, 2) OP-Puro but no treatment/transfection/infection (positive control), 3) OP-Puro treated with the translation inhibitor CHX which serves as a negative control. The controls 1) and 2) are required for data normalization (see [quantification and statistical analysis](#)) and 3) for internal control.

Optional: For drug treatment and viral infection, cells are plated at a desired density according to the experiment timelines. For example, for doxycycline-inducible cell lines, we plate the cells at 20% confluence at 24 h before doxycycline induction, and then induce cells with doxycycline for 24 h before OP-Puro labeling.

OP-Puro labeling

⌚ **Timing:** 2 h

During the OP-Puro labeling step, cells are treated with sufficient medium supplemented with OP-Puro to label newly synthesized proteins. This step introduces a “clickable” alkynyl group into newly synthesized proteins for azide-conjugated fluorophore labeling by click chemistry reaction.

Note: The section for the OP-Puro labeling applies to both confocal microscopy and flow cytometry.

Note: In addition to treatments of interest, we recommend three controls a) no OP-Puro, b) OP-Puro but no treatment/transfection/infection, and c) OP-Puro and the translation inhibitor CHX.

2. For CHX-treated control well c, add CHX to medium at a final concentration of 50 $\mu\text{g}/\text{mL}$ (1:1,000 dilution). Incubate for 15 min at 37°C in a tissue culture incubator.
3. Aspirate media and add 400 μL of pre-warmed cell culture medium (no OP-Puro) to control well a, 400 μL of OP-Puro labeling medium supplemented with 0.4 μL of CHX stock solution (50 $\mu\text{g}/\text{mL}$) to CHX-treated control well c and 400 μL of OP-Puro labeling medium to the remaining wells. Incubate for 1 h at 37°C in a tissue culture incubator. For 12-well plates, the labeling medium is freshly prepared by adding 10 μL of OP-Puro stock solution (20 mM) to 4 mL culture medium.

Note: Cell culture medium should be pre-warmed at 37°C before adding to cells because temperature changes affect protein expression. One hour of OP-Puro incubation is generally sufficient. However, the optimal incubation time for a specific cell line can be determined empirically (0.5–2 h).

Note: The range of OP-Puro concentration can be between 2-50 μM . The optimal OP-Puro concentration for a specific cell line should be determined empirically.

△ CRITICAL: Cell monolayer should be covered with sufficient labeling medium. We recommend using at least 350 μL for 12-well plate.

Proceed to fixation, permeabilization and click chemistry reaction procedures specific for confocal (steps 4–14) or for flow cytometry (steps 15–23).

Fixation, permeabilization and click chemistry reaction (for confocal microscopy)

⌚ Timing: 2 h

After OP-Puro labeling, the cells on coverslips are fixed and permeabilized, followed by click chemistry reaction with an azide-conjugated fluorophore. These steps are performed in a 12-well plate.

4. Aspirate medium and wash cells with ice-cold 1 \times PBS.
5. Fix cells with 500 μL of 1% formaldehyde in 1 \times PBS for 15 min on ice.

Note: Formaldehyde is highly toxic. Always work in a chemical fume hood with proper personal protective equipment and follow safety measures when handling formaldehyde.

6. Wash cells with 1 mL of 1 \times PBS.
7. Permeabilize cells in 500 μL of Permeabilization buffer for 5 min at room temperature.
8. Aspirate Permeabilization buffer.

△ CRITICAL: The saponin-based permeabilization can be reversible. AVOID washing cells before adding click chemistry reaction solution.

9. Add 500 μL of click chemistry reaction solution. Incubate for 30 min at room temperature in the dark. Apply this step to all the samples, including no OP-Puro and CHX-treated control samples.
10. Wash cells with 500 μL of Permeabilization buffer three times.
11. Wash cells with 500 μL of 1 \times PBS.
12. Incubate cells with 500 μL of Hoechst 33342 (10 $\mu\text{g}/\text{mL}$) in 1 \times PBS for 10 min.
13. Wash cells with 500 μL of 1 \times PBS.
14. Mount coverslips on glass microscope slides using ProLong Gold Anti-Fade Reagent. Slides are cured overnight at room temperature before confocal microscopy analysis.

