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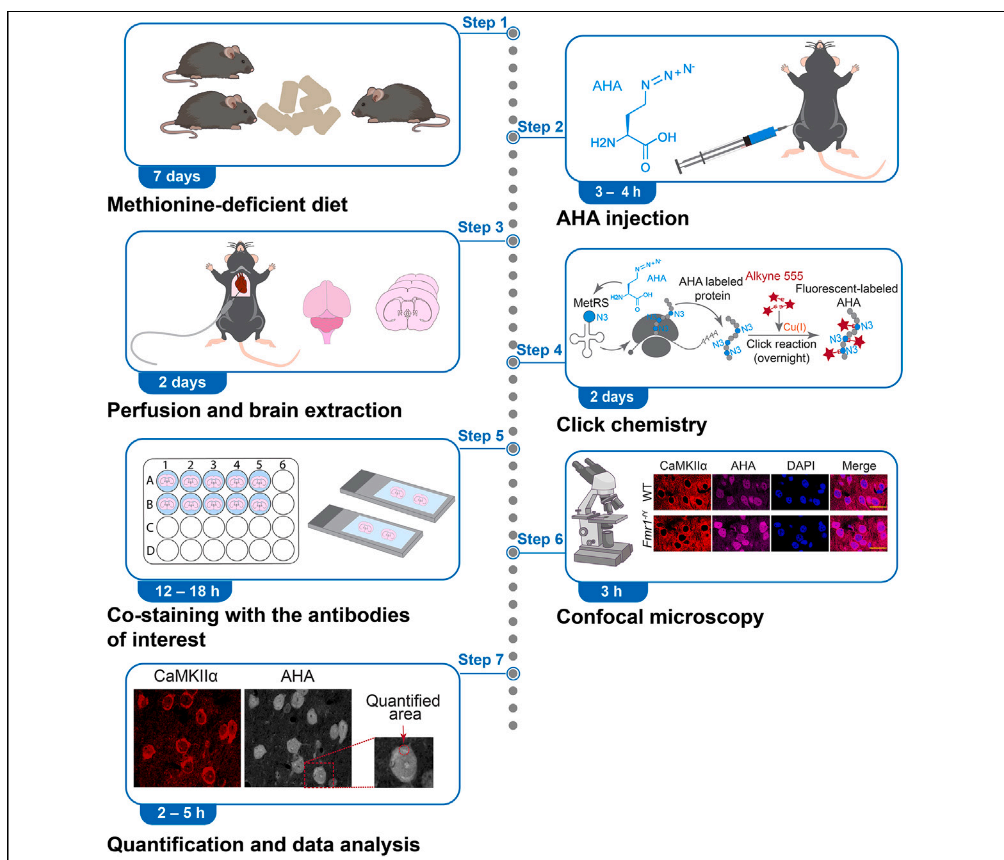
Protocol for measuring protein synthesis in specific cell types in the mouse brain using *in vivo* non-canonical amino acid tagging



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Highlights

Steps for L-azidohomoalanine (AHA) incorporation into the nascent proteins *in vivo*

Cell-type-specific visualization of *de novo* protein synthesis in the brain

AHA signal intensity quantification and analysis in distinct neuronal cell types

The fluorescent non-canonical amino acid tagging (FUNCAT) technique has been used to visualize newly synthesized proteins in cell lines and tissues. Here, we present a protocol for measuring protein synthesis in specific cell types in the mouse brain using *in vivo* FUNCAT. We describe steps for metabolically labeling newly synthesized proteins with azidohomoalanine, which introduces an azide group into the polypeptide. We then detail procedures for binding a fluorophore-conjugated alkyne to the azide group to allow its visualization.

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

Hooshmandi et al., STAR Protocols 5, 102775
March 15, 2024 © 2023 The Authors.
https://doi.org/10.1016/j.xpro.2023.102775

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Protocol for measuring protein synthesis in specific cell types in the mouse brain using *in vivo* non-canonical amino acid tagging

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https://doi.org/10.1016/j.xpro.2023.102775

SUMMARY

The fluorescent non-canonical amino acid tagging (FUNCAT) technique has been used to visualize newly synthesized proteins in cell lines and tissues. Here, we present a protocol for measuring protein synthesis in specific cell types in the mouse brain using *in vivo* FUNCAT. We describe steps for metabolically labeling newly synthesized proteins with azidohomoalanine, which introduces an azide group into the polypeptide. We then detail procedures for binding a fluorophore-conjugated alkyne to the azide group to allow its visualization.
For complete details on the use and execution of this protocol, please refer to tom Dieck et al. (2012)[1] and Hooshmandi et al. (2023).[2]

BEFORE YOU BEGIN

De novo protein synthesis plays a pivotal role in a variety of homeostatic and activity-dependent processes in the nervous system, including development, growth and regeneration, synaptic plasticity, and memory formation.[3] Furthermore, dysregulation of protein synthesis underlies many pathological conditions, such as autism, epilepsy, and chronic pain.[4,5] Protein synthesis is differentially affected in distinct cell types in disease states, requiring methods to assess rates of protein synthesis in a cell-type-specific manner. This is crucial for understanding the roles of protein synthesis in the processes underlying brain function and dysfunction.[6,7]

