Metabolic Labeling with Noncanonical Amino Acids and Visualization by Chemoselective Fluorescent Tagging

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ABSTRACT

Fluorescent labeling of proteins by genetically encoded fluorescent protein tags has enabled an enhanced understanding of cell biological processes but is restricted to the analysis of a limited number of identified proteins. This approach does not permit, e.g., the unbiased visualization of a full proteome in situ. We describe here a fluorescence-based method to follow proteome-wide patterns of newly synthesized proteins in cultured cells, tissue slices, and a whole organism. This technique is compatible with immunohistochemistry and in situ hybridization. Key to this method is the introduction of a small bio-orthogonal reactive group by metabolic labeling. This is accomplished by replacing the amino acid methionine by the azide-bearing methionine surrogate azidohomoalanine (AHA) in a step very similar to classical radioisotope labeling. Subsequently, an alkyne-bearing fluorophore is covalently attached to the group by "click chemistry"—a copper(I)-catalyzed [3+2]azide-alkyne cycloaddition. By similar means, metabolic labeling can also be performed with the alkyne-bearing homopropargylglycine (HPG) and clicked to an azide-functionalized fluorophore. *Curr. Protoc. Cell Biol.* 56:7.11.1-7.11.29.

Keywords: FUNCAT • click chemistry • copper(I)-catalyzed [3+2]azide-alkyne cycloaddition • AHA • HPG • protein synthesis

INTRODUCTION

This unit describes fluorescent noncanonical amino acid tagging (FUNCAT), a recently developed fluorescent labeling method to visualize proteome-wide spatio-temporal patterns of newly synthesized proteins (Beatty and Tirrell, 2008; Roche et al., 2009; Dieterich et al., 2010; Tcherkezian et al., 2010; Hinz et al., 2012). This method complements its nonfluorescent sister technology BONCAT (bio-orthogonal noncanonical amino acid tagging) that enables the tagging of newly synthesized proteins for selective isolation and identification (Dieterich et al., 2006; for detailed BONCAT protocol see Dieterich et al., 2007).

FUNCAT is based on the introduction of small bio-orthogonal, chemically reactive alkyne or azide groups into proteins by means of metabolic labeling with the noncanonical amino acid analogs azidohomoalanine (AHA, azide-bearing) or homopropargylglycine (HPG, alkyne-bearing). Both amino acid analogs are surrogates for methionine and are incorporated into nascent proteins when applied to the extracellular medium and taken up by the cells (Dieterich et al., 2006). Thus, the metabolic labeling step is very similar to classical radioisotope labeling and can be combined with or follow drug treatment

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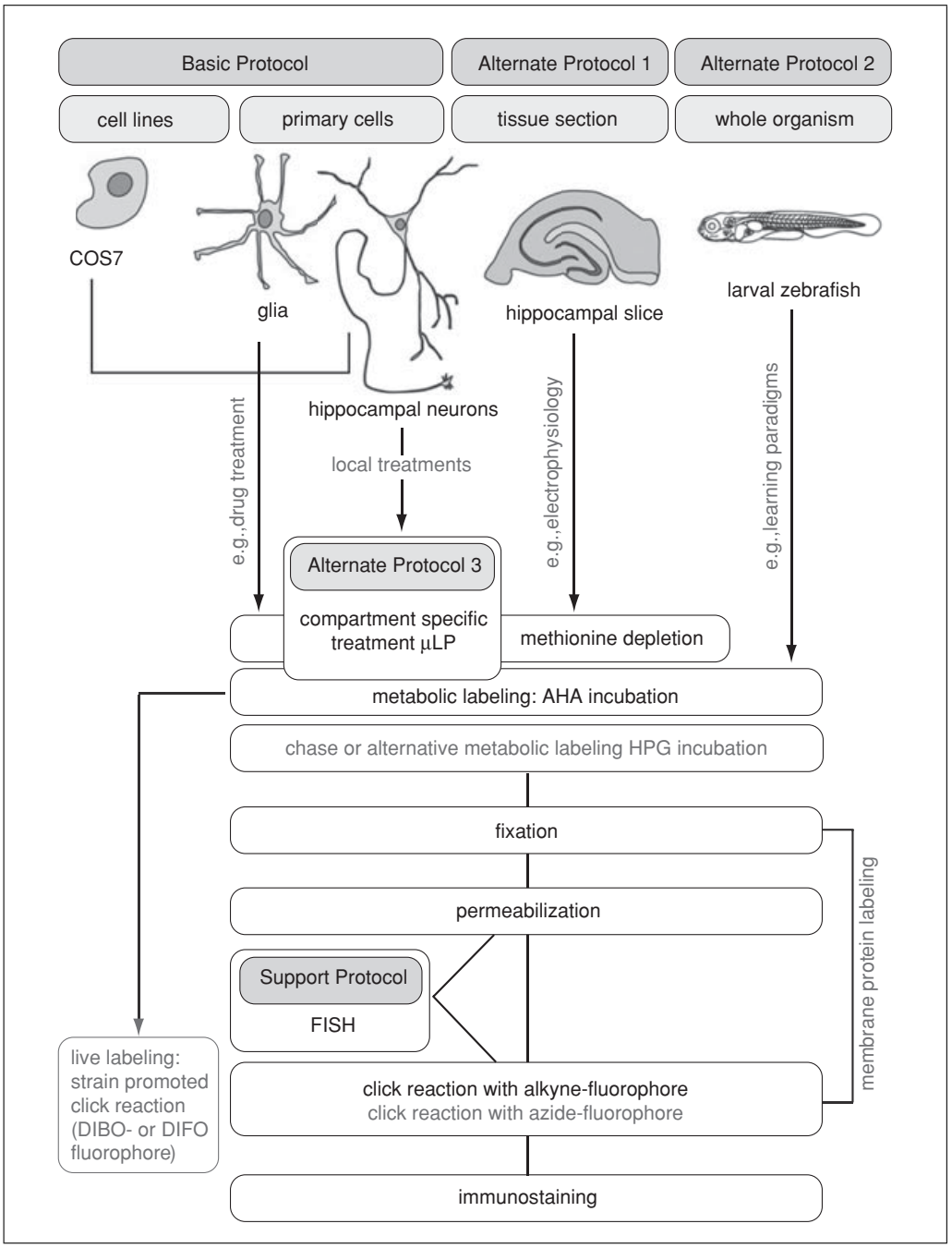

Figure 7.11.1 FUNCAT strategy and protocol overview. The flow chart summarizes the steps of the protocols provided and indicates protocol choice points. Alternatives and options mentioned in the text, but not extensively described, are included to indicate potential extensions (light gray).

or electrophysiological stimulation (Fig. 7.11.1). To increase the fraction of replaced methionine, a methionine depletion step prior to AHA or HPG addition is advisable, and methionine must be absent from the medium during the metabolic labeling reaction. The incorporated azide or alkyne groups, as nonbiological reactive "handles," serve to distinguish newly synthesized proteins from the pre-existing protein fraction before metabolic labeling. Following AHA (or HPG) treatment cells are fixed and a fluorophore is covalently and chemoselectively attached to the introduced functional groups by means of "click chemistry"—a copper(I)-catalyzed [3+2]azide-alkyne cycloaddition (for details and chemistry see Background Information).

STRATEGIC PLANNING

The Basic Protocol describes FUNCAT with AHA metabolic labeling of cultured cell lines and primary cells (COS cells, hippocampal neurons, glial cells) plated on coverslips or glass-bottom dishes, visualization of newly synthesized proteins in fixed cells by chemoselective reaction with a fluorophore-alkyne, and subsequent immunolabeling (Fig. 7.11.1).

Three alternate protocols are provided in the following sections to describe differences in the protocol when applying FUNCAT to hippocampal slices (Alternate Protocol 1), to a whole organism—larval zebrafish—(Alternate Protocol 2); and to hippocampal neurons cultured in microfluidic chamber devices (Alternate Protocol 3; Fig. 7.11.1). The first and second approaches visualize protein synthesis in tissue with intact circuitries; thus they are perfectly suited to combine them with electrophysiology or, as in the case of zebrafish larvae, with behavioral studies. The FUNCAT procedure described in Alternate Protocol 3 is designed to allow compartment-specific treatment of neurons—an approach to study aspects of local protein synthesis or local pharmacological manipulation. Since the method is compatible with immunohistochemistry; all protocols include a section describing post-hoc antibody labeling. The Support Protocol provides a guide to combine FUNCAT with high-resolution fluorescence in situ hybridization (FISH, Fig. 7.11.1). This will be of relevance when bridging the gap between in situ localization of mRNAs, translation, and the newly translated proteome.

The decision about which tissue or cell line to use, which protocol, and the exact conditions to carry out the FUNCAT labeling obviously depends on the biological question of interest. In the protocols provided we give recommendations for appropriate concentrations and incubation times to use—these serve as good starting points as these conditions typically yield robust labeling. In the protocols we indicate the importance of the biological question and discuss several parameters to consider. We also discuss the limitations of this method in the Commentary. Figure 7.11.1 gives an overview of the protocols and shows additional options for further extending experiments (e.g., to live imaging studies).

FUNCAT IN CELL LINES AND PRIMARY CELLS

BASIC PROTOCOL

This protocol describes the metabolic labeling of cultured standard cell lines or cultured primary cells with the azide-bearing noncanonical amino acid azidohomoalanine (AHA) or alternatively the alkyne-bearing amino acid homopropargylglycine (HPG) and the subsequent visualization of labeled proteins using chemoselective fluorescence tagging based on click-chemistry. It is applicable for the examination of new protein synthesis on a cellular level within a specified time frame and specified conditions. Since the fluorescence tagging procedure is performed with fixed and permeabilized cells, newly synthesized proteins of all cell compartments can be visualized.

The protocol is divided into three parts including the metabolic labeling of cells, the FUNCAT-reaction allowing visualization of labeled proteins, and an optional additional immunocytochemistry procedure. Included are basic recommendations and relevant observations for the procedure. This procedure is easy to perform and allows robust and reproducible results in a time frame of about two days.

