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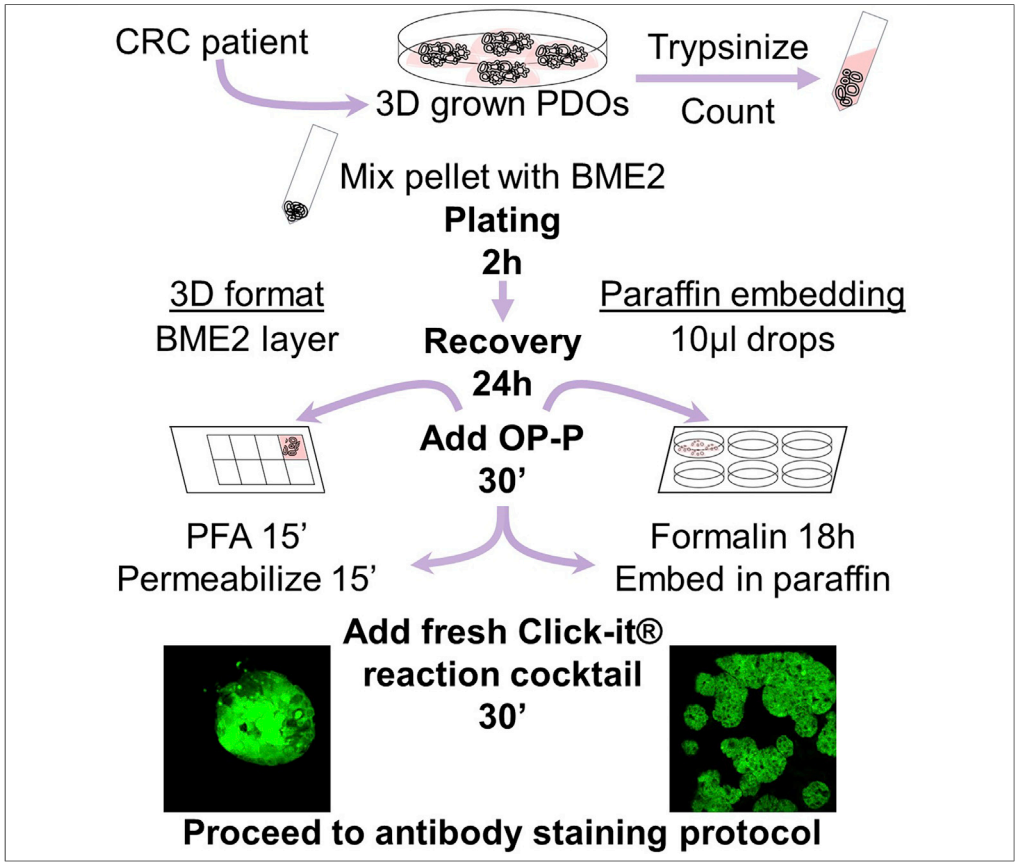
Protocol for Efficient Protein Synthesis Detection by Click Chemistry in Colorectal Cancer Patient-Derived Organoids Grown *In Vitro*



Here, we describe a protocol to detect and visualize protein synthesis by click-chemistry-based immunofluorescence in patient-derived organoids (PDOs) *in vitro*. The protocol uses O-propargyl puromycin (OPP), an analog of puromycin that enters the acceptor site of ribosomes and is incorporated into nascent polypeptides. OPP can be detected by a click chemistry reaction and can be combined with conventional antibody staining. We describe procedures for imaging intact organoids in 3D format or imaging sections of organoids from paraffin blocks.

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HIGHLIGHTS

Protocol allows for protein synthesis visualization in CRC organoids by OPP incorporation

Protein synthesis can be observed within intact organoids in 3D, or in paraffin sections

OPP detection can be combined with antibody staining

Morral et al., STAR Protocols 1, 100103
September 18, 2020
https://doi.org/10.1016/j.xpro.2020.100103

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https://doi.org/10.1016/j.xpro.2020.100103

SUMMARY

Here, we describe a protocol to detect and visualize protein synthesis by click-chemistry-based immunofluorescence in patient-derived organoids (PDOs) *in vitro*. The protocol uses O-propargyl puromycin (OPP), an analog of puromycin that enters the acceptor site of ribosomes and is incorporated into nascent polypeptides. OPP can be detected by a click chemistry reaction and can be combined with conventional antibody staining. We describe procedures for imaging intact organoids in 3D format or imaging sections of organoids from paraffin blocks. For complete details on the use and execution of this protocol, please refer to Morral, Stanisavljevic et al. (2020).