The FUNCAT protocol has been used to measure the nascent protein synthesis in various primary cell cultures and cell lines,[8,9] tissues,[10] and organisms such as zebrafish[11] and mice.[12] In this study, we present the optimized protocol to visualize protein synthesis in the mouse brain, combined with immunohistochemistry using cell-type-specific markers. Unlike the previous protocols that involve the incubation of cultured cells or tissues in AHA-containing solutions,[10,13,14] we injected AHA into live mice, allowing AHA incorporation into the newly synthesized polypeptide chain while the animals are freely moving and exploring their environment.[2] Additionally, we immunostained brain sections from the injected mice for CaMKIIα and GAD67 to measure the AHA signal in excitatory and inhibitory neurons, respectively. In this study, FUNCAT was used to measure protein synthesis in cortical sections from wild-type mice and a mouse model of fragile X syndrome, *Fmr*1 knockout.

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Anisomycin, an inhibitor of protein synthesis, blocked AHA incorporation into the newly synthesized polypeptide chain, demonstrating the validity of this method.

Institutional permissions

All procedures were compliant with the Canadian Council on Animal Care guidelines and approved by McGill University's Animal Care Committee. For researchers who wish to use this protocol, they should comply with their institutional animal welfare policies and obtain the required permissions.

Preparation

⏲ Timing: 1 day

Mice and methionine-deficient diet

1. It is recommended to use 4 to 6 adult mice for each experimental condition.
2. Methionine-deficient chow (Envigo, TD.110208).

Solutions and buffers

⏲ Timing: 2 h

⏲ Timing: 2 h (for step 4)

⏲ Timing: 30 min (for step 5)

⏲ Timing: 3 h (for step 6)

3. Preparation of buffers and solutions for transcardiac perfusion
   a. PFA 4%: Prepare 1 L of PFA 4% according to the following steps.
      i. Add 40 g of paraformaldehyde powder to 800 mL of PBS 1×.
      ii. Stir on heating block at ~60°C in a chemical hood.
      iii. Add 1 mL of 1 N NaOH gradually until you obtain a transparent solution.
      iv. Cool the solution at 20°C–22ºC and filter the undissolved particles.
      v. Adjust the volume to 1 L with PBS 1×.
      vi. Adjust the pH to 7.46 by adding NaOH or HCl.

   Note: 4% PFA can be stored at 20°C–22ºC for one week or at 4°C for up to three weeks.

   b. PBS 1×: Add 100 mL of PBS 10× to 900 mL of ddH_2O.
4. Preparation of AHA and anisomycin.
   a. Click-iT AHA stock: Add 500 μL of sterile 0.9% saline to the 5 mg AHA vial (Click-iT AHA (L-Azidohomoalanine), Thermo Fisher Scientific, Cat No. C10102) and vortex the vial to obtain a transparent solution.

   Note: AHA should be dissolved immediately before injection.

   b. Anisomycin (40 mM): Add 0.1 g of Anisomycin to 1 mL DMSO, vortex, and then add 9 mL ddH_2O.

   Note: The stock solution of anisomycin can be stored for up to four weeks at −20°C.

5. Preparation of click chemistry reagents.

a. Alexa Fluor 555 or 647 Alkyne antibody (2 mM): Add 312.5 μL ddH_2O to the vial of Alkyne antibody.

Note: The stock antibody can be kept for up to six months at −20°C when covered with aluminum to protect it from light.

b. Tris-(2-Carboxyethyl) phosphine, Hydrochloride (TCEP) stock solution (400 mM): Add 1.146 g of TCEP to 10 mL ddH_2O.

Note: The stock solution can be stored for up to three weeks at −20°C.

c. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (triazole ligand) stock solution (200 mM): Add 1.06 g of triazole ligand to 10 mL ddH_2O.

Note: The stock solution can be stored for three weeks at −20°C.

d. Copper(II) sulfate stock solution (200 mM): Add 0.998 g to 20 mL ddH_2O.

Note: The stock solution can be stored for up to one month at 4°C.

6. Preparation of buffers and solutions for IHC.
   a. PBS 1×: Add 100 mL of PBS 10× to 900 mL of ddH_2O.

   Note: PBS 1× can be stored at 20°C–22°C for up to 12 h and at 4°C for up to 4 days.

   b. EDTA (5 mM): Add 0.186 g to 100 mL dd H_2O.

   Note: The stock solution can be stored for up to one month at 4°C.

   c. Blocking solution for click chemistry: For 10 mL of blocking solution add 1 mL of normal goat serum (NGS), 5 μL of Triton X-100, and 500 μL sucrose to 8.5 mL of 1× PBS.