Alternatives: Conventional immunofluorescence staining for proteins of interest can be performed after OP-Puro labeling (step 11). Given that cells are permeabilized by saponin, which only permeabilizes the plasma membrane, additional permeabilization with harsher detergent might be required for some purposes.

Fixation, permeabilization and click chemistry reaction (for flow cytometry)

⌚ Timing: 2 h

After OP-Puro labeling, the cells are fixed and permeabilized in microcentrifuge tubes, followed by click chemistry reaction with an azide-conjugated fluorophore.

Note: In this section, cells are centrifuged for 5 min at 1,000 g at 4°C to collect cell pellets.

15. Aspirate media and wash cells with 1 \times PBS.

16. Harvest cells into 1.5 mL microcentrifuge tubes by trypsinization with trypsin/EDTA solution. Centrifuge and discard the supernatant.
 - a. For HEK293T cells, trypsinization is not required as the cells are readily detached by pipetting up and down in 1× PBS.
17. Wash cells with ice-cold 1× PBS. Centrifuge and discard supernatant.
18. Fix cells with 500 μL of 1% formaldehyde in 1× PBS for 15 min on ice. Centrifuge and discard supernatant.

Note: Formaldehyde is highly toxic. Always work in a chemical fume hood with proper personal protective equipment and follow safety measures when handling formaldehyde.

19. Wash cells with 1 mL of 1× PBS. Centrifuge and discard supernatant carefully.
20. Permeabilize cells in 500 μL of Permeabilization buffer for 5 min at room temperature. Centrifuge and carefully discard supernatant.

△ **CRITICAL:** The saponin-based permeabilization is reversible. AVOID washing the cells before adding click chemistry reaction solution.

21. Resuspend cells in 500 μL of click chemistry reaction solution for 30 min at room temperature in the dark. Apply this step to all the samples, including the no OP-Puro and CHX-treated control samples. Centrifuge and carefully discard supernatant.
22. Wash cells with 500 μL of Permeabilization buffer three times. Centrifuge and carefully discard supernatant.
23. Resuspend cells in 200 μL of FACS buffer and leave on ice in the dark. The samples are ready for flow cytometry analysis.

EXPECTED OUTCOMES

For confocal microscopy analysis, OP-Puro labeling signals are detected in the cytoplasm and nucleus. No OP-Puro control serves as a background signal. CHX-treated control should provide no or low OP-Puro labeling signal. For example, we used this protocol to determine the translation activity in HEK293T cells treated with synthetic 3'-deoxy-3',4'-dideohydro-cytidine (ddhC) nucleoside (Figure 1A) and transfected with SARS-CoV-2 NSP14 (Hsu et al., 2021). We also detected translation activity in Vero E6 cells infected with a SARS-CoV-2 infectious clone expressing a fluorescence protein reporter (SARS-CoV-2-mNG) (Figure 1B) (Hsu et al., 2021). Finally, by combining the protocol with immunofluorescence staining, we determined the translation activity in HEK293T cells expressing the antiviral protein viperin and its catalytically inactive mutant at the single cell level (Hsu et al., 2022).

For flow cytometry analysis, OP-Puro labeling shows a strong fluorescence signal over no OP-Puro control. The no OP-Puro control can serve as a background signal. When normalizing the geometric mean fluorescence intensity (gMFI), CHX-treated control should provide low OP-Puro labeling signals (~10%; see details in [quantification and statistical analysis](#)). For example, we used this protocol to determine the translation activity of HEK293T cells transfected with SARS-CoV-2 NSP14 (Figure 2).

QUANTIFICATION AND STATISTICAL ANALYSIS

We recommend using gMFI values determined by flow cytometry analysis for quantification and statistical analysis. For confocal image analysis, OP-Puro microscopic images should be captured under the same confocal settings (e.g., magnification, zoom factor, laser power, gain). Both manual and automated analyses have been used to quantify OP-Puro signal intensity at single-cell level (Enam et al., 2020; Liu et al., 2012). Briefly, cells of interest are manually selected using the selection tools in ImageJ. For automated analysis, nuclear staining (e.g., Hoechst) is used to determine threshold