BEFORE YOU BEGIN

Cell Culture

Note: This protocol is set up for colorectal cancer (CRC) organoids. The organoid culture protocol is described elsewhere (Calon et al., 2015; Van De Wetering et al., 2015). Although we have not tested the protocol for other organoid types, the OPP incorporation protocol has been extensively used to study protein synthesis in stem cells from other tissues such as hematopoietic stem cells (Signer et al., 2014), muscle stem cells (Zismanov et al., 2016) or skin stem cells (Blanco et al., 2016). Thus, we foresee it can be adapted for other types of organoids.

1. Calculate and thaw the required amount of BME2 (Cultrex® Base Membrane Extract Type 2) the day before plating the organoids. You will need 30 μL per well for 3D format, or 200 μL per well for paraffin-embedded setting. Keep the thawed BME2 vial on ice in a cold room.
2. Pre-warm the culture dish you will use to grow the organoids (8-well culture slide in the case of 3D imaging, or standard 6-well dish for the paraffin embedding setting). You can keep a stock in the incubator if you have enough space, or leave it in the incubator the day before the experiment. If your experiment has several end points, plan to use separate plate for each end point to facilitate the fixation step.

Choosing 3D vs. Paraffin Embedding

The main advantage of the 3D format is that it preserves the organoid structure and cellular organization. We recommend this approach if you seek information about 3D distribution of protein synthesis and you want to generate 3D image reconstruction of the entire organoid morphology (example in Figure 5).

The paraffin-embedded organoid format allows you to obtain several cuts from the same organoid sample and to perform several consecutive stainings on the same experiment. However, depending on the paraffin section, it might be difficult to capture the entire organoid structure. Another advantage of this method is that you can also choose between immunofluorescence (IF) and immunohistochesmistry (IHC) on the organoid sections (example in Figure 6). It is important to consider that not all the antibodies are suitable for paraffin sections when deciding between the two methods. Finally, paraffin blocks can be stored for long periods (years) compared to 3D samples that require to be processed for the click-it reaction and the antibody staining immediately after fixation.

> *Note:* steps 1–6, 14–18, and 30–37 will be relevant for 3D organoids; steps 7–9 and 19–26 will be relevant for paraffin-embedded organoids.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-EphB2 (1:100)	RD Systems	Cat# AF467 RRID: AB_355375
Rabbit anti-CK20 (1:100)	Sigma- Aldrich	Cat# HPA024309 RRID: AB_1852220
Donkey Anti-rabbit 647 (1:500)	Thermo Fisher Scientific	Cat# A31573 RRID: AB_2536183
Donkey Anti-goat 647 (1:500)	Thermo Fisher Scientific	Cat# A21447 RRID: AB_141844
Chemicals, Peptides, and Recombinant Proteins		
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear	Amsbio	3533-010-02
DPBS	Life Technologies	14190169
Triton X-100	Sigma	T8787
BSA	Sigma	10735078001
Tween	Sigma	P1754-25ML
Formaldehyde Solution	Sigma	47608
Formalin Solution 10%	Sigma	HT501128-4L
DAPI	Sigma	D9542-1MG
DAPI-fluoromount G	Southern biotech	0100-20
Antibody diluent (FLEX)	Agilent Dako	K8006
Trypsin	Thermo Fisher Scientific	25300054
Cell Recovery Solution	Corning	354253
O-Propargyl-Puromycin	MedChemExpress/Click Chemistry Tools	HY-15680/1407-5
AFDye 488 Picolyl Azide	Click Chemistry Tools	1276-1
Critical Commercial Assays		
Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit	Thermo Fisher Scientific	C10456

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
Colon organoids	This laboratory	This laboratory
Software and Algorithms		
Leica Application Suite	https://www.leica-microsystems.com/	NA
Image J	NA	https://imagej.net/Fiji
Other		
8-well Culture Slides	Falcon	354108
Slide Cover Glass 24 mm × 50 mm	Deltalab	D102450
Bench orbital rotator	Sigma	Z654515-1EA
Leica Inverted Spectral Confocal Sp5	NA	NA

MATERIALS AND EQUIPMENT

Reagent Preparation

- Fixation buffer: 3.7% formaldehyde in PBS
- Permeabilization buffer: PBS containing 0.5% Triton X-100 in PBS
- Blocking solution: PBS containing 0.2% Triton X-100 + 0.05% Tween + 1% BSA

⚠ CRITICAL: Always use the fume hood and follow safety measures when preparing and handling formaldehyde. It is highly toxic, carcinogenic, and because it can cause skin irritation, it is recommendable to wear protective clothes, gloves, as well as face mask.