Materials

Adherent cells from primary cell preparation or cell lines grown on:
- 18-mm glass coverslips in a 12-well culture plate *or*
- 12-mm glass coverslips in a 24-well culture plate *or*
- MatTek glass-bottom dishes

Methionine-free media [e.g., HBS (see recipe) or methionine-free DMEM with supplements (see recipe) or methionine-free Hibernate A with B27 (see recipe)]

100 mM AHA (see recipe)
100 mM methionine (see recipe)
40 mM anisomycin (see recipe)
Ice
PBS-MC (see recipe)
Fixation solutions: e.g., PFA-sucrose (see recipe) *or* PLP (see recipe)
Phosphate-buffered saline (PBS; see recipe), pH 7.4
B-Block (see recipe)
PBS-Tx (see recipe), optional
Phosphate-buffered saline (PBS; see recipe for 10× PBS), pH 7.8
200 mM TBTA in dimethyl sulfoxide (DMSO), triazole ligand (see recipe)
500 mM TCEP in H_2O (see recipe)
2 mM fluorophore-alkyne-tag in DMSO (see recipe)
200 mM $CuSO_4$ in H_2O (see recipe)
FUNCAT wash buffer (see recipe)
Primary antibody (e.g., rabbit anti-MAP2 *or* mouse anti-MAP2)
C-Block (see recipe), optional
Secondary antibody, fluorophore-coupled (e.g., goat anti-rabbit-Alexa 647 *or* goat anti-mouse Alexa 647)
1 mg/ml DAPI (see recipe)
Mounting medium: e.g., Mowiol *or* Fluoromount *or* Aquapolymount

Vortex mixer
Horizontal shaker
FUNCAT incubation plate (see special equipment, Fig. 7.11.2A) *or* MatTek overhead incubation support (see special equipment, Fig. 7.11.2B)
Microscopic slides

Label newly synthesized proteins with AHA

Day 1

1. Wash the cells on coverslips with HBS or methionine-free medium prewarmed to 37°C.

   Densities 10,000 to 40,000 for hippocampal neurons in MatTek dishes or 24-well plate, cell lines and glial cells 80% confluency.

   Use the following volumes for incubation steps and washes: 500 µl for 12-mm coverslips/24-well plate and MatTek dishes and 1 ml for 18-mm coverslips/12-well plate.

Figure 7.11.2 *(appears on next page)* Special reagents and materials described in the FUNCAT protocols. (**A**) FUNCAT incubation plate made from a 24-well cell culture plate with paraffin drops for upside-down incubation of circular coverslips. (**B**) Tube-lid support filled with modeling clay and sealed with two-component epoxy glue for upside-down incubation of MatTek glass-bottom dishes. (**C**) Standard microfluidic chamber for compartmentalized neuron incubation. Neurons are plated on the cell body chamber side through the connected wells 1. They extend axons and dendrites into the microgrooves but only axons grow the whole 900-µm distance through the microgrooves and reach the axon chamber that is accessible via the connected wells 2. Dendrites usually stop growing at a 200- to 300-µm distance within the microgrooves. The cell body and axon chamber can be fluidically isolated; therefore, compartments can be incubated with different solutions. (**D**) The microfluidic local perfusion (µLP) version of the microfluidic chamber has a perfusion channel perpendicular to the microgrooves (Taylor et al., 2010). It is located at a distance from the cell body chamber where dendrites still populate the microgrooves. Thus, perfusion via the perfusion channel allows one to manipulate selectively a proportion of dendrites and axons. (**E**) The microfluidic chambers are assembled on coverslips where the cells attach. After metabolic labeling, the PDMS part of the chamber is removed and the cells are fixed and processed further on the coverslip. Upside-down incubation for the click reaction is performed on Parafilm with unilateral silicone spacer support in a humidified chamber.

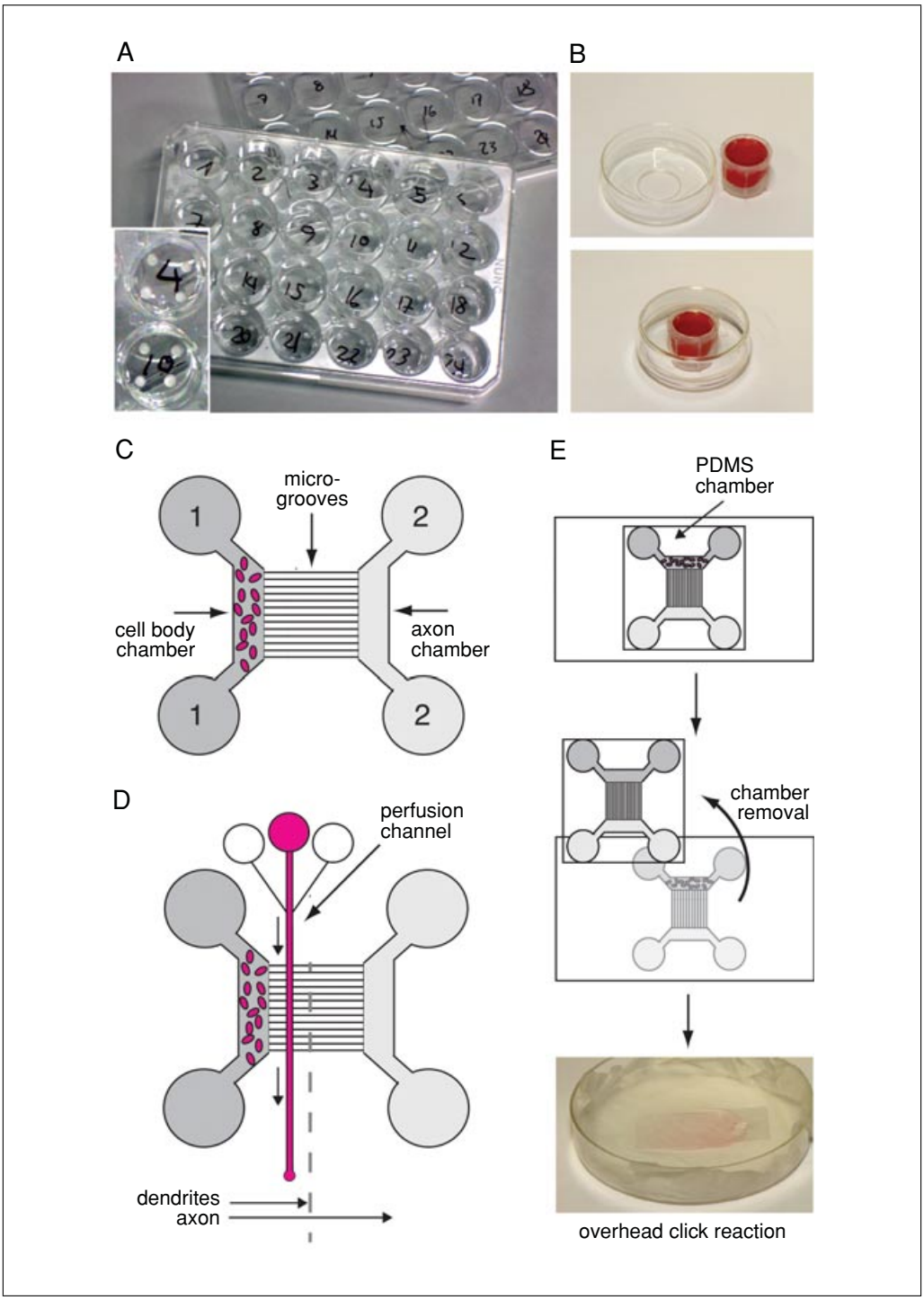

Figure 7.11.2 *(legend appears on previous page)*

Choose labeling medium according to cell type and duration of labeling time. For cell lines, methionine-free DMEM can be used, and for primary neuronal cultures, the methionine-free Hibernate A medium can be used. Supplementing the medium with serum or similar additives will not reduce labeling efficiency. For labeling times up to 1 hr, 1× HBS is sufficient. Longer labeling procedures demand methionine-free medium to supply cells with additional nutrients. Incubate neurons preferentially with methionine-free Hibernate A supplemented with B27.

2. Incubate the cells with 1× HBS or methionine-free medium for 20 to 30 min using appropriate culture conditions for the cell type used.

3. Dilute AHA from the stock solution to a final concentration of 4 mM AHA in methionine-free medium and add any other desired stimulus/drug. Incubate the cells for 2 hr (or any other appropriate time) using culture conditions according to the cell type. To evaluate background labeling, treat control cells with 4 mM methionine and/or 4 mM AHA in presence of a protein synthesis inhibitor (e.g., 40 μM anisomycin).

   AHA is relatively expensive but can easily be synthesized (Link et al., 2007) using a standard rotary evaporator. Make sure that there are no precipitates or filter fibers in the stock solution when using self-synthesized AHA since they lead to fluorescent precipitate signal later. Centrifugation or filtration clears the stock solution.

   Depending on the biological question, use AHA at other concentrations. We suggest 4 mM AHA as an initial concentration, as it leads to robust labeling in a time frame of minutes to hours. When adjusting incubation conditions to the question of interest, first vary incubation times and as a second step vary concentrations.

   Alternatively, AHA can be replaced by HPG (or added in a second incubation phase). In this case, an azide-bearing fluorescent tag has to be used in the click reaction step (step 10d).

4. *Optional:* Incubate with 1× HBS or methionine-free medium with 4 mM methionine for 15 min.

   A chase of AHA with methionine reduces background when expected integration of AHA is low and the signal after fluorescent tagging is weak.

5. Place the dishes on ice. Wash the dishes briefly two times, each time with ice-cold PBS-MC.

6. Fix with PFA-sucrose for 20 min at room temperature or PLP for 2 min at 37°C and 20 min on ice.

   Gentle agitation ensures even fixation.

7. Wash three times, each time with PBS, pH 7.4, for 10 min at room temperature.

   At this point, coverslips can be stored in PBS, pH 7.4, at 4°C for several days.

Perform chemo-selective fluorescent tagging

8. Incubate the cells on coverslips in B-Block with Triton X-100 for 1.5 hr at room temperature to permeabilize the cells and block unspecific binding sites.

   Both B-Block and C-Block work well and either block is used and only changed if antibodies do not work in the respective block, e.g. we use C-Block that contains goat serum preferentially with secondary antibodies developed in goat to achieve optimal blocking. However, this blocking solution cannot be used with primary antibodies developed in goat.

   Make sure that B-Block is supplemented with Triton X-100 for this step. Alternatively, incubate the cells with PBS-Tx for 15 min and block before immunocytochemistry

   Cells have to be permeabilized at this step; therefore, Triton X-100 permeabilization needs to be carried out here. We found that blocking (B-Block) helps at this step to reduce FUNCAT labeling background but is not entirely necessary. However, if only PBS-Tx is used at this step for permeabilization, the unspecific binding sites have to be blocked later for the antibody binding, because for the antibody steps blocking is indeed necessary.

   We use PBS-Tx in experiments where we compare labeling with (PBS-Tx) or without (PBS-MC) permeabilization to estimate the difference between total newly synthesized and preferentially membrane-localized newly synthesized proteins.

9. Wash three times, each time with PBS, pH 7.8, for 10 min.

   During the washing steps prepare the TCEP and $CuSO_4$ solutions and prewarm aliquots of TBTA and fluorescent tag to room temperature.

10. Assemble the FUNCAT reaction protected from light (e.g., cover with a box or aluminum foil) and without interruption at room temperature in the following order:

    a. 5 ml PBS, pH 7.8.

       The PBS, pH 7.8, should be at room temperature to prevent precipitation of the TBTA. Use freshly diluted PBS from the 10× PBS stock solution.

    b. Add 5 µl of 200 mM TBTA stock solution (1:1000) and vortex at high speed for 10 sec.