   Note: The blocking solution for click chemistry must be freshly prepared prior to this step.

   d. Blocking solution for co-staining IHC: Add 100 μL of normal goat serum (NGS) to 10 mL of 1× PBS.

   Note: The blocking solution must be freshly prepared prior to this step.

   e. FUNCAT wash buffer: For 100 mL of wash buffer add 10 mL of 0.5 mM EDTA and 1 mL of Tween-20 in 89 mL of 1× PBS.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CaMKII-α (6G9) mouse mAb, 1:800 dilution	Cell Signaling Technology	Cat No. 50049; RRID: AB_2721906
Anti-GAD67 antibody, 1:5,000 dilution	Sigma-Aldrich	Cat No. MAB5406; RRID: AB_2278725
Alexa Fluor 647 alkyne, 1:500 dilution	Thermo Fisher Scientific	Cat No. A10278
Alexa Fluor 555 alkyne, 1:500 dilution	Thermo Fisher Scientific	Cat No. A20013

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Anisomycin	Sigma-Aldrich	Cat No. A9789
Copper(II) sulfate	Thermo Fisher Scientific	Cat No. 033308.22
Click-iT AHA (L-azidohomoalanine)	Thermo Fisher Scientific	Cat No. C10102
Tris-(2-carboxyethyl) phosphine, hydrochloride (TCEP)	Thermo Fisher Scientific	Cat No. T2556
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine	Sigma-Aldrich	Cas No. 510758-28-8
Ethylenediaminetetraacetic acid (EDTA)	BioShop	Cas No. 6381-92-6
Methionine-deficient diet	Envigo	TD.110208
Normal goat serum	Thermo Fisher Scientific	Cat No. PCN5000
Paraformaldehyde (PFA)	Sigma-Aldrich	Cas No. 30525-89-4
Isoflurane	Fresenius Kabi	CP0406V2
Mounting medium with DAPI	Abcam	Cat No. ab104139
Anisomycin	Sigma-Aldrich	Cat No. A9789
Copper(II) sulfate	Thermo Fisher Scientific	Cat No. 033308.22
Experimental models: Organisms/strains		
Mouse; *Fmr1*$^{-/y}$, 6–8 weeks old, male mice	Jackson Laboratory	Stock No. 003025
Mouse; C57BL/6J, 6–8 weeks old, male mice	Jackson Laboratory	Stock No: 000664
Software and algorithms		
Zeiss LSM 880 with Airyscan confocal laser scanning microscope	Zeiss	RRID: SCR_020925
Leica VT1200S vibratome	Leica Biosystems	RRID: SCR_018453
ChemiDoc imaging system	Bio-Rad	RRID: SCR_019037
ImageJ	ImageJ	RRID: SCR_003070
GraphPad Prism	NA	RRID: SCR_002798
Leica VT1200S vibratome	Leica Biosystems	RRID: SCR_018453
Other		
Vibratome	Leica Biosystems	NA
Confocal microscope	Zeiss	NA
Mounting media containing DAPI	Thermo Fisher Scientific	Lot: 2305183
Mounting slides and coverslips	Thermo Fisher Scientific	Cat No: 22-037-246
Vortex	Cole-Parmer	UZ-04724-05
Rocking lab shaker	Thermo Fisher Scientific	Cat No. 88861021
Water bath	Cole-Parmer	UZ-12105-86
Peristaltic perfusion pump	Mandel	GA-F117800

MATERIALS AND EQUIPMENT

AHA		
Reagent	Final concentration	Amount
Click-iT AHA (L-Azidohomoalanine)	10 μg/μL	300 μL per adult mouse with 30 g weight
Saline	0.9%	Add 500 μL to a vial
Total	NA	500 μL

Note: The stock solution can be stored at 4°C for up to one day.

Anisomycin		
Reagent	Final concentration	Amount
Anisomycin	40 mM	0.1 g
DMSO	100×	1 mL
ddH_2O	NA	9 mL
Total	NA	10 mL

Note: The stock solution of anisomycin can be stored for up to four weeks at −20°C.

Alexa Fluor 647 or 555 Alkyne		
Reagent	Final concentration	Amount
Alexa Fluor 647 or 555 Alkyne	2 μM	2 μL per 1 mL of solution
ddH_2O	NA	312.5 μL per vial
Total	NA	314 μL

Note: The stock can be stored at −20°C for up to six months.

Tris-(2-Carboxyethyl) phosphine, Hydrochloride (TCEP)		
Reagent	Final concentration	Amount
Tris-(2-Carboxyethyl) phosphine, Hydrochloride (TCEP)	400 μM	1.146 g
ddH_2O	NA	8.854 mL
Total	NA	10 mL

Note: The stock solution can be stored for up to three weeks at −20°C.

Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (triazole ligand)		
Reagent	Final concentration	Amount
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (triazole ligand)	200 mM	1.06 g
ddH_2O	NA	8.94 mL
Total	NA	10 mL

Note: The stock solution can be stored for three weeks at −20°C.