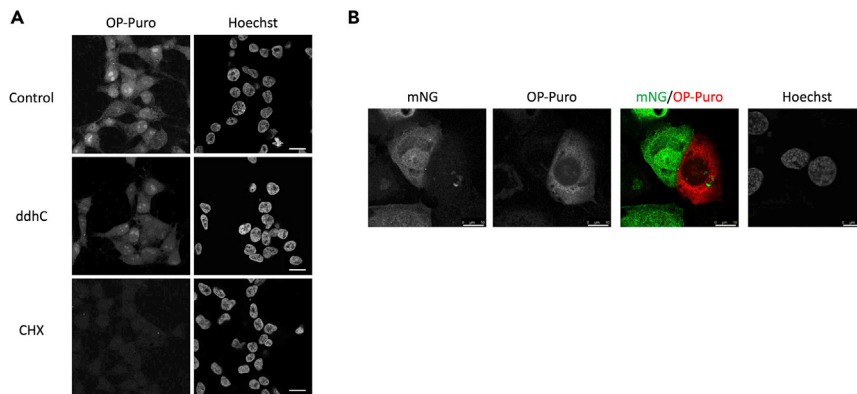


Figure 1. Confocal microscopy analysis of OP-Puro labeling

(A) Synthetic ddhC nucleoside induces translation inhibition. HEK293T cells were treated with 1 mM synthetic 3'-deoxy-3',4'-didehydro-cytidine (ddhC) nucleoside for 24 h, stained by OP-Puro labeling with Azide-fluor 488 and analyzed by confocal microscopy. Scale bars, 20 μ m. CHX, cycloheximide.

(B) SARS-CoV-2 infection inhibits cellular translation. Vero E6 cells were infected with recombinant SARS-CoV-2 expressing mNeonGreen (mNG) (22) at an MOI of 0.5 for 24 h. Cells were stained by OP-Puro labeling with AZDye 647 Azide and analyzed by confocal microscopy. Scale bars, 10 μ m.

and cell boundaries (watershed method). The fluorescence intensity of each cell is measured as the mean of the selected pixel values. A local background for each cell is measured and subtracted from the intensity of the corresponding cell.

To normalize OP-Puro intensities between 0 and 100%, the no OP-Puro control is used to define the baseline (0%) and a positive control sample (e.g., with OP-Puro but no treatment/transfection/infection) subtracted by the no OP-Puro control is defined as 100%. We use the following equation to calculate the normalized OP-Puro intensity of a given sample,

$$OP - Puro \text{ intensity (\%)} = \frac{(OPP_x - OPP_{min})}{(OPP_{max} - OPP_{min})}$$

where,

OPP_x = gMFI of OP-Puro intensity of a sample of interest.

OPP_{min} = gMFI of OP-Puro intensity of no OP-Puro control.

OPP_{max} = gMFI of OP-Puro intensity of OP-Puro but no treatment/transfection/infection (positive control).

We recommend using at least three biological repeats for statistical analysis.

LIMITATIONS

A concern has been raised that puromycin labeling is not sensitive enough to measure translation inhibition under glucose starvation (Marciano et al., 2018). Although we did not test this protocol to measure translation under glucose starvation, it is likely that OP-Puro labeling might be skewed in this scenario. Therefore, we recommend using OP-Puro labeling along with additional translation/protein synthesis assays based on different detection mechanisms.

Consistent with the literature, we observe the OP-Puro signal in both the nucleus and cytoplasm. Given that OP-Puro labeled proteins are released from the ribosomes, the OP-Puro signal does not represent the sites of subcellular translation (Enam et al., 2020). Additional translation assays

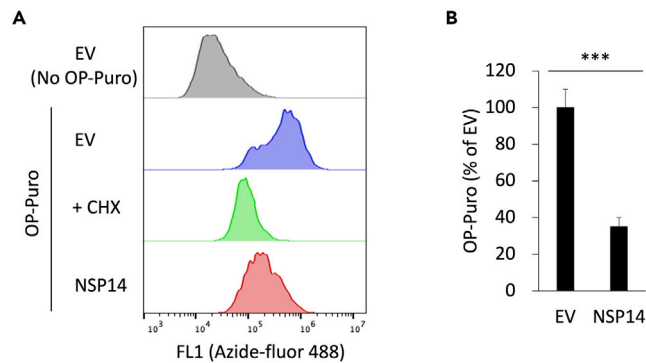


Figure 2. Flow cytometry analysis of OP-Puro labeling

(A) Histograms of HEK293T cells transfected with SARS-CoV-2 NSP14 for 24 h and stained by OP-Puro labeling with Azide-fluor 488. EV, empty vector. CHX, cycloheximide.