       After addition of TBTA the solution will turn milky.

    c. Add 5 µl of 500 mM TCEP stock solution (1:1000) and vortex at high speed for 10 sec.

    d. Add 0.5 µl to 1 µl of 2 mM fluorophore-alkyne-tag (1:5000 to 1:10,000) and vortex at high speed for 10 sec.

       After addition of the fluorophore-alkyne-tag the solution sometimes clears slightly.

    e. Add 5 µl of the 200 mM $CuSO_4$ stock solution (1:1000) and vortex at high speed for 30 sec.

       The solution turns clear. If precipitates from the fluorescent tag are observed on the coverslips, filter the FUNCAT reaction mix through 0.22-µm pore-size filters at this point.

       This example is given for 5 ml of FUNCAT reaction mix and needs to be scaled up or down according to the experimental needs.

11. Incubate the cells upside-down in the FUNCAT reaction mix overnight at room temperature with gentle agitation in a horizontal shaker. Protect from light (by covering with a box) in all subsequent steps.

    We strongly recommend carrying out the incubation in the FUNCAT reaction mix upside-down, since otherwise the reaction solution will form fluorescent precipitates on the coverslips.

    For overhead incubation of coverslips, the reaction mix is added to the FUNCAT incubation plate (Fig. 7.11.2A) and coverslips are placed on the paraffin dots with the cells facing the solution. Make sure that the coverslip is completely covered with the reaction mix. Prevent air bubbles.

    For overhead incubation of MatTek dishes, apply a thin layer of silicone grease to the edge of the MatTek incubation support. Fill the support with 300 µl reaction mix to yield a positive meniscus for the solution and place the MatTek dish carefully on top with cells facing down (Fig. 7.11.2B). Gently press and turn in order to seal the chamber with the grease.

Day 2

12. Wash three times, each time with FUNCAT wash buffer for 10 min at room temperature.

    For washing steps, turn the coverslips so that the cells are facing up again.

13. Wash twice, each time with PBS, pH 7.4, for 10 min at room temperature.

Perform immunocytochemistry

14. Incubate with primary antibody (e.g., rabbit-anti-MAP2 or mouse-anti-MAP2 at 1:1000) in B-Block or C-Block for 1 to 2 hr at room temperature or overnight at 4°C.

    Do not use C-Block with goat primary antibodies. Preferentially use C-Block with secondary antibodies developed in goat. B-Block can be used universally. Other blocking reagents might be used—test what is best for your antibody combination.

The choice of incubation length will depend on the experimental planning (an overnight incubation can be used as a pause point) and might depend on how the antibodies perform best. In our hands, for most antibodies, both conditions give the same result and the decision is merely a matter of timing the experiment—including a pause point or not.

Overall fluorescence background might be increased due to $CuSO_4$ in the FUNCAT reaction. If experiments include weakly overexpressed GFP or RFP fusion proteins, it is advisable to enhance the fluorescence signal by using immunocytochemistry.

15. Wash three times, each time with PBS, pH 7.4, for 10 min at room temperature.
16. Incubate with secondary antibody (e.g., goat-anti-rabbit Alexa 647 1:1000 or goat-anti-mouse Alexa 647 at 1:1000) in B-Block or C-Block for 0.5 to 2 hr at room temperature.
17. Wash twice, each time with PBS, pH 7.4, for 10 min each at room temperature.
18. *Optional:* To label nuclei, dilute DAPI stock 1:1000 in PBS and incubate the cells for 3 min at room temperature.

    If labeling cell nuclei for orientation is of interest, add this step.

19. Wash three times, each time with PBS, pH 7.4, for 5 min at room temperature.
20. Mount on microscope slides using mounting medium.
21. Store up to 5 weeks at 4°C until imaged.

ALTERNATE PROTOCOL 1

FUNCAT IN HIPPOCAMPAL SLICES

FUNCAT labeling is applicable to intact tissues such as acute hippocampal slices. This protocol summarizes steps for fluorescence detection of proteins in acute hippocampal slices that are newly synthesized during an incubation period of up to several hours after dissection. This protocol uses AHA metabolic labeling and immunohistochemistry in acute hippocampal slices and is accomplished within 3 days. The same protocol can be used to label organotypic slice cultures.

Materials

Acute 450-μm hippocampal slices sectioned on a vibratome or tissue slicer (for protocol see Madison and Edson, 2001)
Ringer's solution, carbogenated (see recipe)
100 mM AHA (see recipe)
100 mM methionine (see recipe)
40 mM anisomycin (see recipe)
PBS-MC (see recipe)
Fixation solutions: e.g., PFA-sucrose (see recipe) *or* PLP (see recipe)
3% agarose solution (see recipe)
PBS, pH 7.8 (see recipe)
Superglue
B-Block (see recipe)
PBS-Tx (see recipe)
200 mM TBTA in dimethyl sulfoxide (DMSO), triazole ligand (see recipe)
500 mM TCEP in H_2O (see recipe)
2 mM fluorophore-alkyne-tag in DMSO
200 mM $CuSO_4$ in H_2O (see recipe)
FUNCAT wash buffer (see recipe)
PBS-T, pH 7.4 (see recipe)
Primary antibody (e.g., goat anti-rabbit-Alexa 647 *or* goat anti-mouse Alexa 647)
Secondary antibody, fluorophore-coupled (e.g., goat anti-rabbit Alexa 647)

1 mg/ml DAPI (see recipe)
Mounting medium: e.g., Mowiol *or* Fluoromount *or* Aquapolymount

Whatman filter paper no. 1
35-mm tissue culture dishes
Interface recovery chamber (Madison and Edson, 2001)
Submerged incubation chamber (tissue slice chamber, Harvard Apparatus)
Water bath
Horizontal shaker
Hot plate magnetic stirrer
Artist brushes
Stereomicroscope
Scalpel
Forceps
Vibratome Leica VT1200S
24-well plates
15-ml Falcon tubes
Vortex mixer
Microscope slides
Coverslips

Metabolically label newly synthesized proteins in slices with AHA

Day 1

1. Incubate acute hippocampal slices on Whatman filter paper (no. 1) moistened with Ringer's solution on a 35-mm tissue culture dish in an interface recovery chamber for 1.5 hr at room temperature.

2. Dilute AHA from stock solution to 4 mM AHA in Ringer's solution. Transfer the slices into a submerged chamber filled with oxygenated 4 mM AHA and incubate for 4 hr (or any other desired time) at 32°C. Use 4 mM methionine and 4 mM AHA/40 μM anisomycin in Ringer's solution as controls. Place the submerged incubation chamber in water bath to maintain a temperature of 32°C.

   Incubation times of 1 to 4 hr lead to increased labeling with the indicated concentration in this protocol. Vary time and concentration as needed.

   HPG can be used instead of or to chase AHA. Note that in this case an azide-bearing fluorescent tag must be used in step 13.d.

3. Wash once with PBS-MC.

   Use enough PBS-MC to cover the sections.

4. Fix with PFA-sucrose for 20 min at room temperature.

   The horizontal shaker is used in all subsequent incubation and washing steps.

   Use enough PFA-sucrose to cover the sections.

5. Wash once with PBS-MC.

   Use enough PBS-MC to cover the sections.

6. Embed the fixed slices in 3% agarose.

   Prepare 3% low-gelling temperature agarose in PBS, pH 7.8. Keep the agarose liquid by stirring it on a hot plate magnetic stirrer at 50°C. Pour some of the agarose into a prewarmed glass dish. Briefly wash the slices in agarose to get rid of excess PBS from the washing steps. Add a few drops of agarose into the lid of a 1.5-ml microcentrifuge tube. Quickly transfer one slice into the agarose while it is still liquid using fine brushes. Straighten the slice in the agarose under a stereomicroscope. Place the agarose-embedded slice on ice for 5 min.

7. Cut the agarose block containing the slice.

   Remove the agarose block from the lid and cut a small cuboid along the six planes of the slice using a fine scalpel and fine forceps to hold the agarose block. Make sure that there is enough agarose left around the slice (500 μm) to glue the agarose block to the support in the next step.

8. Mount the agarose-embedded slice.

   Apply a little bit of superglue to the stage of the vibratome and immediately place the agarose-embedded slice on top using fine forceps. Let the glue harden for 10 sec.

   Place the stage with the mounted block in the vibratome chamber filled with PBS, pH 7.8.

9. Prepare 50-μm vibratome sections from the agarose-embedded slices.

   Use vibratome settings of 0.01 mm/sec speed and 0.8 mm amplitude.

10. Transfer the re-sectioned slices into 24-well plates filled with PBS, pH 7.8.

11. Permeabilize the slices with 0.5% Triton X-100 in B-Block overnight at room temperature.

    For this step and all following steps involving reagents or solutions, use 500 μl for 12-mm coverslips/24-well plate and MatTek dishes and 1 ml for 18-mm coverslips/12-well plate. This step and all subsequent steps are carried out at room temperature.

Perform chemo-selective fluorescence tagging

Day 2

12. Wash the slices three times, each time with 0.1% Triton X-100 in PBS (PBS-Tx), pH 7.8

13. Assemble the FUNCAT reaction, protected from light, without interruption in the following order in a 15-ml Falcon tube:

    a. 5 ml PBS, pH 7.8.

       The PBS, pH 7.8, should be at room temperature to prevent precipitation of the TBTA. Use freshly diluted PBS from the 10× PBS stock solution.

    b. Add 5 μl of 200 mM TBTA stock solution (1:1000) and vortex at high speed for 10 sec.

       After addition of TBTA the solution will turn milky.

    c. Add 5 μl of 500 mM TCEP stock solution (1:1000) and vortex at high speed for 10 sec.

    d. Add 5 μl of 2 mM fluorophore-alkyne-tag (1:1000) and vortex at high speed for 10 sec.

       After addition of the fluorophore-alkyne-tag, the solution sometimes clears slightly.

    e. Add 5 μl of the 200 mM $CuSO_4$ stock solution (1:1000) and vortex at high speed for 30 sec.

       The solution turns clear. If precipitates from the fluorescent tag are observed at later steps, filter the FUNCAT reaction mix through 0.22-μm pore-size filters at this point.

       This example is given for 5 ml FUNCAT reaction mix and needs to be scaled according to the experimental needs.

14. Incubate each slice in 250 μl of the FUNCAT reaction mix overnight at room temperature. Protect from light in all subsequent steps.

    Free-floating slices can be incubated in multi-well dishes and do not need overhead incubation. Cu(I) precipitates predominantly adhere to coated dishes.