Alternatives: Thermo Fisher OPP detection kit; some of the reagents provided with the kit can be purchased individually from other vendors or prepared from scratch in the lab.

- OPP
  OPP from MedChemExpress or from Click Chemistry Tools is custom-synthesized and shipped as a white powder that should be kept at 4°C. Once reconstituted in PBS (20 mM), OPP should be aliquoted and stored at −20°C for up to 24 months.
- Alexa picolyl azide
  Different Alexa picolyl azide colors can be purchased from Click Chemistry Tools (https://clickchemistrytools.com/product/afdye-488-picolyl-azide/). We have tested and used this azide. The same company also provides a detection kit, but we have not tested it.

Alternatives: A detailed protocol for making click reaction from scratch can be found elsewhere (Calve et al., 2016) We did not test this protocol.

STEP-BY-STEP METHOD DETAILS

Organoid Plating – 3D Format

Timing: 2 h

Here we describe how to plate the organoids, preserving their morphological and cellular organization in order to acquire high-resolution confocal images for 3D reconstruction.

Note: For this protocol, we plate the organoids in a thin layer of BME2 (example in Figure 2A) instead of the standard drops. This makes the organoids more accessible to the stain, since

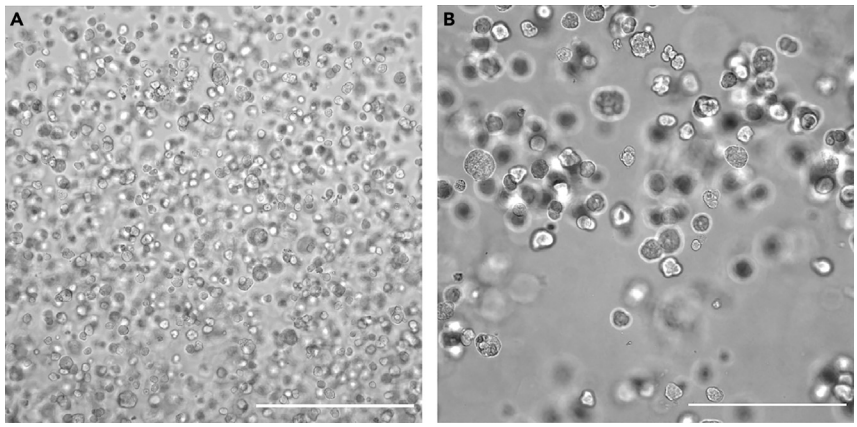

Figure 1. Representative Image of Single Cells and Small Organoids

(A) Single cell suspension generated by trypsinization.

(B) Small organoids generated by dissolving the BME2 with Cell Recovery Solution. Scale bars: 500 μm.

the thickness of the BME2 drop may prevent the reagents from reaching the inner parts, leaving the organoids unlabeled. Using a layer instead of a drop decreases the thickness, facilitating the imaging process.

Note: Plate the organoids at least one day before the experiment and let them recover for 12–16 h. We recommend plating between 5–7 × 10^4 cells per well. You can let them to grow for a couple of days in order to see crypt-like structures with cellular heterogeneity (stem and differentiated cells).

Note: Place the 8-well culture slide with the organoids in a humid chamber to avoid evaporation of the organoid culture medium.

1. Plate the organoids in an 8-well culture slide; you have two options depending on your experimental conditions:
   a. Plate the organoids in a single cell format: Remove the medium and resuspend the drops containing the organoids directly with pre-warmed Trypsin. You can do this in the same plate where the organoids were grown. We recommend using 1.5 mL of Trypsin per one 6-well. Incubate 5 to 15 min at 37°C, checking regularly under the microscope the state of the organoids. Pipet up and down using p1000 first, and then switch to p200 in order to dissolve the BME2 and break the organoids down to a single cell format (Figure 1A). Confirm under the microscope that you have achieved single cell format, transfer the mixture to a 15mL tube and add 5 mL of medium. Centrifuge and wash once resuspending the pellet in 5 mL of medium. Count the cells and plate the organoids in the 8-well culture slide.
   b. Plate the organoids as a small organoids: If you do not want to generate single cells, we recommend to resuspend the drops containing the organoids in Cell Recovery Solution that will allow you to dissolve the BME2 while preserving the organoid structure (Figure 1B). Use 1.5 mL of Cell Recovery Solution per one 6-well and gently disaggregate the drops using p1000. Transfer the mixture into a 15 mL tube and keep it on ice for 30 min. After this incubation period, gently mix with p1000, add 5 mL of cold PBS and centrifuge the mixture. Wash the pellet with cold PBS and centrifuge again to obtain the pellet. In order to have an estimation of the number of cells you have, we suggest taking an aliquot of your organoid suspension before the incubation with Cell Recovery Solution and bringing it to a single cell state using Trypsin, counting, and extrapolating the result.

