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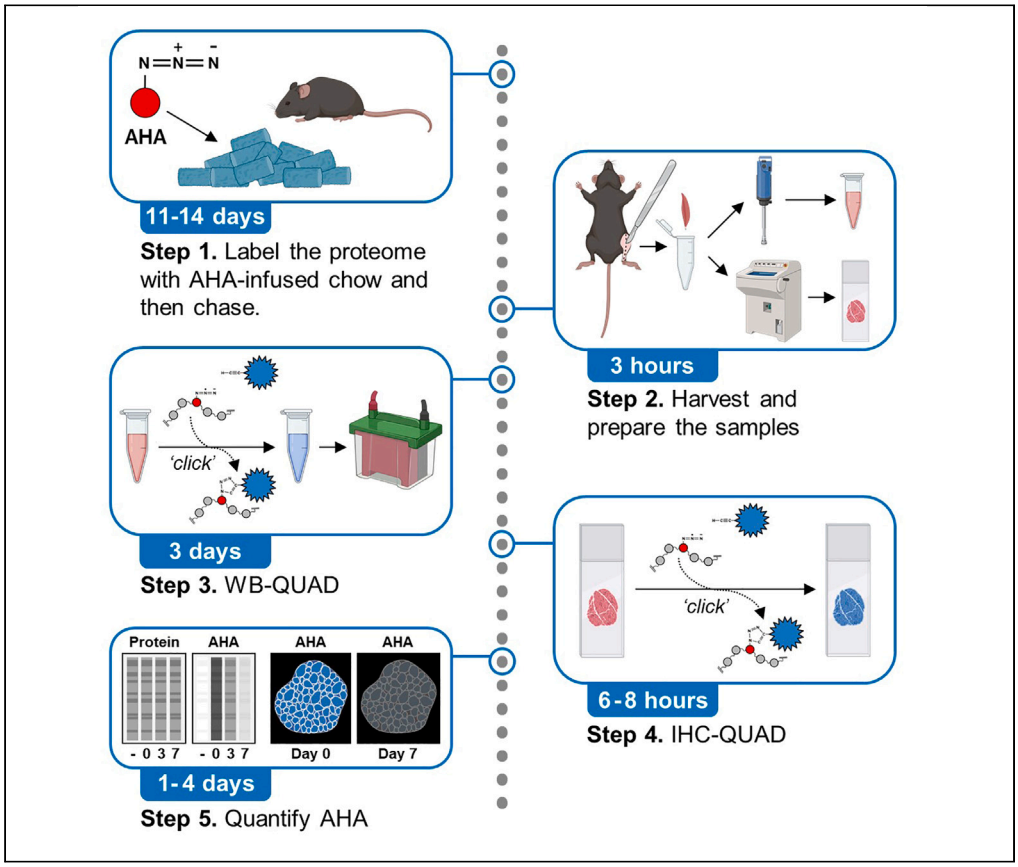
Protocol for quantifying the *in vivo* rate of protein degradation in mice using a pulse-chase technique



The ability to measure the *in vivo* rate of protein degradation is a major limitation in numerous fields of biology. Here, we present a protocol for quantifying this rate in mice using a pulse-chase technique that utilizes an azide-bearing non-canonical amino acid called azidohomoalanine (AHA). We describe steps for using chow containing AHA to pulse-label the animal's proteome. We then detail the quantification of AHA-labeled proteins in whole-tissue lysates or histological sections using a copper-catalyzed azide-alkyne cycloaddition *'click'* reaction.

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

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Highlights

A pulse-chase approach for visualizing the *in vivo* rate of protein degradation

Quantify protein degradation rate in whole-tissue lysate and histological sections

Label proteins *in vivo* using specially formulated AHA chow

Use 'click' chemistry with Western blotting and IHC to identify labeled proteins

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Protocol for quantifying the *in vivo* rate of protein degradation in mice using a pulse-chase technique

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SUMMARY

The ability to measure the *in vivo* rate of protein degradation is a major limitation in numerous fields of biology. Here, we present a protocol for quantifying this rate in mice using a pulse-chase technique that utilizes an azide-bearing non-canonical amino acid called azidohomoalanine (AHA). We describe steps for using chow containing AHA to pulse-label the animal's proteome. We then detail the quantification of AHA-labeled proteins in whole-tissue lysates or histological sections using a copper-catalyzed azide-alkyne cycloaddition *'click'* reaction.