Copper (II) sulfate		
Reagent	Final concentration	Amount
Copper(II) sulfate	200 mM	0.998 g
ddH_2O	NA	19.002 mL
Total	NA	20 mL

Note: The stock solution can be stored for up to one month at 4°C.

PBS 1×		
Reagent	Final concentration	Amount
PBS 10×	10×	100 mL
ddH_2O	NA	900 mL
Total	NA	1 L

Note: The solution can be stored at 4°C for up to a few days.

PFA 4%		
Reagent	Final concentration	Amount
PFA	4%	40 g
PBS	1×	960 mL
Total	NA	1 L

Note: The solution can be stored at 4°C for up to three weeks.

EDTA		
Reagent	Final concentration	Amount
EDTA	5 mM	0.186 g
ddH_2O	NA	100 mL
Total	NA	100 mL

Note: The stock solution can be stored for up to one month at 4°C.

Blocking solution for click chemistry reaction		
Reagent	Final concentration	Amount
Triton X-100	0.05%	5 μL
Normal goat serum	10%	1 mL
Sucrose	5%	0.5 mL
PBS	1×	8.5 mL
Triton X-100	0.05%	5 μL
Total	NA	10 mL

Note: The blocking solution for click chemistry must be freshly prepared.

Blocking solution for co-staining IHC		
Reagent	Final concentration	Amount
Normal goat serum	1%	100 μL
PBS	1×	9.9 mL
Total	NA	10 mL

Note: The blocking solution must be freshly prepared.

FUNCAT wash buffer		
Reagent	Final concentration	Amount
EDTA	0.5 mM	10 mL
Tween-20	20×	1 mL
PBS	1×	89 mL
Total	NA	100 mL

Note: The wash buffer can be stored at 4°C for up to two weeks.

STEP-BY-STEP METHOD DETAILS

Part 1. Methionine-deficient diet

Timing: 7 days

The goal of this step is to reduce the amount of available methionine in cells.

1. Put the mice on a methionine-deficient diet for seven days prior to AHA injection to enhance competitive incorporation of the AHA into the nascent polypeptide chain.

Part 2. AHA injection

Timing: 3–4 h (for one mouse)

This section aims to incorporate AHA, a methionine analog, into the newly synthesized polypeptide chain.

2. Dissolve the AHA (Click-iT AHA (L-Azidohomoalanine), Thermo Fisher Scientific, Cat No. C10102) in sterile 0.9% saline solution.
   a. Add 500 μL of sterile 0.9% saline to the 5 mg AHA vial.
   b. Vortex the vial to obtain a transparent solution at 10 μg/μL final concentration.
3. Inject the AHA (100 μg/ gram of body weight) intraperitoneally (i.p.) and return the mice to their home cages for 3 h.

   Note: To confirm the specificity of the AHA incorporation, inject another group of mice with a protein synthesis inhibitor, anisomycin (dissolve anisomycin in saline 0.9% to obtain a 40 μM solution and inject at 100 mg/kg (i.p.) one hour before the AHA injection).

   ⚠ CRITICAL: As stress can potentially affect protein synthesis, minimize the animal's stress level by returning the mouse to its home cage immediately after AHA injection and keep the cage in a quiet and well-ventilated place until perfusion step.

Part 3. Perfusion and brain extraction

⏲ Timing: 2 days

The purpose of this step is to prepare the brains for click chemistry and immunohistology experiments.

4. Anesthetize the mouse using anesthesia machine.
   a. Place the mouse in an anesthesia induction chamber where the mouse inhales a mixture of oxygen (flowmeter is set at 0.8–1.5 L/min) and 3%–5% isoflurane.
   b. Once the mouse is unconscious, transfer it to the nose cone connected to the anesthesia machine to maintain a stable level of anesthesia.
   c. Adjust the oxygen flowmeter to 0.4–0.8 L/min and the isoflurane to 2%–2.5%.
   d. Assess the anesthesia level by pinching the tail or toe with a pair of tweezers. The perfusion can be started only if the surgical level of anesthesia is met.

   ⚠ CRITICAL: Ensure the mice are deeply anesthetized and reached the surgical level.

5. Perfuse the mice transcardially with 4% PFA (refer to Wu et al.[15])
   a. Place the anesthetized mouse on its back and tape the four paws to the perfusion board.
   b. Expose the heart with surgical incisions.
   c. Insert the 28-gauge needle into the heart from the apex of the left ventricle.
   d. Perfuse the mouse with 10 mL 1× PBS using a peristaltic pump with 30 RPM (~2 mL/5 s) flow rate and then switch the 1× PBS to PFA 4% and keep perfusing with 50–100 mL of PFA, depending on the stiffness of the tail and limbs.
6. Extract the brain.
   a. Cut and peel the skin and muscles to expose the skull.
   b. While holding the skull firmly with forceps, cut through the skull to expose the brain.
   c. After detaching the cranial nerves and blood vessels, gently scoop out the brain.
   d. Post-fix the extracted brain in 4% PFA for 12–18 h at 20°C–22°C.