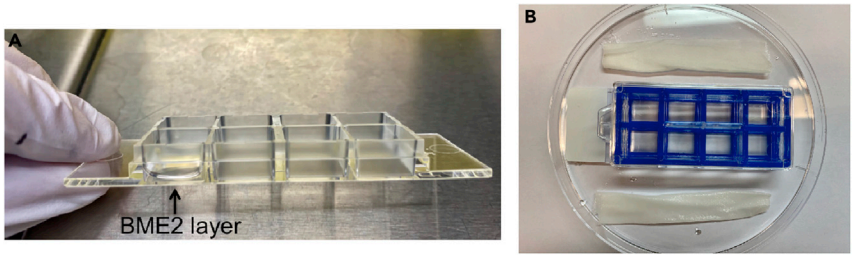

Figure 2. 8-Well Culture Slide to Plate the BME2 Layer Containing the Organoids

(A) Representative image of an 8-well culture slide with a BME2 layer containing the organoids (first well on the left). (B) During the incubation periods, place the culture slide within a humid chamber to avoid evaporation. Notice the wet tissues on both sides of the slide.

2. Resuspend the single cell or the small organoids pellet with BME2 by gently pipetting up and down. The amount of BME2 required would vary depending on the number of wells you need to plate. There is no need to perform this step on ice if the procedure is done quickly.

⚠ CRITICAL: Avoid bubble formation when resuspending the organoid pellet with BME2.

3. Distribute 20 μL of the mixture per well in a layer format (Figure 2A). With the help of apipette, make sure the mixture is evenly distributed and that it reaches all the walls and corners of the well. Avoid bubbles.

⚠ CRITICAL: Make sure to distribute the BME2 uniformly over the well when plating the organoids in the 8-well culture slides.

4. Place the 8-well culture slide in the incubator at 37°C. Because of its shortness, this step does not require a humid chamber. A minimum of 30 min is required for a proper solidification. This period of time can be extended for up to 1 h.
5. Add 400 μL of the appropriate growth medium to each well on top of the BME2 layer containing the organoids.
6. Place the 8-well culture slide within a humid chamber and let the organoids recover between 12–16 h in the incubator at 37°C, 5% CO_2 (Figure 2B).

Organoid Plating – Paraffin Embedding

Timing: 2 h

Here we describe the procedure to plate the organoids for paraffin embedding purpose. In this case, the organoids are plated in regular 6-well plates in a drop format (Figure 3).

Note: The number of organoids per well can vary depending on the experiment. Is important to calculate the number of wells you will plate before you start the experiment.

Note: To have enough material, at least one full well of a 6-well plate is needed per condition (approximately 1 million of cells).

Note: Plate the organoids at least one the day before the experiment and let them recover for 12–16 h.

7. Plate the organoids in BME2 drops following the same guidelines we provided before. You can also plate single cells or small organoids. If you cannot plate the organoids immediately after you

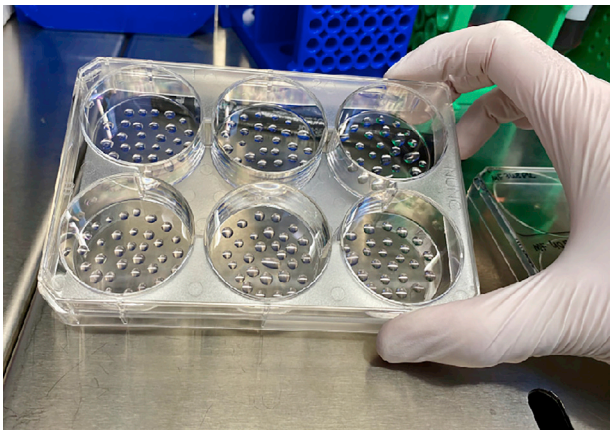

Figure 3. Organoids Plated in Drop Format in a 6-Well Plate
For the paraffin embedding protocol, organoids are plated in drop format in a 6-well plate.

mix them with BME2, the mixture must be kept on ice until you use it, or the BME2 will solidify. If you are using a 6-well plate format, we recommend using 200 μL of BME2 per well distributed uniformly in a form of 10 μL drops (20 drops/well).