For complete details on the use and execution of this protocol, please refer to Steinert et al. (2023).[1]

BEFORE YOU BEGIN

The methods outlined in this protocol describe the steps necessary to use the tibialis anterior (TA) muscles of 8-12-week-old mice for the assessment of protein degradation with Western blot quantification of AHA degradation (WB-QUAD) and immunohistochemical (IHC) quantification of AHA degradation (IHC-QUAD). The same methods can be used to examine protein degradation in other tissues throughout the body and, in theory, can be used in various types of animals of any age.

Institutional permissions

The use of mice in this protocol was approved by and performed in accordance with the regulations of the University of Wisconsin – Madison's Institutional Animal Care and Use Committee. Before beginning, approval must be obtained from the appropriate entities at each researcher's institution.

Breed or purchase mice

Timing: 1 week to 6+ months

1. Acquire mice that are 8–12 weeks old.
   a. If ordering mice from a commercial vendor, be sure to allow enough time for the shipment to arrive and for the mice to acclimate to their new surroundings for at least 48 h.
   b. If breeding mice to use for these experiments, allow ample time to breed for the desired genetic mutations.

Note: In order to quantify the time-dependent loss of AHA-labeled proteins, at least 4 mice in each of the three experimental groups (e.g., Day 0, Day 3, and Day 7 following the pulse with

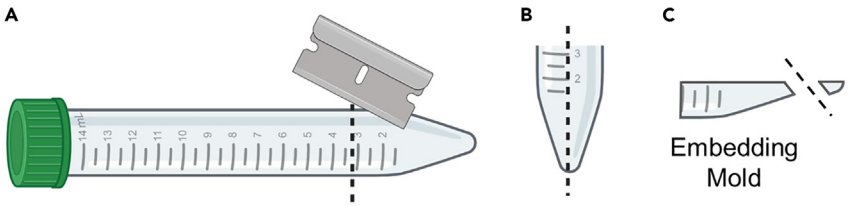

Figure 1. Construction of an embedding mold for freezing muscles in OCT

AHA) should be employed. At a minimum, two experimental time points must be assessed including an immediate post-pulse group (Day 0) and at least one chase group (e.g., Day 3 or Day 7 following the pulse). There should also be an animal of the same age, genetic background, etc. that is placed on the control chow during the pulse period as tissues from this animal will be used as a negative control for the experimental procedures.

Acquisition of low-methionine and AHA-infused chow

Timing: 8+ weeks

2. Order low-methionine (0.2% L-methionine) chow and AHA-infused (0.2% L-azidohomoalanine) chow in non-irradiated pelleted form, packaged separately in 1 kg bags, at least 8 weeks prior to starting the experiment.

Note: Store the unopened chow at 4°C or lower. Chow should be used within 6 months of the manufacture date.

Make the embedding mold that will be used for IHC muscle freezing

Timing: 15–30 min

3. To make an embedding mold that can be used to freeze muscles in optimal cutting temperature compound (OCT), cut off the bottom 3 cm (tapered end) of a 15 mL conical tube (Figure 1A).
   a. Cut the bottom section longitudinally in half (Figure 1B).
   b. Remove a small portion of the rounded bottom tip so that when the OCT is frozen, forceps can be used to push the block out of the large end of the embedding mold (Figure 1C).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Peroxidase-conjugated streptavidin (1:100000 dilution)	Jackson ImmunoResearch (West Grove, PA, USA)	ID: 016-030-084
Anti-Laminin (1:500 dilution)	Sigma-Aldrich (St. Louis, MO, USA)	ID: L9393
Alexa Fluor 488 Goat Anti-Rabbit IgG Secondary (1:5000 dilution)	Invitrogen (Waltham, MA, USA)	ID: A11008
Chemicals, peptides, and recombinant proteins		
Urea	Sigma-Aldrich (St. Louis, MO, USA)	ID: 9510-OP
Tris base	Thermo Fisher Scientific (Waltham, MA, USA)	ID: BP152-5
Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS)	Avantor delivered by VWR (Radnor, PA, USA)	ID: 21-031-CV
Triton-X 100	Thermo Fisher Scientific (Waltham, MA, USA)	ID: ICN807423