Immunohistochemistry

Day 3

15. Wash the slices three times, each time with FUNCAT wash buffer for 20 min at room temperature.
16. Wash the slices twice, each time with PBS-T for 10 min at room temperature.
17. Incubate with primary antibody in B-Block for 2 hr at room temperature.
18. Wash the slices three times, each time with PBS-T for 15 min at room temperature.
19. Incubate with secondary antibody in B-Block including DAPI 1:1000 for 1 hr at room temperature.
20. Wash the slices three times, each time with PBS-T for 15 min at room temperature.
21. Wash the slices twice, each time with PBS, pH 7.4, for 10 min at room temperature.
22. Mount on microscope slides with Mowiol or Fluoromount and cover with coverslips.
23. Store up to 5 weeks at 4°C until imaged.

FUNCAT IN LARVAL ZEBRAFISH

ALTERNATE PROTOCOL 2

Metabolic labeling with AHA to visualize areas of new protein synthesis is also applicable to the larval zebrafish. Nacre zebrafish lack melanophores and, therefore, enable direct imaging e.g., of the nervous system without prior dissection. AHA has been found not to be toxic to the live organism at the concentration described here (Hinz et al., 2012); however, longer incubations than compared to cell culture and hippocampal slices are necessary to allow for diffusion of AHA into the tissue and incorporation into newly synthesized proteins. High levels of fluorescence have been found especially in the tail muscles and the liver; however, visualization of differential protein synthesis was also possible in the spinal cord and nervous system. This protocol is accomplished within ∼1 week.

Materials

4 to 6 days post-fertilization (dpf) larval zebrafish
E3 embryo medium (see recipe)
100 mM AHA (see recipe)
5 mg/ml puromycin (see recipe)
Ice
PFA-sucrose (see recipe)
Methanol
PBS-T (pH 7.4 and 7.8; see recipe)
PBDTT (see recipe)
Proteinase K (10 μg/ml)
Z-Block (see recipe)
200 mM TBTA in DMSO, triazole ligand (see recipe)
500 mM TCEP in H_2O (see recipe)
2 mM fluorophore-alkyne-tag in DMSO (see recipe)
200 mM $CuSO_4$ in H_2O (see recipe)
0.5 M EDTA
Primary antibody
Secondary antibody, fluorophore-coupled
0.6% agarose

50-mm petri dishes
1.5-ml microcentrifuge tubes

Vortex mixer
Rotary shaker
MatTek dish
Stereomicroscope
Microwave

Metabolic labeling of newly synthesized proteins in larval zebrafish with AHA

Day 1

1. Incubate 4 to 6 dpf larval zebrafish in 7.5 ml E3 embryo medium (pH 7.0 to 7.6) or E3 embryo medium (pH 7.0 to 7.6) containing 4 mM AHA at 26° to 28°C in a 50-mm petri dish.

   Incubation times of 24 hr or more lead to prominent labeling. Incubation times of more than 72 hr are not recommended. Puromycin (5 μg/ml, dilute from 1000× stock) controls can be carried out for up to 48 hr.

Day 2

2. Wash the larval zebrafish three times, each time for 5 min with 10 ml E3 embryo medium at room temperature.
3. Transfer the larvae into a 1.5-ml microcentrifuge tube and anesthetize for 30 min to 1 hr on ice.

   Do not use more than ∼5 larvae in each tube.

4. Remove the remaining E3 embryo medium and replace with 500 μl room temperature PFA-sucrose fixative. Incubate for 3 hr at room temperature and invert the tube every 30 min.
5. Wash the larval zebrafish briefly in 100% methanol at room temperature.
6. Incubate in 500 μl of 100% methanol overnight at −20°C.

   At this step, samples can be stored for prolonged time periods.

Perform chemo-selective fluorescence tagging

Day 3

7. Rehydrate the larvae by successive 5-min washes in 75% methanol in PBS-T, 50% methanol in PBS-T, 25% methanol in PBS-T, and PBS-T, pH 7.4.
8. Wash twice, each time for 5 min with 500 μl PBDTT at room temperature.
9. Permeabilize the sample by incubating in 500 μl of 10 μg/ml Proteinase K in PBS-T, pH 7.4, for 1 hr at room temperature.

   Do not exceed 1 hr incubation, as tissue will deteriorate rapidly.

10. Wash briefly in 500 μl PBS-T, pH 7.4, and immediately post-fix for 20 min in 500 μl PFA-sucrose at room temperature.
11. Wash twice, each time with 500 μl PBS-T, pH 7.4.
12. Wash three times, each time with 500 μl PBDTT for 5 min.
13. Incubate in 500 μl Z-Block for 3 hr at 4°C.
14. Wash three times, each time with 500 μl PBS-T, pH 7.8, for 10 to 15 min at room temperature.

15. Assemble the FUNCAT reaction mix, protected from light, without interruption in the following order in a 1.5-ml microcentrifuge tube:

    a. 1 ml PBS-T, pH 7.8.

       The PBS-T, pH 7.8, should be at room temperature to prevent precipitation of the TBTA. Use freshly diluted PBS from the 10× PBS stock solution.

    b. Add 1 μl of 200 mM TBTA stock solution (1:1000) and vortex at high speed for 10 sec.

       After addition of TBTA the solution will turn slightly milky but TBTA should not precipitate.

    c. Add 1 μl of 500 mM TCEP stock solution (1:1000) and vortex at high speed for 10 sec.

    d. Add 2.5 μl of 2 mM fluorophore-alkyne-tag (1:400) and vortex at high speed for 10 sec.

       After addition of the fluorophore-alkyne-tag the solution sometimes clears slightly.

    e. Add 1 μl of the 200 mM $CuSO_4$ stock solution (1:1000) and vortex at high speed for 30 sec.

       The solution turns clear. If precipitates from the fluorescent tag are observed at later stages, filter the FUNCAT reaction mix through 0.22-μm pore-size filters at this point.

16. Incubate each set of five larvae in 0.5 ml FUNCAT reaction mix overnight at room temperature with agitation.

Immunohistochemistry

Day 4

17. Wash four times, each time with 500 μl PBDTT + 0.5 mM EDTA for 30 min at room temperature.

18. Incubate in primary antibody in 20% Z-Block overnight at 4°C.

Day 5

19. Wash four times, each time with 500 μl PBDTT for 30 min at room temperature.

20. Incubate in secondary antibody in 20% Z-Block overnight at 4°C.

Day 6

21. Wash twice, each time with 500 μl PBDTT for 1 hr at room temperature.

22. Wash four times, each time with 500 μl PBDTT for 30 min at room temperature.

23. Wash with 500 μl PBS, pH 7.4, for 15 min at room temperature.

24. Prepare a 0.6% agarose solution in E3 embryo medium, heating the solution in the microwave to dissolve the agarose. Let cool slightly. Mount the samples in 0.6% agarose on MatTek dishes, using a stereomicroscope to position and orient the samples.

    When mounting larvae, place the area of interest directly against the glass area of the MatTek dish.

FUNCAT IN MICROFLUIDIC CHAMBERS

ALTERNATE PROTOCOL 3

In order to approach visualization of newly synthesized proteins in combination with either compartmentalized labeling or compartment specific treatment of neurons, we use FUNCAT in microfluidic chamber devices (Taylor et al., 2010). The use of the chambers allows the compartment specific addition of the amino acid surrogate and/or

drugs. This protocol describes the variations made to the Basic Protocol to investigate sub-compartments.

This alternate protocol describes metabolic labeling of hippocampal neurons with AHA via different compartments of a standard microfluidic or μLP (microfluidic local perfusion) chamber (Fig. 7.11.2C,D) and indicates putative changes, manipulations with drugs, and pitfalls. Of note, due to potential intracellular diffusion of AHA and some drugs, time scales have to be figured out individually. Experiments designed to study local protein synthesis might need laser-assisted transection of dendrites and axons. This method is under development and the protocol serves as a basis to approach visualization of local protein synthesis.

***Additional Materials** (also see Basic Protocol)*

Methionine-free Hibernate A (Brain Bits) with B27 (see recipe)
Primary hippocampal neurons cultured in microfluidic chambers (Taylor et al., 2010)
100 mM AHA (see recipe)
100 mM methionine (see recipe)
Ice
40 mM anisomycin (see recipe)
PBS-MC (see recipe)
Fixation solutions: e.g., PFA-sucrose (see recipe) *or* PLP (see recipe)
PBS-Tx (see recipe)
C-Block (see recipe)
PBS, pH 7.8 (see recipe)

Microfluidic chambers (Taylor et al., 2010; Fig. 7.11.2C,D; available from Xona Microfluidics LLC: SDN900 or μLP)
Syringe pump
37°C incubator
Spacer (approximately 3 × 1 × 1–mm^3)
Humidified chamber

Metabolic labeling of newly synthesized proteins in microfluidic chambers

Day 1

1. Wash the cell body chamber two times with 300 μl prewarmed methionine-free Hibernate A.

   Aspirate medium from both connected cell body wells (wells 1 in Fig. 7.11.2C,D) carefully with a pipet. To achieve full replacement of the standard culture medium and to avoid clogging by air bubbles, immediately apply washing solution to only one of the connected wells and allow for flow through. Remove the first wash from both wells and repeat. Use the same procedure also for axon wells (next step, wells 2 in Fig. 7.11.2C,D) and all subsequent solution replacements.

2. Wash the axon chamber twice, each time with 300 μl prewarmed methionine-free Hibernate A.

3. Replace the medium in all perfusion channel wells with 75 μl prewarmed (37°C) methionine-free Hibernate A.

4. Connect syringe pump tubing to the channel outlet. Set the pump to withdrawal mode with 0.1 ml/hr.

   The perfusion channel needs 5 min to be filled with the respective solutions with these settings. For incubations, place the chambers back into the incubator and pump

constantly. Otherwise, in this chamber type, solutions will diffuse extracellularly to other compartments. Fluidic isolation can be checked by adding different fluorescent dyes, such as Alexa 488 hydrazide, to the various compartments (e.g., Taylor et al., 2010).

5. Incubate the cells with methionine-free Hibernate A for 20 to 30 min at 37°C in the incubator for methionine depletion.

6. Prepare prewarmed solutions with AHA in methionine-free Hibernate A and drug solutions, if applicable, and replace in the desired wells.

   The concentration of AHA depends on the purpose of the experiment as does the incubation time. We recommend 4 mM AHA for 2 hr as starting conditions as those give robust labeling in cell bodies no matter where AHA is applied. Control experiments with 4 mM methionine and 4 mM AHA and 40 μM anisomycin are recommended to estimate the extent of background labeling.

   Replace solutions using the method indicated above (see step 1, annotation) in the desired compartment. In all other compartments, keep methionine-free Hibernate A. The design of the experiment might require replacements in several wells e.g., addition of AHA to the axon wells, addition of a protein synthesis inhibitor to the cell body chamber, and addition of a glutamate receptor antagonist to the perfusion channel. Preferably do not stop perfusion when changing incubation solutions. Replace solutions quickly in the respective wells. To prevent diffusion by differences in hydrostatic pressure load wells 1 and 2 (Fig. 7.11.2) with similar volumes.