   Note: For each brain, add 7–10 mL of 4% PFA. Keep the extracted brain in 7–10 mL of 4% PFA for 12–18 h at 20°C–22°C.

7. Using the vibratome, cut the brains to obtain 40 μm thickness coronal sections.

a. Prepare the ice-cold 1× PBS. Prepare 1 L of ice-cold 1× PBS by placing it at −20°C for 20–30 min.
b. Prepare the brain. Locate the cerebellum while the brain dorsal side is up and make a transverse cut rostral to the cerebellum and discard the caudal part.
c. Prepare the vibratome.
   i. Prepare the vibratome by filling the slicing chamber with ice-cold 1× PBS and adding ice to the tray surrounding the chamber.
   ii. Mount a razor blade on the blade holder of the vibratome.
   iii. Set the vibratome cutting thickness at 40 μm.
   iv. Set the vibratome speed to 0.36 mm/s and amplitude to 1.20 mm.
d. Start cutting.
   i. Mount the brain onto the cutting stage of the vibratome using super glue.

Note: The brain's rostral side is up and the dorsal side is facing the blade.

   ii. Start the vibratome and slowly lower the cutting blade into the 1× PBS solution until it touches the brain's surface.
   iii. Start cutting the brain.
   iv. Collect the coronal sections in a 24-well plate filled with 1× PBS.

Note: transfers the free-floating sections using a paint brush to the wells pre-filled with 1 mL of 1× PBS.

8. Select three sections per cortical area/animal and transfer them to a new 24-well plate.

Note: Cortical sections have been used in this protocol. Sections from other areas such as hippocampus and amygdala can be collected based on the area of interest.

9. Wash the sections 6 times for 5 min each with 500 μL PBS on rocking shaker at 50 RPM at the 20°C–22°C.

Part 4. Click chemistry

Timing: 2 days

At this stage, click reactions are used to label the azide group of AHA with the alkyne chemical group present in the fluorescent alkyne antibody, allowing for subsequent visualization.

10. Block the sections for 12–18 h at 4°C in 500 μL of blocking buffer containing 0.05% Triton X-100, 10% normal goat serum, and 5% sucrose in PBS.

Note: The blocking solution for click chemistry must be freshly prepared.

Note: the blocking step should be performed on orbital shaker with gentle agitation at 20 RPM.

11. Perform 6 washes of 5 min using PBS on rocking shaker with gentile agitation (20–30 RPM).

Note: aggressive washing (shaking at more than 50 RPM) at this step can interfere with the blocking quality.

12. Incubate the sections in 500 μL of click buffer for 12–18 h at 4°C and keep the plate on a shaker at 20 RPM speed, based on the following protocol and order.

⚠ CRITICAL: Add the reagents according to the following order. In click chemistry reactions, the order of the reagent addition ensures that all necessary components are present in the correct form at the appropriate time for the reaction to proceed effectively. An essential step in the click reaction involves adding TRIzol and TCEP before $CuSO_4$. This is a critical step because TRIzol and TCEP play a key role in reducing copper (II) ions (Cu^{2+}) to copper (I) ions (Cu^+). These copper (I) ions then catalyze and facilitate the reaction.

a. Prepare fresh PBS at 20°C–22°C.
b. Add 1 μL of 200 mM TRIzol per 1 mL of click buffer. [Vortex the buffer with high speed for 10 s].
c. Add 1 μL of 500 mM TCEP per 1 mL of click buffer. [Vortex the buffer with high speed for 10 s].
d. Add 1 μL of 2 mM fluorescent Alexa Fluor 647 alkyne per 1 mL of click buffer. [Vortex the buffer with high speed for 10 s].

Note: There are a variety of Alexa Fluor alkyne antibodies with different wavelengths such as 488, 555, and 647 nm. Consider the wavelength of interest based on the channel you plan to image.

e. Add 1 μL of 200 mM $CuSO_4$ per 1 mL of click buffer. [Vortex the buffer with high speed for 30 s].

Note: Protect the click buffer from light. All reagents should be prepared fresh before the click step.

Part 5. Co-staining with the antibodies of interest

⏲ Timing: 12–18 h

The goal of this step is to label different neuronal types using primary antibodies. Use antibodies against CaMKIIα and GAD67 to label excitatory and inhibitory neurons, respectively.

13. Wash the sections after the click step 10 times for 5 min each with 500 μL of the "FUNCAT wash buffer" consisting of 0.5 mM EDTA and 1% Tween-20 in PBS on rocking shaker (50 RPM).

Note: The wash buffer can be stored at 4°C for up to two weeks.

14. Transfer the sections to a new 24-well plate and block the sections with 500 μL of the immunohistochemistry (IHC) blocking buffer (1% normal goat serum in PBS) for 1 h at 20°C–22°C on an orbital shaker (20 RPM).