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Albumin, bovine serum (heat-shock treated) (BSA)	Thermo Fisher Scientific (Waltham, MA, USA)	ID: BP1600100
β-Glycerophosphate disodium salt pentahydrate	Santa Cruz Biotechnology, Inc. (Dallas, TX, USA)	ID: sc-203323
Sodium fluoride	Thermo Fisher Scientific (Waltham, MA, USA)	ID: S299-100
Leupeptin	Avantor delivered by VWR (Radnor, PA, USA)	ID: 25608-930
Sodium orthovanadate	Thermo Fisher Scientific (Waltham, MA, USA)	ID: ICN15966410
Phenylmethyl sulfonyl fluoride (PMSF)	Thermo Fisher Scientific (Waltham, MA, USA)	ID: PI-36978
DMSO	Thermo Fisher Scientific (Waltham, MA, USA)	ID: PI20684
Low-methionine chow	Cambridge Isotope Laboratories (Tewksbury, MA, USA)	ID: MF-UNLABELED-MET
AHA-infused chow	Cambridge Isotope Laboratories (Tewksbury, MA, USA)	ID: MF-AHA
19% Protein Extruded Rodent Diet	Inotiv, Inc. (West Lafayette, IN, USA)	ID: 2019
Optimal cutting temperature (OCT) compound	Sakura Finetek (USA)	ID: 25608-930
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (TBTA)	Sigma-Aldrich (St. Louis, MO, USA)	ID: 678937-50MG
Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)	Sigma-Aldrich (St. Louis, MO, USA)	ID: 580560-1GM
Copper (II) sulfate, anhydrous	Alfa Aesar (Haverhill, MA, USA)	ID: AA33308-02
Biotin-PEG4 Alkyne	Sigma-Aldrich (St. Louis, MO, USA)	ID: 764213-10MG
AZDye 594 – Alkyne	Click Chemistry Tools (Scottsdale, AZ, USA)	ID: 1297-1
Glycerol	Fisher Scientific (Waltham, MA, USA)	ID: AC15892-0010
Sodium dodecyl sulfate (SDS)	Fisher Scientific (Waltham, MA, USA)	ID: S539-500
Bromophenol blue	Fisher Scientific (Waltham, MA, USA)	ID: AC151350250
DL-dithiothreitol (DTT)	Fisher Scientific (Waltham, MA, USA)	ID: BP1725
Non-fat dry milk	Fisher Scientific (Waltham, MA, USA)	ID: NC9121673
Glycine	Fisher Scientific (Waltham, MA, USA)	ID: BP381-5
Methanol	Thermo Fisher Scientific (Waltham, MA, USA)	ID: A9354
Sodium chloride	Fisher Scientific (Waltham, MA, USA)	ID: BP358-10
Tween-20	Fisher Scientific (Waltham, MA, USA)	ID: BP337-500
Critical commercial assays		
DC Protein Assay Kit	Life Science (Hercules, CA, USA)	ID: 5000112
No-Stain Kit	Invitrogen (Waltham, MA, USA)	ID: A44449
Experimental models: Organisms/strains		
Mouse: wild-type C57BL/6; 8–12 weeks old; male or female	Jackson Laboratories (Bar Harbor, MA, USA)	Stock# 000664
Software and algorithms		
VisionWorksLS	Analytik Jena AG (UVP) (Upland, CA, USA)	N/A
Nikon NIS-Elements D software	Nikon Instruments (Melville, NY, USA)	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer
NIH ImageJ Fiji	National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin)	https://imagej.net/software/fiji/
Other		
Superfrost Plus Microscope Slides	Fisher Scientific (Waltham, MA, USA)	ID: 12-550-15
1.5 mL microfuge tubes	Avantor delivered by VWR (Radnor, PA, USA)	ID: 10011-700
15 mL conical tubes	Avantor delivered by VWR (Radnor, PA, USA)	ID: 89039-664
14 mL snap cap Falcon tubes	Fisher Scientific (Waltham, MA, USA)	ID: 14-959-11B

MATERIALS AND EQUIPMENT

- Buffer A: dissolve 0.5% Triton-X and 0.5% BSA in DPBS.

Buffer can be stored at 4°C for up to 3 days.

- 5% milk in Tris-Buffered Saline with 0.1% Tween-20 (TBST): dissolve 5 g of powdered non-fat dry milk in 90 mL of TBST. Adjust total volume to 100 mL with TBST.

Buffer can be stored at 4°C for up to 3 days.

- Bromophenol Blue (0.34% m/v): dissolve 34 mg of powdered Bromophenol blue in 10 mL of diH_2O.

Buffer can be stored at 20°C–22°C for up to one year.