7. Incubate for the desired time (starting point 2 hr) with chambers placed in the incubator with constant pumping and perfusion.

   Adjust time and drug/AHA concentrations according to the biological question.

8. Place the dishes on ice and gently remove the PDMS part of the chamber without displacing dendrites (Fig. 7.11.2E).

9. Wash the coverslips immediately two times, each time with 1 ml ice-cold PBS-MC.

10. Wash once with 0.5 ml fixation solution, and then fix for 20 min at room temperature with 0.5 ml PFA-sucrose or PLP.

11. Wash once with 0.5 ml PBS-Tx and then permeabilize with 0.5 ml PBS-Tx for 15 min at room temperature.

    If preferentially newly synthesized membrane proteins are intended to be labeled, this step can be omitted. Note that this significantly reduces the signal obtained later. For this purpose, blocking buffer in the next step has to be without detergent and cells have to be permeabilized with PBS-Tx and blocked before immunocytochemistry and after FUNCAT reaction.

Chemo-selective fluorescence tagging

12. Incubate in 0.5 ml C-Block for 1 hr at room temperature.

13. Wash twice, each time for 5 min with 1 ml PBS, pH 7.8.

14. Prepare 1 ml FUNCAT reaction mix per coverslip as described in the Basic Protocol (step 10a-d).

15. Incubate the coverslip upside down in the click reaction mix overnight at room temperature.

    Pipet 1 ml FUNCAT reaction mix on Parafilm in a humidified chamber, position a small spacer (made from silicone or any other inert material) next to the drop and place the coverslip upside down with one edge on the Parafilm and one resting on the spacer (Fig. 7.11.2E). This way the solution evenly distributes under the coverslip, the cells are covered but not destroyed. Protect from light.

Immunohistochemistry

Day 2

16. Wash twice, each time for 5 min with 1 ml PBS-Tx.
17. Wash once for 5 min with 1 ml PBS.
18. Proceed with DAPI staining or immunohistochemistry section of Basic Protocol (see steps 14 to 21).

SUPPORT PROTOCOL

COMBINATION OF FUNCAT WITH HIGH-RESOLUTION FISH

This protocol describes the steps necessary to combine FUNCAT with high-resolution fluorescence in situ hybridization using the Affymetrix QG ViewRNA method based on branched DNA in situ hybridization (Player et al., 2001).

***Additional Materials** (also see Basic Protocol and Alternate Protocol 3)*

Cells (see Basic Protocol or Alternate Protocol 3)
Affymetrix QG ViewRNA HC Screening Assay kit containing stock solutions for buffers; prepare the following buffers according to the manufacturer's recommendations:
  Working detergent solution
  Working protease stop buffer
  Working probe set diluent
  RNAView wash buffer
  Working storage buffer
  Working amplifier diluent
  Working label probe diluent
Affymetrix QG ViewRNA Probe set for mRNA of interest
Affymetrix QG ViewRNA HC Screening Signal Amplification kit (divide into 10-µl aliquots when thawing for the first time and use aliquots up to three times):
  Preamplifier
  Amplifier
  Label probe
Hybridization oven (40°C)
Humidified chamber
Thermomixer, heating block (40°C)

Day 1

Metabolic labeling with AHA

1. Perform metabolic labeling with AHA as described in the Basic Protocol (steps 1 to 3) or Alternate Protocol 3 (steps 1 to 9).
2. Wash twice, each time with PBS, pH 7.4, to stop metabolic labeling.
3. Fix with PLP for 30 min at room temperature.

   Avoid RNase contamination of the samples. Wear gloves, change them frequently, and use RNase-free water and PBS.

   For washing steps in 24-well plates or MatTek dishes, use ≥300 µl; for incubation steps, 150 µl.

Hybridize the probe set

4. Wash three times, each time with PBS at room temperature.
5. Permeabilize 3 min with 150 µl working detergent solution at room temperature.

6. Wash three times, each time with PBS at room temperature.

   The manufacturer recommends a proteinase K digestion step that is not performed in this protocol to avoid degradation of the newly synthesized proteins.

7. Equilibrate in 150 µl working protease stop buffer up to 30 min until the probe set is prepared at room temperature.

   Dilute the probe set 1:100 in working probe set diluent prewarmed to 40°C.

8. Wash the cells once with PBS at room temperature.

9. Incubate with 150 µl probe set mixture for 3 hr at 40°C in a hybridization oven.

   Create a humidified chamber to avoid evaporation: seal 24-well plates with Parafilm before placing into the oven. Place MatTek dishes on a tray with tissues soaked in water, slip it into a plastic bag, and seal it before transfer to 40°C.

10. Wash three times, each time with RNAView wash buffer at room temperature.

11. *Optional:* Store in working storage buffer overnight at 4°C.

Day 2

Amplify hybridized probe set

12. Wash twice, each time with RNAView wash buffer at room temperature.

13. Incubate with PreAmp mixture 1 hr at 40°C in a humidified chamber in the hybridization oven.

    Dilute PreAmp 1:100 in working amplifier diluent prewarmed to 40°C in the thermomixer heat block.

14. Wash three times, each time with RNAView wash buffer at room temperature.

15. Incubate with Amp mixture 1 hr at 40°C in a humidified chamber.

    Dilute Amp 1:100 in working amplifier diluent prewarmed to 40°C.

16. Wash three times, each time with RNAView wash buffer at room temperature.

17. Incubate with Label Probe mixture 1 hr at 40°C in a humidified chamber.

    Dilute label probes 1:100 in working label probe diluent prewarmed to 40°C.

    Choose the label probe fluorophore appropriate for combination with the fluorophores used to be clicked to AHA and coupled to the antibodies used for immunocytochemistry. Protect from light.

18. Wash three times, each time with RNAView wash buffer at room temperature.

Chemo-selective fluorescence tagging

19. Wash three times, each time with PBS-MC at room temperature.

20. Block 1 hr with C-Block at room temperature.

21. Prepare FUNCAT reaction mix and proceed at step 10 of the Basic Protocol.

    It is possible to perform both chemoselective labeling and immunohistochemistry after FISH. Use RNase-free reagents and the shortest possible incubation times. If the signal to noise ratio allows, shorten the click reaction to 2 hr.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose solution

Dissolve low-gelling temperature agarose in 1× PBS, pH 7.8, to a final concentration of 3% by heating on a hot plate magnetic stirrer. Prepare fresh.

AHA or HPG or methionine

Dissolve AHA (L-azidohomoalanine, Invitrogen) or HPG (L-homopropargylglycine, Invitrogen) or methionine in 1× HBS or in methionine-free medium to a final concentration of 100 mM. Store up to 1 week at 4°C.

Anisomycin stock solution

Dilute anisomycin (Tocris) to a final concentration of 40 mM in DMSO. Divide into 10-µl aliquots. Store aliquots up to 1 month at −20°C.

B-Block

Dissolve the following in 1× PBS, pH 7.4:
10% normal horse serum
5% sucrose
2% bovine serum albumin (BSA)
Filter with 0.22-µm pore size sterile filters
Store up to 12 months at −20°C
Just prior to use add Triton X-100 to 0.1% for cell permeabilization when needed

C-Block

Dissolve the following in 1× PBS, pH 7.4:
4% normal goat serum
Filter with 0.22-µm pore size sterile filters
Store up to 12 months at −20°C

$CuSO_4$ solution

Dissolve $CuSO_4$ to a final concentration of 200 mM in distilled water
Prepare solution fresh

DAPI stock solution (1000×)

Dissolve 1 mg/ml DAPI in distilled water
Divide into 500-µl aliquots
Store aliquots up to 1 year at −20°C or up to 1 month at 4°C
Dilute just before use 1:1000 in phosphate-buffered saline (PBS) or other respective incubation solution

E3 embryo medium 1×

5 mM NaCl
0.17 mM KCl
0.33 mM $CaCl_2$
0.33 mM $MgSO_4$
Store up to 3 days at room temperature

Fluorescent tag

Fluorophore-alkyne or -azide tags are commercially available from Invitrogen or Click Chemistry Tools (e.g., AlexaFluor for 488-nm, 555-nm, 594-nm and 647-nm excitation wavelength from Invitrogen)

continued

Dissolve the tag in dimethyl sulfoxide (DMSO) to a final concentration of 2 mM
Divide into 20-µl aliquots
Store up to 12 months at −20°C

Working aliquots can be stored up to 3 months at 4°C, protected from light.

FUNCAT incubation plate

To prepare FUNCAT incubation plates use a 12-well or 24-well culture plate according to the coverslip size used. Heat paraffin (e.g., Granopent "P", Carl Roth) in a glass beaker on a hot plate with a temperature of 150°C. Dip a glass pipet into the fluid paraffin and make four paraffin dots of the size of a pin per well. The plates can be cleaned easily and reused many times (Fig. 7.11.2A).

FUNCAT-wash buffer

Dissolve in PBS, pH 7.8
0.5 mM EDTA
1% Tween-20

HBS, 2×

238 mM NaCl
10 mM KCl
4 mM $CaCl_2$ (from 1 M stock solution)
4 mM $MgCl_2$ (from 1 M stock solution)
60 mM glucose
20 mM HEPES
Adjust pH to 7.35 with a few drops of 1 N NaOH
Filter through sterile 0.22-µm pore size filters
Store up to 2 months at 4°C

Alternatively, divide into aliquots and store up to 6 months at −20°C. For 1× HBS mix 1 + 1 with deionized water.

MatTek overhead incubation support

Fill the lid of a 5-ml round-bottom tube with toy modeling clay. Leave a 2- to 3-mm space to the edge and seal the surface with a layer made from two-component epoxy glue. The created well should hold 300 µl solution (Fig,. 7.11.2B).

Methionine-free medium

For DMEM minus methionine, minus cysteine, minus glutamine, add L-cysteine to a final concentration of 0.201 mM, add glutamine, serum and other supplements necessary for cultivation of cell culture lines. Filter through 0.22-µm pore size sterile filters. For methionine-free Hibernate A, add supplements necessary for cultivation of primary cell cultures. The use of B27 (Invitrogen, 1:50) for cultivation of neuronal cultures is advisable. Filter through 0.22-µm pore size sterile filters.

1× PB, pH 7.4

21.71 g $Na_2HPO_4 \cdot 7\ H_2O$
2.62 g $NaH_2PO_4 \cdot H_2O$
Adjust volume to 1 liter with distilled water
Store up to 6 months at 4°C

PBS-DTT

Dissolve 1% dimethyl sulfoxide (DMSO), 0.5% Triton X-100, and 0.1% Tween-20 in 1× PBS, pH 7.4 (see recipe)
Prepare fresh

PBS, 10×

1.37 M NaCl
27 mM KCl
43 mM $Na_2HPO_4.7H_2O$
14 mM KH_2PO_4
Add distilled water up to 900 ml
Adjust pH to 7.8 for click reactions and 7.4 for all other uses with a few drops of 1 N NaOH
Adjust volume to 1 liter with distilled water
Store up to 6 months at room temperature

For 1× PBS, use 1 part of 10× PBS and 9 parts of distilled water; adjust pH if necessary.