⚠ CRITICAL: If the experiment requires a high numbers of wells, or if you have little experience handling BME2, we recommend aliquoting the suspension of organoids in BME2 in order to avoid solidification. If using an Eppendorf, hold it by the lid, the warmth of your hands may speed up the process of BME2 solidification.

8. Allow the drops to solidify in the incubator at 37°C for 30 min before adding the medium. A minimum of 30 min is required for a proper solidification. This period of time can be extended up to 1 h. We recommend using 2 to 3 mL of growth medium per well for the 6-well plate format. You can reduce the volume down to 1 mL for short incubations, such as during OPP incubation (1–2 h maximum).
9. Let the organoids recover for 12–16 h in the incubator at 37°C, 5% CO_2.

OPP Incorporation– 3D Format and Paraffin Embedding

Timing: 1 h

During the OPP incorporation step, organoids are kept with regular growth medium in order to let the compound be incorporated into newly synthesized proteins.

Note: The steps for the OPP incorporation are shared for both the 3D format and the paraffin embedding protocols. 30 min of OPP incubation should be sufficient to visualize its incorporation into nascent proteins. However, the incubation time can be extended depending on the organoid line.

10. Preparation of **OPP working solution**:
    a) Dilute the OPP reagent (from 20 mM stock) 1:1,000 in organoid culture medium to prepare a 20 μM final working solution. Prepare it fresh for every experiment.

⚠ CRITICAL: The range of concentration for OPP is between 2 μM to 20 μM. We recommend you to test and determine the optimal concentration yourself.

11. Remove the organoid containing medium carefully and add 300 μL of OPP working solution per well for the 8-well culture slides or 1 mL per well for the 6-well plate. Alternatively, add OPP (1:1,000) directly to the well whenever you want to avoid the effect of fresh medium addition on your culture, or in the case there is a pre-treatment with a drug you wish to keep.
12. Incubate the organoids for 30 min at 37°C, or under optimal conditions for the organoid type.

⚠ CRITICAL: OPP incorporation into nascent polypeptides halts translation elongation and leads to the generation of truncated peptides that can be targeted for degradation. It has been shown the maximum peak of OPP incorporation is detected 1 h after administration (Liu et al., 2012; Signer et al., 2014). However, we already detected OPP with 30 min incubation period in our organoid model. Thus, we recommend incubation periods between 30 min and 1 h in order to maximize detection and minimize the influence of differences in degradation activity and eventually cell death. We highly recommend testing different incubation periods and adjust it to the organoid or cell line used before the final experiment.

13. Proceed to fixation and permeabilization procedures specific for the 3D format (steps 14–18) or for the paraffin embedding (steps 19–26).

Fixation and Permeabilization– 3D Format

Timing: 2 h

After OPP incorporation, the organoids are fixed and permeabilized, followed by OPP detection and antibody staining.

14. After incubation, remove the medium containing the OPP and wash the cells carefully once with PBS. Remove PBS.
15. **Fixation**: This step must be done in the fume hood! Add 300 μL of freshly prepared 3.7% Formaldehyde. Incubate for 15 min at 21°C–23°C.
16. Remove the fixative inside the fume hood and wash twice with PBS.

⚠ CRITICAL: After fixation, the BME2 can lose consistency. Therefore, from this step onwards is critical to be careful when removing any liquid from the 8-well culture slide. We recommend tilting the 8-well chamber, placing the tip of the pipette in one of the well corners and removing the solution slowly in order to prevent any loss of BME2 containing the organoids. A p1000 pipette tip with a cut end can help.

17. **Permeabilization**: Add 300 μL of permeabilization buffer. Incubate 20 min at 21°C.
18. Remove the permeabilization buffer and wash twice with PBS.

Fixation– Paraffin Samples

Timing: 2 days

Here we describe how to perform the fixation of the drops containing the organoids and the material required for the embedding into paraffin blocks.

Note: We recommend performing the fixation of the BME2 drops directly in the same 6-well plate.