Note: The blocking solution must be freshly prepared.

15. Incubate the blocked sections in the 500 μL of the primary antibody of interest for 12–18 h at 4°C on an orbital shaker.

Note: The primary antibody should be selected based on the cell types of interest. For example, we used antibodies against CaMKIIα (1:800 dilution, in 1× PBS and 2% NGS) and GAD67 (1:5000 dilution, in 1× PBS and 2% NGS) to identify the excitatory and inhibitory neurons, respectively.

16. Wash the sections 3 times for 5 min each with 500 μL PBS on rocking shaker.

17. Incubate the sections in 500 μL of corresponding secondary antibody (such as Alexa Fluor 488, 1:500 dilution) at 20°C–22°C on orbital shaker for 1 h.

   ⚠ CRITICAL: The Alexa Fluor antibody required for this step differs from the Alexa Fluor antibody required for the click chemistry step. Click chemistry requires Alexa Fluor to have an alkyne group in order for the reaction to proceed.

18. Wash the sections 3 times for 5 min each at 20°C–22°C with 500 μL of PBS.
19. Prepare the glass slides, coverslips, and mounting media.
20. Mount the sections on the slides and cover each section with 20 μL of DAPI-containing mounting media.
21. Place the coverslips, apply the sealant (nail polish), and allow the slides to dry at 20°C–22°C for 2 h and then keep them at 4°C in darkness.

Part 6. Confocal microscopy

Timing: 3 h

In this part, tissue sections will be imaged using confocal microscopy.

22. Image the slides using a confocal microscope. For better resolution and reliable quantification of the AHA signal, use the 63× oil objective in Z-stack mode to capture images.
    a. Use the 63× oil objective and ensure the objective lens is clean and properly aligned.
    b. Place one slide on the microscope stage, adjust the stage and the pinhole size for confocal microscopy.
    c. Choose lasers with appropriate wavelengths of interest and select proper emission and excitation filters to allow specific wavelengths to pass through.
    d. Adjust laser power to an optimal level.
    e. Choose the Z-stacks mode and start imaging. From each region of interest, such as the prefrontal cortex or the hippocampus, stacks of 15–20 optical sections spaced at 2–2.5 μm intervals are taken.

   ⚠ CRITICAL: Avoid the high laser power as it can lead to photobleaching.

23. Image at least 3 sections per area (prefrontal cortex) for each mouse.

   Note: Depending on the number of sections, the imaging step may take less or more time.

Part 7. Quantification and data analysis

Timing: 2–5 h

This step aims to quantify the AHA signal in the cytoplasm of different neurons to assess the rate of *de novo* protein synthesis.

Merge all Z-stacks using Image >> Stacks >> Z project >> Max intensity on ImageJ.

24. Measure the integrated density of the AHA signal in the cytoplasm with the guidance of the neuronal marker and DAPI.
    a. Open the merged image using ImageJ.
    b. Follow the following steps on ImageJ software. Color >> Image >> Channels tool >> select color mode.
    c. Select the marker channel. For example, the CAMKIIα channel (red)

d. Using the marker, select one neuron.
e. Make a region of interest (ROI) that fits inside the cytoplasm of the neuron.
f. The aim is to measure the AHA signal in the cytoplasm of neurons. One effective technique to ensure that the AHA signal is measured in the cytoplasm, and not in the nucleus, is to confirm that the ROI does not overlap with the nuclear stain, DAPI. Therefore, select the DAPI channel alongside the AHA channel, and only if there is no overlap, the DAPI channel will be deselected and the AHA signal will be measured. Relocate the ROI if it is not appropriately located within the cytoplasm.
g. Select the AHA channel and deselect the marker and DAPI channels before measuring the integrated density of the AHA.

Note: to measure the integrated density in ImageJ, select: Analyze >> Set measurement >> Integrated density >> Ctrl + M.

Continue this procedure with the next neuron until you have quantified the desired number of neurons.

⚠ CRITICAL: Once ROI is defined for the first image, it is critical to maintain the same dimensions of ROI across all images and experimental groups. To ensure that the signal quantification remains comparable across different images, the ROI dimensions must be consistent. To achieve this, save your ROI dimensions by using the Ctrl + T key command on your computer. Quantify the AHA signal in 20–40 excitatory or 10 to 20 inhibitory neurons.

25. Normalize and analyze the data.

Divide all values for the control and *Fmr1*$^{-/y}$ groups by the control's average, then multiply the result by 100 to normalize the data. The normalized numbers will then be subjected to a student's t-test statistical analysis.

Note: Restrict your quantification to the cytoplasm and average the values from each mouse (please refer to quantification and statistical analysis section). This step may take less or more time depending on the number of images.