PBS-MC, 1×

Dissolve the following in 1× PBS pH 7.4:
1 mM $MgCl_2$
0.1 mM $CaCl_2$
Prepare fresh

Dilute $MgCl_2$ and $CaCl_2$ from 1 M stock solutions.

PBS-T

Dissolve 0.1% Tween-20 in 1× PBS
Adjust pH to 7.4 or 7.8 with a few drops of 1 N NaOH
Prepare fresh

PBS-Tx

Dissolve 0.1% Triton X-100 in 1× PBS
Prepare fresh

PFA-Sucrose

PBS-MC (see recipe) supplemented with:
4% PFA (Stock: 16% w/v, Alfa Aesar)
4% (w/v) sucrose
Prepare fresh

PFA-Sucrose for larval zebrafish

1× PBS, pH 7.4, supplemented with:
4% PFA (Stock: 16% w/v, Alfa Aesar)
3% (w/v) sucrose
Store up to 1 week at 4°C

PLP-fix

Solution A: Lysine phosphate buffer
Add 3.66 g lysine hydrochloride to 100 ml distilled water
Adjust pH to 7.4 by adding 0.1 M Na_2HPO_4
Double the volume of the solution by adding the appropriate amount of 1× PB (see recipe)

Solution B: Paraformaldehyde
0.54 g glucose
10 ml of 16% (w/) paraformaldehyde solution (Alfa Aesar)
Mix 3 parts of solution A with 1 part of solution B
Add sodium *m*-periodate to a final concentration of 0.01 M (0.21 g/100 ml)
Store up to 1 week at 4°C

Puromycin (1000× stock)

Dissolve 5 mg/ml puromycin in distilled water
Divide into 10- to 25-µl aliquots
Store up to 1 month at −20°C

Ringer's solution

119 mM NaCl
2.5 mM KCl
1.3 mM $MgSO_4$
2.5 mM $CaCl_2$
1.0 mM NaH_2PO_4
26.2 mM $NaHCO_3$
11.0 mM glucose
Adjust pH to 7.35 with 1 N NaOH
Make sure that $CaCl_2$ is completely dissolved before the next compounds are added.
Store up to 1week at 4°C

Start carbogenating the solution 10 to 15 min before perfusion of hippocampal slices.

TBTA

Dissolve TBTA {Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine} in anhydrous, freshly opened dimethyl sulfoxide (DMSO) to a final concentration of 200 mM
Divide into 10- or 20-µl aliquots
Store aliquots up to 6 month at −20°C

Avoid repeated freezing and thawing of aliquots (no more than 3 times).

TCEP

Dissolve TCEP-HCl [Tris-(2-carboxyethyl)phosphine hydrochloride] to a final concentration of 500 mM in distilled water

Prepare solution fresh directly before use

Z-Block

Dissolve in PBDTT:
5% BSA
10% normal goat serum
Filter through 0.22-µm pore size sterile filters
Store up to 1 year at −20°C

COMMENTARY

Background Information

Comparison with other methods

Fluorescent labeling of proteins by genetically encoded fluorescent protein tags pioneered by GFP opened a new era in understanding cell biological processes by visualization of spatio-temporal patterns in protein distribution (Chalfie et al., 1994; Heim et al., 1994). One drawback of this approach is the relatively big size of the tag, which in some cases affects the folding and behavior of the proteins of interest. Another limitation became obvious with the focus of studies turning to a systems biological point of view. With the genetically encoded fluorescent tagging approach the analysis is restricted to a limited number of known proteins at a given time.

Metabolic labeling of the proteome with either radioisotope- or stable isotope-tagged amino acids are powerful methods to quantify or identify and compare proteome-wide changes in combination with biochemistry and mass spectrometry, respectively (Hu et al., 2004; Cox and Mann, 2011). Since the nature of the label does not influence biological

processes, it is perfectly suited to reflect physiological conditions. In contrast, these methods are not well-suited for either the purification of the newly synthesized protein pool or the in situ visualization within the cell. The conversion of radioactivity into a visual signal by exposure to film emulsion is time-consuming and difficult to combine with other imaging methods, and cannot be extended to live imaging. BONCAT and FUNCAT fill these gaps. FUNCAT is a fluorescence-based method to follow proteome-wide patterns of newly synthesized proteins in situ and is compatible with immunohistochemistry and in situ hybridization. Introduction of noncanonical amino acids with small, bioorthogonal chemical handles enables a multitude of ligation options (Fig. 7.11.3 A,B), e.g., to fluorophores for visualization (FUNCAT), biotin for purification and mass spectrometry (BONCAT), but is not limited to those. Thus, the elegance in this approach lies in the versatility of the method.

Mechanism and chemistry

As described above, the introduction of a small bio-orthogonal reactive handle is accomplished by metabolic labeling similar to classical radioisotope labeling. Methionine is replaced in the medium by the azide- or alkyne-bearing methionine surrogates AHA or HPG (Fig. 7.11.3A). Both noncanonical amino acids are taken up by cellular amino acid transporters—mainly by LAT1 (Fig. 7.11.3C; Kanai et al., 1998). Key to this methodology is that not only transporters but also endogenous methionyl tRNA synthetase (MetRS)—the enzyme charging methionine onto its tRNA—accepts AHA and HPG as substrates, although with lower efficiency than methionine (Kiick et al., 2002). Once charged onto the tRNA, incorporation of the amino acid analogs into nascent proteins is straightforward. Thus, during metabolic labeling newly synthesized proteins are endowed with new functionalities, namely azide (AHA) or alkyne (HPG) groups that differentiate them from the pre-existing protein pool (Fig. 7.11.3C). If AHA and HPG are applied sequentially (or potentially also at two distinct places), two different subpopulations of proteins are labeled (Dieterich et al., 2010).

After incorporation into newly synthesized proteins, the functional groups are visualized by fluorophores in a reaction based on "click chemistry"—a copper(I)-catalyzed [3+2]azide-alkyne cycloaddition (Fig. 7.11.3B,C; Rostovtsev et al., 2002; Tornoe et al., 2002). To this end, the fluorophore has to be functionalized by the respective counterpart. AHA reacts with alkyne-bearing fluorescent tags; HPG is clicked to azide carriers. The catalyzing Cu(I) is produced directly in the reaction from TCEP and Cu(II) and the triazole ligand TBTA serves the dual purpose of both activating the Cu(I) ion for catalysis and protecting it from disproportionation, thus improving the kinetics of the bioconjugation and also allowing for long reaction times required in some protocols (for further details see Prescher and Bertozzi, 2005; Dieterich and Link, 2009; Dieterich, 2010).

Limitations and extensions

Beside the versatility of the method in general, the introduction of bioorthogonal groups by AHA and HPG have the advantage of minimal interference with protein folding, trafficking and function (Dieterich et al., 2006) due to the small-size chemical tags and, thus, the likely close reflection of physiological conditions. The conditions given in the protocols give robust labeling (Figs. 7.11.4-7.11.5) but may need adaptation to an individual cell type or a particular question—see the Critical Parameters and Troubleshooting section.

A prerequisite for a protein to be labeled by this method is the presence of at least one naturally occurring methionine in the protein, and, secondly, that this be replaced by the surrogate amino acid during protein synthesis. Even if the number of potential replacement sites is not a severe limitation since e.g., for the zebrafish genome a fraction of 99.99% percent of proteins was calculated to fulfill this prerequisite and 99.98% of all protein entries of a human protein database contain at least a single methionine (Dieterich et al., 2006; Hinz et al., 2012), the replacement fraction sometimes is. The factors most prominently influencing the fraction of methionine replacement are the competition with the internal methionine pool and the incubation time. With long incubation periods, eventually a steady state level will be reached, but never full replacement. The shorter the metabolic labeling time the more important it will be to reduce competition by methionine. Methionine depletion prior to the AHA metabolic labeling decreases competition by methionine for charging onto its tRNA, but also provides a nonphysiological situation to the cell. Long metabolic labeling times might become an issue when cells, in particular neurons, do not tolerate or react to long incubations in artificial medium or when intended local applications are counteracted by intracellular distribution or diffusion of the amino acids or drugs. In microfluidic