19. Wash the wells with PBS.

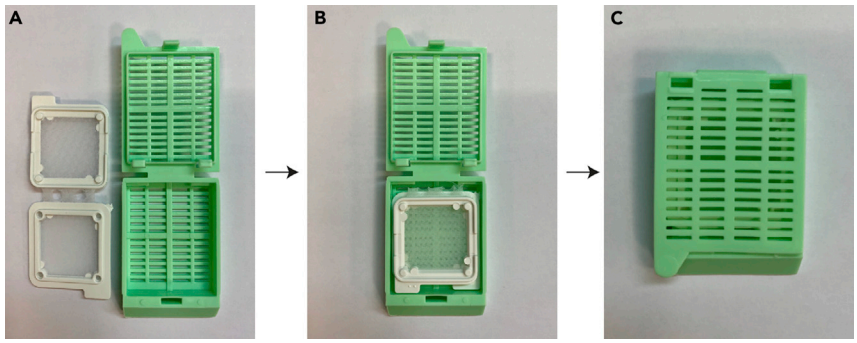

Figure 4. Microcassette and Cassette Used to Embed the Organoid Pellets in Paraffin

(A, B and C) After fixation, drops containing the organoids are placed within the microcassette (white) which will be placed inside the normal cassette (green).

20. From this step onward, sterile conditions are no longer required. Inside the fume hood, carefully add 3 mL of Formalin per well and leave the plates inside the hood for 2h at 21°C. Use tin foil to protect the plates from light.
21. Wash the wells once with PBS. Remove PBS.
22. Add again Formalin to the wells. Seal the plates with Parafilm and incubate for 16–18 h at 4°C, protected from light.
23. Wash once with PBS. Remove PBS.
24. Tilt the plates and remove the liquid, and, by gently pushing with the p1000 tip, detach entire drops from the plate.
25. Collect the drops as gently as possible using a p1000 pipette with a tip cut at an angle (to make it wider). Transfer the drops to a microcassette, and put the microcassette inside a normal cassette (Figure 4).

26. Place the cassette in a vessel containing PBS and keep it fully submerged. Proceed to standard protocol for paraffin embedding.

⚠ CRITICAL: All steps except the 12–16 h incubation must be performed inside the fume hood. There are several ways to transport the drops from a plate into a microcassette, including using a cell scrapper to detach the drops.

OPP Detection: Click-it Reaction

Timing: 1 h

In this section we describe how to prepare the Click-it OPP reaction cocktail for a successful OPP detection.

Note: The steps for OPP detection are shared for both the 3D and the paraffin embedding protocols. In the case of the 3D format, the click reaction will take place inside the 8-well culture slide. For the paraffin-embedded organoids, if you have followed the standard protocol for immunofluorescence using paraffin-embedded samples, you should stop before the incubation with the primary antibody and perform the click-it reaction prior to it. Even if you do not plan to use any primary antibody, you should still perform all the steps of rehydration and antigen retrieval of your organoid paraffin sections.

27. Prepare the **Click-it OPP reaction cocktail** following the Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit from Thermo Fisher Scientific.

a. Prepare all the reagents required for the Click-it OPP reaction cocktail following the kit protocol (steps 1.1–1.4)
b. Follow the recipe provided in Table 2 of Thermo Fisher'skit protocol to prepare the Click-it OPP reaction cocktail (steps 5.1 and 5.2).

⚠ CRITICAL: The Click-it OPP reaction cocktail needs to be prepared fresh every time and must be used immediately. When mixing the different components of the click-it cocktail is crucial to follow the same order as they are listed in the table provided in the kit instructions.

28. Add 150µl of Click-it OPP reaction cocktail per well for the 3D setting and incubate for 30 min at 21°C. Alternatively, incubate the slides carrying paraffin cuts, as you would normally incubate with the antibody. Volumes should be adjusted according to your needs. Make sure to protect the sample from light from this step forward.
29. After the click-it reaction, wash twice with PBS. After this step, you can already proceed to analyze your samples. Alternatively, you can continue with immunofluorescence protocol.

Alternatives: You can use a different color of Alexa Fluor picolyl azide. Alexa picolyl azide can be purchased from different vendors, the one we tested is listed in the troubleshooting section below.

Blocking and Antibody Staining-3D Format

Timing: 2 days

Note: For the paraffin-embedded organoids, continue with your standard immunohistochemistry or immunofluorescence protocol for paraffin sections.