EXPECTED OUTCOMES

The FUNCAT protocol is designed to incorporate AHA with a small orthogonal group into nascent proteins. The combination of AHA labeling and immunohistochemistry for cell type markers allows for a visualization of the newly synthesized proteins in specific cell types. Neuronal markers, such as CaMKIIα and GAD67, enable measurement of the AHA signal, representing protein synthesis, not only in excitatory and inhibitory neurons but also in various neuronal compartments. We have used this protocol to visualize and quantify nascent protein synthesis in excitatory and inhibitory neurons in the brain of wild-type (WT) and *Fmr1*$^{-/y}$ mice[2] (Figures 1A and 1B). AHA labeling is blocked by the protein synthesis inhibitor, anisomycin, indicating the sensitivity of the protocol (Figure 2). Anisomycin was dissolved in saline 0.9% to obtain a 40 μM solution. Mice then received an intraperitoneal injection of 100 mg/kg of anisomycin or an equal volume of saline 1 h before the AHA injection.

QUANTIFICATION AND STATISTICAL ANALYSIS

We use the Airyscan super-resolution module (SR) of the confocal microscope (Zeiss, LSM880) to capture AHA signal. Using the Z-stack mode, stacks of 15–20 optical sections at 63× magnification are taken. All stacks are superimposed using the ImageJ software, employing the maximum intensity algorithm. Three sections from each mouse brain area are imaged and quantified. For CaMKIIα-positive excitatory neurons, 20–40 neurons per section and for GAD67-positive inhibitory neurons 10–20 neurons per section are quantified. All data for either excitatory or inhibitory neurons

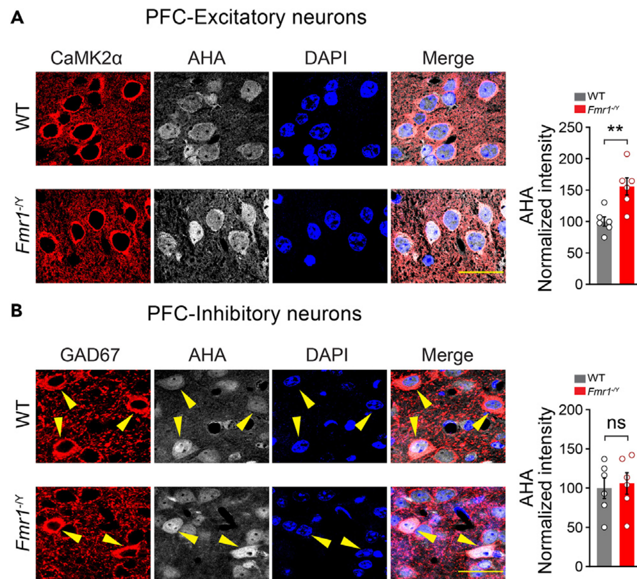

Figure 1. AHA incorporation (gray) in excitatory (CaMKIIα-positive, red in A) and inhibitory (GAD67-positive, red in B) neurons

(A and B) Representative immunofluorescent images and quantification from the prefrontal cortex (PFC) of WT (n = 6) and *Fmr1*$^{-/y}$ mice (n = 6) in excitatory (CAMKIIα-positive, t (10) = 3.601, p = 0.0048) and inhibitory (GAD67-positive, t (10) = 0.3130, $p > 0.05$) neurons. Yellow arrows mark inhibitory neurons. Each data point represents an individual animal. All data are presented as mean ± SEM **$p < 0.01$, and ns, not significant, Student's t-test. Scale bars, 25 μm.

are averaged to obtain a single AHA signal value for each mouse. These values are used to conduct statistical analysis.

> *Note:* Quantification of the AHA signal should be restricted to the neuronal cytoplasm with the guidance of the cytoplasmic cell type marker and DAPI signal to distinguish between neuronal cytoplasm and nucleus. The parameters for image acquisition (mode, laser intensity and PMT gain, number of optical sections in each z-stack, and etc.) and analysis (dimension of the region of interest (ROI), number of quantified cells, and brain area) must be consistent across all experimental groups.

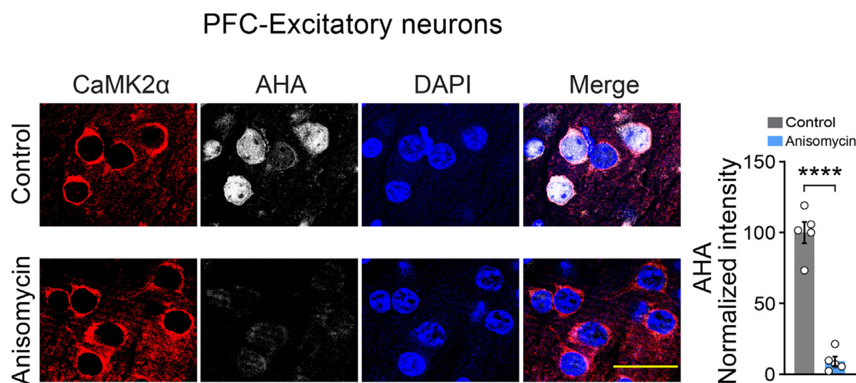

Figure 2. Anisomycin blocks AHA incorporation into newly synthesized proteins

Representative immunofluorescent images and quantification show AHA (gray) incorporation in excitatory (CaMKIIα-positive, red) neurons in the prefrontal cortex (PFC) of anisomycin-pretreated (n = 5) as compared to control mice (n = 5) (t (8) = 11.11, $p < 0.0001$). Each data point represents an individual animal. All data are presented as mean ± SEM ****$p < 0.0001$, Student's t-test. Scale bar, 25 μm.