(B) Data represent the normalized mean fluorescence intensity between cells transfected with SARS-CoV-2 NSP14. Data are shown as mean \pm SD of three biological repeats. *** $p < 0.001$ by unpaired Student's t test.

focusing on subcellular translation are required for spatial translation studies (Jagannathan et al., 2011).

This protocol detects general cellular translation efficiency. Like other protein synthesis assays using puromycin and methionine analogs, the protocol cannot be used to determine protein synthesis of individual proteins since it is design to measure global translation. Puromycin and methionine analogs are incorporated into translated protein, introducing a “clickable” alkyne or azide for further fluorophore conjugation. However, pretreatment with methionine-free media is required to deplete free methionine for methionine analog labeling. To examine the rate of protein synthesis of specific proteins of interest, we recommend using radioactive [35 S]-methionine pulse labeling and immunoprecipitation for your target proteins. Radioactive [35 S]-methionine provides a superior sensitivity and signal-to-noise ratio. Since the labeling density corresponds to the methionine content of proteins, this approach cannot be used to study proteins without methionine. If large-scale comparison is necessary, we recommend using ribosome profiling or quantitative proteomics.

We do not recommend directly comparing OP-Puro intensities between cell lines with different genetic backgrounds, or between different time points. If a time course is necessary, we recommend normalizing OP-Puro signal with a control condition and no OP-Puro treatment along with each time points.

TROUBLESHOOTING

Problem 1

No or low signal detected for all samples including control cells.

Potential solution

- Cells are overconfluent or cell density is too high. Reduce cell plating density.
- Cells are not healthy. Check cell viability and cell culture for contamination.
- Use freshly made solutions.
- Insufficient OP-Puro labeling. When working with a new cell line, we recommend that you optimize the OP-Puro concentration and incubation time (0.5–2 h).

Problem 2

High signal detected for all samples including negative control cells.

Potential solution

- Wash cells thoroughly with Permeabilization buffer after staining. If the problem persists, we recommend decreasing the concentration of azide-conjugated fluorophore in click chemistry reaction solution. We usually use less AZDye 647 Azide which provides a strong fluorescence signal.

Problem 3

Multimodal signal detected by flow cytometry analysis.

Potential solution

- This could be a result of the nature of cell heterogeneity (due to mixture of different cell types, cell cycle stages, etc.). Cell synchronization and additional cell gating strategy for detection of the cell of interest might improve the signal.
- Cells in suspension form clumps. For labeling in Eppendorf tubes, ensure cells are fully resuspended by vortexing after each resuspension steps. Pipetting might be required for large cell clumps.
- Incomplete saponin permeabilization. Ensure cells are fully resuspended in Permeabilization buffer. Saponin-based permeabilization can be reversible. AVOID washing cells before adding click chemistry reaction solution.
- If the problem persists, we recommend increasing labeling medium volume.

Problem 4

Uneven signal detected on a coverslip by confocal microscopy analysis.

Potential solution

- For labeling on coverslips, ensure the labeling medium covers the surface and gently rock the plate every 15 min to make sure the cells are covered. If the problem persists, we recommend increasing labeling medium volume.

Problem 5

Inconsistent results between biological replicates.

Potential solution

- Translation is sensitive to cell culture conditions. Keep cell culture conditions consistent, including temperature, CO₂ level, serum-free medium composition and the lot of fetal bovine serum (FBS).
- Cells are not healthy. Check cell viability and cell culture for contamination. If cells are overconfluent or cell density is too high on the day of labeling, discard the cells and plate less cells.
- Use freshly made solutions.
- Insufficient OP-Puro labeling. See [problem 1](#), [2](#), [3](#), and [4](#).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Cresswell (peter.cresswell@yale.edu).

Materials availability

This study did not generate new reagents.

Data and code availability

This study did not generate new datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

J.H., M.L.R., and J.P. performed research; J.H. analyzed data; and J.H., M.L.R., J.P., and P.C. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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