30. Blocking: Add 300 µL of blocking solution and incubate 1h at 21°C.
31. Prepare 150 µL of the primary antibody dilution per well in a standard antibody diluent or in a blocking solution. Different primary antibodies can be combined together. (See the Key Resources Table for the antibodies we have used). Add to each well the desired combination of the antibodies and incubate for 12–16 h at 4°C in humid conditions, protected from light.
32. After incubation, wash each well 3 times for 5 min with 300 µL PBS. Place the chamber slide on a benchtop orbital shaker set to 60 RPM during each wash.
33. Dilute the corresponding secondary antibodies using a standard antibody diluent or in a blocking solution and add 150 µL to each well. Incubate for 1 h at 21°C protected from light.
34. *Optional:* To visualize DNA/nuclei prepare a solution of DAPI (1µg/mL) in PBS. Remove the secondary antibody,add 300 µL to each well and incubate for 5–10 min.
    a. Alternatively, you can use Fluoromount G mounting medium, which already contains DAPI and this step can be skipped.
35. Wash each well 3 times with 300 µL PBS.
36. Remove the PBS and detach the upper part using the removal kit provided with the chambers.
37. Using a p200 pipette tip with the tip cut off add a small amount of Fluorescent DAPI mounting medium over each specimen (~20–30 µL each). Place a cover slip over the slide, avoiding bubbles. Use clear nail polish to seal the sides of the cover glass to the slide. Allow to harden for 12–16 h at 21°C in the dark.

EXPECTED OUTCOMES

- Following the 3D setting protocol the organoids retain their morphology. Z-stacks can be acquired using a confocal microscope and further processed to generate a 3D reconstruction of the full organoid structure. OPP staining is detected in the nucleus and cytoplasm in cells that have incorporated the compound. In this example, CK20 antibody staining marks the cytoplasm of differentiated progenitors intermingled within the organoid. If DAPI was used, the

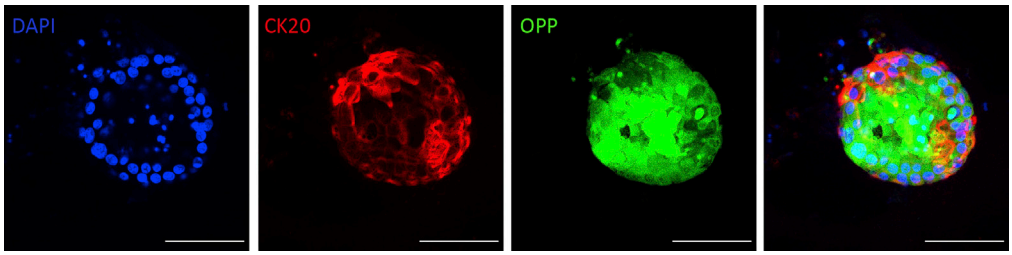

Figure 5. Immunofluorescence of CK20 (Red) and OPP (Green) in Colorectal Cancer Organoids in 3D Format
Colorectal cancer organoids plated in 8-well culture slides were incubated 30 min with OPP *in vitro* followed by click-it reaction and antibody staining. Images were taken using a Leica inverted confocal SP5 microscope. Scale bars: 50 μm.

nuclei of individual cells were also labeled, facilitating the observation of the entire structure of the organoids (Figure 5).

- Following the paraffin-embedded protocol, single images of the paraffin cuts can be acquired either by conventional confocal microscopy, or by scanning the entire slide using a digital slide scanner. In this case, the resolution of the organoid morphology is less accurate, but is a useful setting to visualize and quantify overall changes in protein synthesis. In this example, OPP staining is combined with the cell membrane receptor EPHB2, a stem cell marker. DAPI is also included in the staining to visualize the nuclei of the cells. (Figure 6).

LIMITATIONS

The protocol uses a click-chemistry based technique to compare overall protein synthesis rate. If your aim is to compare levels of particular proteins, this technique is not suitable. The time window we use for the labelling is short, and thus, only polypeptides synthetized during this period will be detected. Secondly, OPP incorporation halts the translation in a random fashion and the entire protein most likely will not be produced, preventing further comparison of individual protein levels. For such experiments, we encourage you to use HPG or AHA, click chemistry based analogs of methionine, that will be incorporated into fully functional proteins.

This is a qualitative assay and we do not recommend you to compare OPP intensity levels between different organoid types, nor between experiments where click reaction was not performed simultaneously.

For the 3D setting, we recommend proceeding immediately to OPP detection steps because of the fragility of the BME layer. For this reason, time course experiments should be performed in such a way that the end point is the same for all conditions, which may limit your options.

Not all primary antibodies work for paraffin sections, and you will have to test each antibody before combining it with click reaction.

Although we only tested colorectal cancer organoids, we foresee that in organoids derived from tissues with low protein turnover rate OPP might be difficult to detect.