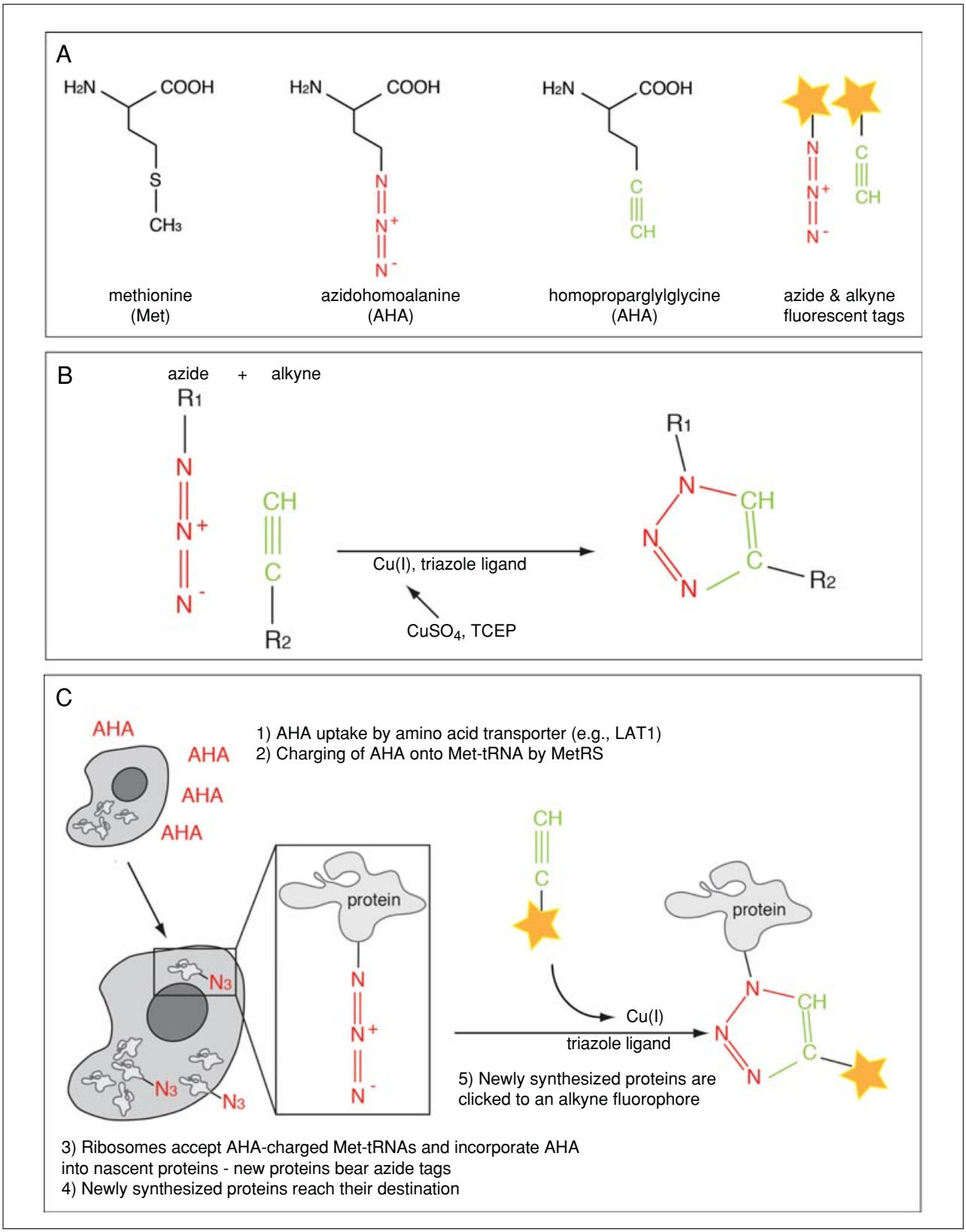

Figure 7.11.3 FUNCAT: chemistry and principle. (**A**) The chemical structures of the noncanonical amino acids AHA (azide-bearing) and HPG (alkyne-bearing) are similar to methionine (Met). A variety of azide- or alkyne-functionalized fluorophores (A) are available to covalently ligate a fluorophore to the noncanonical amino acids by Cu(I)-catalyzed azide + alkyne [3+2]-cycloaddition (**B**). The Cu(I) catalyst is produced in the reaction mixture from Cu(II) and TCEP and is stabilized by the triazole ligand (TBTA). (**C**) Explanation of FUNCAT procedure steps during metabolic labeling and click reaction.

chambers we have found that after 1 hr—regardless from which compartment AHA is loaded—the noncanonical amino acid reached the cell body even when fluidic isolation is intact (Fig. 7.11.5B,C).

The advantage that FUNCAT labels newly synthesized proteins on a proteome-wide level might turn into a disadvantage when, for instance, only a subpopulation of cells is of interest and the signal in other cell types creates a "background." New developments aim to restrict the metabolic labeling. Expression of mutant MetRS versions that accept a longer-chain homolog of AHA-ANL (azidonorleucine)—that in turn is not a substrate of the endogenous MetRS, are a potential way to genetically control the metabolic labeling (Ngo et al., 2009). Genetic manipulation of the MetRS instead of the protein of interest directly, as in fluorescent protein-tagged approaches, rules out problems and restrictions due to overexpression and the limited capacity for genetically encoding several tags at the same time.

The fact that $CuSO_4$ is toxic to cells limits the use of the protocols described here to manipulations that are analyzed after fixation of the cells or tissue of interest. Recent developments to apply this technique also in live cells make use of the fact that [3+2]azide-alkyne cycloaddition not only can be catalyzed by Cu(I) but also is achieved by strain promotion (Agard et al., 2004). The embedding of the alkyne moiety into a cyclooctene structure with sidechains that promote strain as in DIBO (dibenzocyclooctyne) or DIFO (difluorinated cyclooctyne) thus enables a copper-free click reaction. To date, the poor membrane permeability of the reagents limit the application to labeling of newly synthesized membrane proteins (Dieterich et al., 2010), but efforts are in progress to extend the repertoire of reagents to enable the live visualization of intracellular proteins (Beatty et al., 2010, 2011).

Critical Parameters & Troubleshooting

The conditions given in the protocols should lead to robust labeling (Figs. 7.11.4-7.11.5). Problems that could arise and their possible solutions are listed in Table 7.11.1. However, the starting times and concentrations suggested here might not be optimal for all biological questions. Adaptations of parameters in the protocol should consider the following: protein synthesis rates differ between cell types. Incorporation of the amino acid surrogates into post-mitotic cells, like neurons, is lower than in dividing cells. The choice of the labeling medium should also be considered. The ideal labeling medium with respect to cell health and physiological state would be the respective fully complemented and conditioned culturing medium free of methionine. Unfortunately, this is not possible in most cases. While cell lines usually tolerate an incubation in 1× HBS or unsupplemented media well, this is certainly not the case for neurons. We use methionine-free Hibernate A supplemented with B27 for neurons and found that leaving out B27 already for short incubations compromises the neurons. We recommend to test if cells tolerate the incubation conditions of choice before performing a metabolic labeling experiment.

When adjusting the incubation conditions for FUNCAT experiments in microfluidic chambers, factors that might be critical and have to be controlled for are, e.g., extracellular and intracellular diffusion of drugs or amino

Figure 7.11.4 *(appears on next page)* Example results. Representative FUNCAT experiments in (**A**) COS7 cells, (**B**) glial cells, (**C**) cultured hippocampal neurons, and (**D**) acute hippocampal slices using different fluorescent tags. (A) COS cells incubated with 4 mM AHA for 1 hr, clicked to Alexa594-alkyne, labeled for actin with Alexa488-phalloidin and DAPI to stain nuclei. In the presence of the protein synthesis inhibitor anisomycin (40 μM), the FUNCAT signal is significantly reduced (present only at low levels in the nucleus) and when AHA is replaced by Met no FUNCAT signal is visible. (B) Primary astrocytes treated with 4 mM AHA, clicked to TxRed-alkyne and stained for GFAP (two left panels) and the respective anisomycin control (two right panels). (C) Increasing the duration of 4 mM AHA incubation increases the FUNCAT signal (AHA, Tamra-alkyne and Met control) in hippocampal neurons stained for the neuron marker MAP2 and the presynaptic protein synaptophysin. FUNCAT signal (as fire lookup table in lower panel) is clearly visible in soma and dendrites after 2 hr. After 6 hr, there is also ample labeling of synaptic sites. (D) Micrograph of area CA1 from a FUNCAT experiment in an acute hippocampal slice incubated for 4 hr with 4 mM AHA (left panel) and clicked to Alexa488-alkyne and the respective Met control (right panel). For better visualization, orientation slices are stained with the neuron marker MAP2. DAPI labeling in the AHA slice shows that the FUNCAT signal in pyramidal cells is higher than in other cells dispersed in the neurophil layer. Scale bars 20 μm (A), 10 μm (B,C), 100 μm (D).