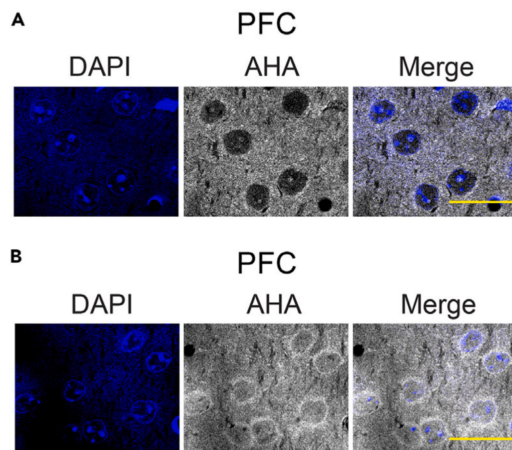

Figure 3. Common problems of *in vivo* fluorescent non-canonical amino acid tagging (FUNCAT) protocol
(A and B) Representative immunofluorescent images show AHA incorporation (gray) in excitatory neurons in the prefrontal cortex. (A) Weak or no AHA signal, and (B) high AHA background signal. Scale bars, 25 μm.

LIMITATIONS

FUNCAT is a valuable tool for studying the rate of protein synthesis and the localization of newly synthesized proteins within different cellular compartments. However, it is critical to recognize the limitations of this technique. The incorporation of the non-canonical amino acid AHA into the polypeptide chain may be affected by variations in methionine content across different proteins. This inconsistency can result in relatively weak signal intensity emitted from proteins with low methionine content and marginally affect the accuracy and sensitivity of the AHA visualization.

The second limitation of the protocol is that the AHA signal will not be detectable if the AHA administration is unsuccessful. This issue can be addressed by improving the accuracy of injections. Additionally, administering AHA bilaterally can minimize the chances of failed intraperitoneal injection. Furthermore, click chemistry involves numerous intricate steps and requires several days to complete. This extended duration may damage the cell processes such as dendrites and axons, potentially influencing the AHA signal in these cellular compartments.

TROUBLESHOOTING

Problem 1

The most frequent problem encountered during the AHA incorporation protocol is a strong fluorescent background, which may be accompanied by either a strong or weak AHA signal (Figures 3A and 3B) (related to part 4, step 10 and part 5, step 13).

Potential solution

- Insufficient blocking, due to short blocking time or high blocking temperature, is the primary cause of the strong background. To resolve this problem, consider using fresh reagents for making a blocking solution and also blocking the sections for 12–18 h at 4°C.
- The second cause could be inadequate washing after the click chemistry step. To address this issue, wash 10 times on a rolling shaker at 50 RPM speed. Ensure that you are using the washing buffer, not PBS.

Problem 2

Weak or no AHA signal, following the click chemistry step (related to part 4, step 12).

Potential solution

- If the AHA signal is absent or very weak, the click step might not react at all. In this case, use freshly made reagents for the click buffer. For example, prepare fresh copper sulfate, TRIzol, and TECP.

- In addition, consider adding the reagents based on the mentioned order and vortexing the solution properly.

Problem 3
AHA signal intensity is not consistent along the brain section (related to part 6, step 22 and part 3, step 7).

In this issue, the AHA signal is strong in one area of the image and weak in another area.

Potential solution

- Clean the objective lens properly.
- The blade of the vibratome does not cut straight, thus calibrate the blade holder.

Problem 4
High intensity of AHA signal in the nucleus (related to part 6, step 22 and part 4, step 10).

Potential solution

- Reduce the laser power for DAPI channel.
- Calculate the amount of Triton X-100 added to the blocking solution for click chemistry and make sure you have added 0.05%.

Problem 5
The CAMKIIα and GAD67 signals are absent, despite the normal AHA signal (related to steps 14 and 15 of part 5).

Potential solution

- The antibody concentration needs to be optimized.
- The blocking solution for co-staining IHC is not fresh.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Arkady Khoutorsky (arkady.khoutorsky@mcgill.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This work was supported by a grant from the Simons Foundation (SFARI, award #611773) and the Canadian Institutes of Health Research (CIHR, PJT-162412) to A.K. M.H. was supported by the Brain Canada and Transforming Autism Care Consortium (TACC) graduate fellowships.

AUTHOR CONTRIBUTIONS

M.H. and A.K. wrote the initial draft of the manuscript and created the figures. Everyone else edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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