TROUBLESHOOTING

Problem

For the 3D setting, after the fixation and permeabilization steps, the BME2 can be degraded and you can lose material during the following washes and incubation steps.

Potential Solution

We highly recommend plating a high number of organoids per each well. For the 3D setting, around 50 to 70.000 cells per well is the standard amount.

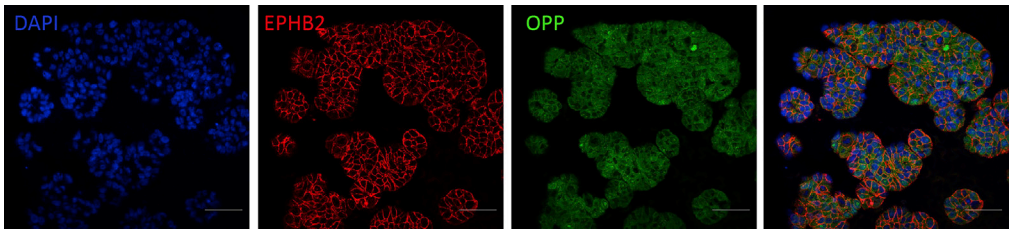

Figure 6. Immunofluorescence of EPHB2 (Red) and OPP (Green) in Paraffin-Embedded Colorectal Cancer Organoids
Colorectal cancer organoids were incubated during 30 min with OPP *in vitro* and embedded into paraffin blocks. Click-it reaction and antibody staining was performed on the paraffin cuts and visualized using a Leica inverted confocal SP5 microscope. Scale bars: 50 μm.

Problem
Organoids cannot be detected in the paraffin cuts.

Potential Solution
Since the starting amount of the organoid material placed inside the micro cassette is low, we also highly recommend placing at least one full well (1 million cells) of fixed drops per microcassette. You will probably have to perform several cuts of the paraffin block until you reach the organoids. Use the position of the microcassette as a starting guide when you cut the paraffin.

Problem
OPP is not detected.

Potential Solution
The click-it reaction might not have worked for several reasons:

Make sure you prepare the click-it reaction cocktail fresh following the recipe provided in the manufacturer protocol and that you mix all the components in the correct order. All reagents should be brought to 21°C before the use. Once prepared, the reaction mixture must be used immediately. Make sure you incubate the click-it reaction at 21°C protected from light. Make sure you are using a correct picolyl azide dye. In case there are several click reaction kits in use in your lab, make sure that you are not using an incorrect kit, or incorrect combination of the reagents. Another potential explanation would be that your permeabilization protocol has not worked and the azide dye has not penetrated into the cell.

The cause for no OPP signal can also be the OPP concentration or the incubation time. In our experiments, we used the highest concentration from the range recommended by Thermo Fisher, and we did not test higher ones. Incubation time can be extended, we tested incubations for up to 2h.

Problem
No signal detected from the antibody used in the combination with click-it reaction.

Potential Solution
For the 3D setting, the standard protocol for click-it reaction includes permeabilization step with Triton X-100. This may not be suitable for all the antibodies. Thermo Fisher protocol for OPP allows the use of alternative permeabilization methods, and we recommend testing the reaction with the permeabilization most suited for the antibody you wish to use and finding the optimal conditions before your final experiment.

In the case of the paraffin sections, not all antibodies are suitable for paraffin slides and you should check this information for your antibody. We do not foresee any possible incompatibility with the

click-it reaction itself and the antibody staining, except the proper choice of fluorophore combination where spectra do not overlap. We recommend testing the antibodies without click-it reaction and finding the optimal conditions before you proceed to include the click-it reaction step.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eduard Batlle (eduard.batlle@irbbarcelona.org).

Materials Availability

CRC organoids used in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

This study did not generate new datasets or code.

ACKNOWLEDGMENTS

We thank all members of the Batlle laboratory and IRB Barcelona core facilities for extensive support; Giorgio Stassi and the HUB (Netherlands) for sharing PDO7 and PDO-p18 CRC organoids, respectively. C.M. held La Caixa predoctoral fellowships and J.S. held a Juan de la Cierva postdoctoral fellowship from the MINECO. This work has been supported by grants from ERC (ERC AdvG 340176), WWCR (grant 12_1209) and AGAUR (2017 SGR 698).

AUTHOR CONTRIBUTIONS

E.B. supervised the study. C.M. and J.S. wrote the manuscript. C.M. and J.S. designed and performed the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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