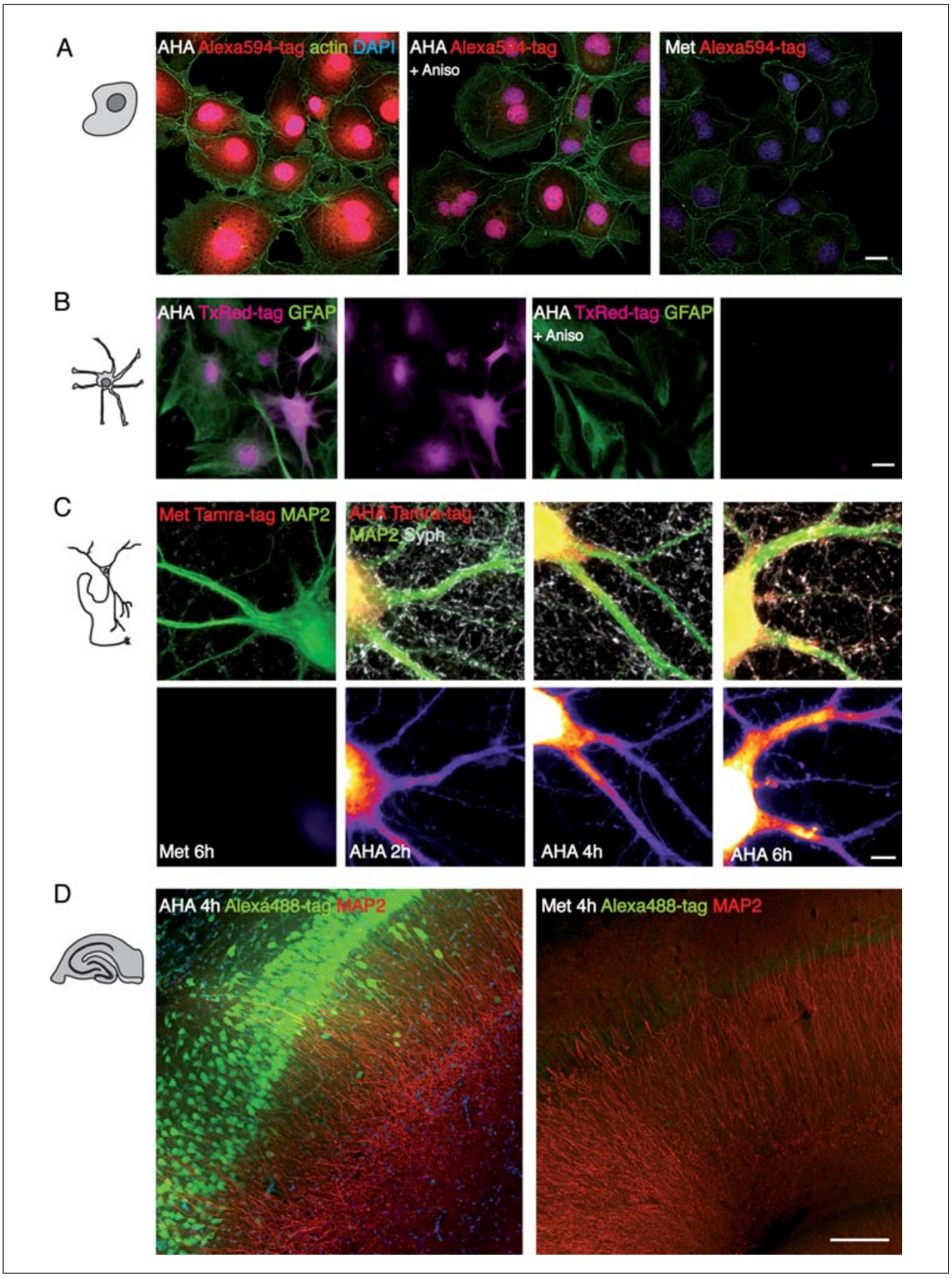

Figure 7.11.4 *(legend appears on previous page)*

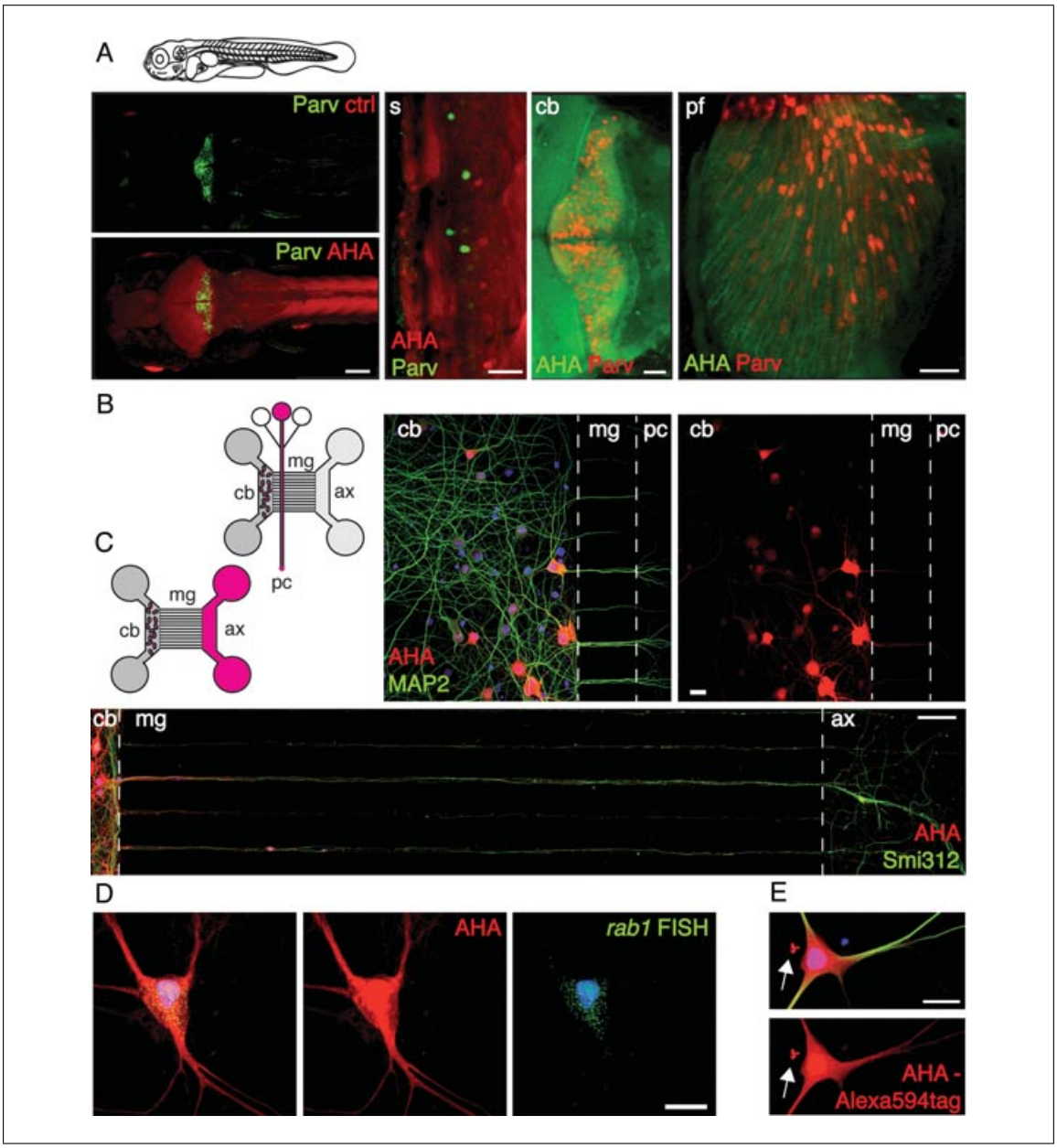

Figure 7.11.5 Expected results. (**A**) FUNCAT in whole 7 dpf zebrafish larvae after 72 hr AHA labeling was combined with antibody labeling for parvalbumin. A dorsal view of the head of larvae incubated without (ctrl) and with 4 mM AHA, clicked to Alexa594-tag and stained for parvalbumin, shows the specificity and low background of the FUNCAT labeling (A, left panel). Higher magnifications of different regions—a lateral view of the spinal cord (s, Alexa594-tag), dorsal view of the cerebellum (cb, Alexa488-tag), and a lateral view of the pectoral fin (pf, Alexa488-tag) show that within the tissue cell populations show differences in FUNCAT signal and can be identified by antibody staining. (B,C). Application of 4 mM AHA for 2 hr via the perfusion channel (pc) in a μLP chamber (**B**) or 1 hr via the axon chamber (ax) of a microfluidic chamber without perfusion channel (**C**). Both conditions lead to signal in the cell body compartment (cb) in the soma of cells (cb) that send neurites to the respective compartment indicating that axons and dendrites are capable of AHA uptake. MAP2 positive cells (B) that do not extend dendrites through the microgrooves (mg) to the perfusion channel (pc) are not intensely labeled. In a well-grown chamber culture usually ~30% to 50% of the neurons in a distance of 150 μm from the microgrooves are labeled when AHA is loaded from the perfusion channel. (**D**) High-resolution FISH for Rab1 mRNA combined with FUNCAT in a hippocampal neuron. After FISH the click reaction was performed for only 2 hr instead of overnight for maximal preservation of the FISH signal. (**E**) Fluorescent precipitates (arrow) appear in the samples and make analysis difficult when the FUNCAT click reaction is not performed overhead or grease from sealing the MatTek dishes on the incubation support during overhead click reaction spills into the sample. Scale bars 100 μm (A, left panel), 25 μm (A, other panels), 20 μm (B, D), 50 μm (C).

Table 7.11.1 Troubleshooting

Problem	Cause	Potential solution
No or low FUNCAT signal	No alkyne-azide reaction	Make sure that you use an alkyne-bearing fluorophore for ligation to AHA and azide-bearing fluorophores for ligation to HP
	pH of click reaction mix	Click reaction works best with a slightly basic pH of 7.6-7.8
	Click reaction reagents added in the wrong order to the reaction mix	It is critical to add the reagents in the correct order, quickly and with vigorous vortexing
	Fluorophore bleaching	Starting with the assembly of the FUNCAT reaction mixture, protect both the mixture and samples from light
	TCEP not working, no Cu(I) formation	Dissolve TCEP immediately prior to reaction assembly—if this does not help, purchase a new batch of TCEP
	Fluorescent tag stock solution not properly dissolved	Always use fresh and preferably unopened DMSO to prepare the fluorescent tag stock solution. If the solution is not immediately clear upon dissolving in DMSO, it is unusable. Store tag in aliquots at −20°C; do not freeze-thaw more than three times.
	Triazole ligand (TBTA) not working	Always use fresh and preferably unopened DMSO to prepare the ligand stock solution. The ligand solution should become clear and with a light yellow color after vortexing. Store small aliquots of the dissolved ligand at −20°C; do not freeze-thaw more than three times.
High background signal in methionine or protein synthesis inhibitor controls	Nonspecific binding of fluorescent tag	Block unreacted aldehyde groups from PFA fixation with 0.1M glycine in PBS. Block other unspecific binding sites with a BSA-containing blocking buffers prior to FUNCAT reaction. Increase the number and duration of washing steps. Try a different fluorescent tag. Synthesize the fluorescent tag with water-soluble polyethylene glycol linker.
	Protein synthesis inhibitor not working	Store in aliquots at −20°C; do not freeze-thaw more than three times. If this does not help, use a new batch.
	Autofluorescence of tissue/cells	Try a different fluorescent tag
Fluorescent precipitates form	Copper/tag precipitate from click reaction	It is critical to add the reagents in the correct order, quickly and with vigorous vortexing; also, prewarm the reagents to room temperature. Carry out the click reaction upside down at room temperature. Filter the reaction mixture through a 0.22-μm filter. Decrease the $CuSO_4$ concentration (perform dilution series).

continued

Table 7.11.1 Troubleshooting, *continued*

Problem	Cause	Potential solution
	Leaking grease from sealing support chambers	Take care that no excess grease is used to seal the MatTek dish on the support and avoid spills into the incubation solution. Use less or no grease. Incubate instead in a humidified chamber.
FISH signal not visible	RNA degradation	Use RNase-free reagents. Shorten the incubation times, e.g., perform click reaction for only 2 hr instead of overnight.
	Background from FUNCAT signal	Change the combination of fluorophores for FISH and fluorescent tag

acid analogs, uptake capacity of the respective cellular compartment for AHA (neurites of different neuron types might have different amino acid transporter densities), and the time needed for newly synthesized proteins to reach their final destination. From our experience, it is crucial to control every microfluidic chamber for the quality of the cultured neurons and ensure that dendrites and axons populate the microgrooves evenly without any cell debris clogging the microgrooves.

When combining this protocol with FISH, any source of RNase contamination should be avoided after the fixation step. Click reaction time, blocking steps, and antibody incubation steps can be shortened. Of note, we do not use proteinase K treatment in this FISH protocol (in contrast to the manufacturer's recommendation). We avoid proteinase K in order to preserve the integrity of newly synthesized proteins and enable the combination with immunocytochemistry. The procedure leads to clear and highly localized in situ signals with every antisense probe set we used so far.

Anticipated Results

Application of the protocols should result in fluorescent labeling of cells and tissue that is clearly distinguishable from background labeling as assessed with a methionine-incubated control or when compared to a sample treated with AHA in the presence of a protein synthesis inhibitor. Typical example results with immunostaining are shown in Figures 7.11.4 and 7.11.5. In our experience, we face detection limits in hippocampal neurons when we lower concentrations of AHA to less than 100 μM or limit incubation times to <10 min. These limits depend on the cell types used and should be analyzed by comparison with the respective controls.

Time Considerations

The Basic Protocol is usually accomplished within 2 days. One day is needed for metabolic labeling, with the exact length depending on the incubation time. Fixation, blocking, and preparation for the FUNCAT reaction need approximately 2 hr. The click reaction itself is carried out overnight but can—with concomitant loss of signal intensity—be shortened to few hours. The next day, optional immunocytochemistry requires an additional ∼5 hr. If FISH (Support Protocol) is included in the procedure, the first day includes, after metabolic labeling, (depending on desired time), fixation, and permeabilization (approximately 1 hr in total), a 3 hr probe set hybridization. Next, the protocol has an overnight storage step that can be omitted. The remainder of the FISH protocol is accomplished in 4 hr (3 hr of them incubation time) before switching back to the FUNCAT basic protocol (FUNCAT reaction mix incubation).

Alternate Protocol 1 (FUNCAT in hippocampal slices) is carried out within 3 days. The most time-consuming part compared to the other protocols is the re-sectioning of the agarose-embedded slices on day 1, which takes ∼1 hr per slice.

Alternate Protocol 2 (FUNCAT in larval zebrafish) needs longer incubations to ensure penetration of reagents into the whole organism. The whole protocol from metabolic labeling to immunohistochemistry takes one week.

Alternate Protocol 3 (FUNCAT in microfluidic chambers) is comparable in time to the Basic Protocol.

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