- Urea/Tris Lysis Buffer: combine 4.8 g urea and 0.5 mL of 1 M Tris (pH 8.0) and adjust the total volume to 10 mL with diH_2O.

Make fresh buffer for every use. The dissolution of urea is an endothermic reaction, so the solution will become cold. The solution can be gently heated to 20–25°C to aid complete dissolution. Refrain from heating above 30°C because cyanates that are detrimental to proteins may form.[2] It may take 30–45 min of agitation to fully dissolve the urea.

Urea/Tris Lysis Buffer + Inhibitors: volume per sample

Reagent	Amount	Final concentration
Urea/Tris Lysis Buffer	694.75 µL	
β-glycerophosphate (600 mM) in diH_2O	31.25 µL	25 mM
Sodium fluoride (1 M) in diH_2O	18.75 µL	25 mM
Leupeptin (10 mg/mL) in diH_2O	0.75 µL	10 µg/mL
Sodium orthovanadate (1 M) in diH_2O	0.75 µL	1 mM
PMSF (0.2 M) in isopropanol	3.75 µL	1 mM
Total	**750 µL**	

Buffer should be made fresh for every use, for multiple reactions, multiply the 'amount' by the number of samples.

Note: On average, TA muscles are approximately 40 mg. This mass will vary based on age, strain, and interventions.

⚠ CRITICAL: Add PMSF last and use buffer within 15 min of this addition due to the short half-life of PMSF in an aqueous solution.

WB-QUAD *'Click'* Reaction Master Mix: volume per sample

Reagent	Amount	Final concentration
DPBS	18.125 µL	
TBTA (20 mM) in DMSO	1.275 µL	944 µM
Copper sulfate (100 mM) in diH_2O	2 µL	7.407 mM
Biotin PEG4-alkyne (5 mM) in DMSO	1.6 µL	296 µM
TCEP (50 mM) in diH2O	4 µL	7.404 mM
Total	**27 µL**	

Buffer should be made fresh immediately before use. For multiple reactions, multiply the 'amount' by the number of samples.

Note: Reagents should be added in the order listed with vortexing between each addition.

Note: It has been recommended that fresh TCEP be used to maximize *'click'* reaction efficiency and avoid precipitation of the copper during the reaction.[3] However, we have successfully completed numerous *'click'* reactions with aliquots of stock TCEP that had been stored at −20°C for several weeks.

IHC-QUAD *'Click'* Reaction Master Mix: volume per sample		
Reagent	Amount	Final concentration
DPBS	45.25 μL	
TBTA (20 mM) in DMSO	0.25 μL	0.1 mM
Copper sulfate (100 mM) in diH_2O	0.5 μL	1 mM
AZDye 594 Alkyne (50 μM) in DMSO	3 μL	3 μM
TCEP (50 mM) in diH_2O	1 μL	1 mM
Total	**50 μL**	
Buffer should be made fresh immediately before use. For multiple reactions, multiply the 'amount' by the number of samples.		

Note: Reagents should be added in the order listed with vortexing between each addition.

Note: It has been recommended that fresh TCEP be used to maximize *'click'* reaction efficiency and avoid precipitation of the copper during the reaction.[3] However, we have successfully completed numerous *'click'* reactions with aliquots of stock TCEP that had been stored at −20°C for several weeks.

4x Laemmli Buffer		
Reagent	Amount	Final concentration
Glycerol	4 mL	40%
Tris Base (1 M) pH 6.8 in diH_2O	2 mL	200 mM
SDS	800 mg	8%
Bromophenol Blue (0.34% m/v in diH_2O)	1.0 mL	0.034%
diH_2O	0.4 mL	
Add immediately before use:		
DTT (2 M)	2 mL	0.4 M
Total	**10 mL**	
A stock solution of the first 5 reagents can be made and stored for up to 2 years.		

⚠ CRITICAL: DTT should be added immediately before use. Any remaining buffer containing DTT should be discarded and a fresh batch should be made prior to the next use.

Tris-Glycine Running Buffer		
Reagent	Amount	Final concentration
Tris Base	3 g	25 mM
Glycine	14.4 g	192 mM
SDS	1 g	3.47 mM
diH_2O	9812 mL	
Total	**1 L**	
The first 3 ingredients can also be combined at higher concentrations to make a 10× stock solution, and this solution can be stored at 4°C.		

Transfer Buffer		
Reagent	Amount	Final concentration
Tris Base	4 g	33 mM
Glycine	10.4 g	139 mM
diH_2O	785 mL	
Methanol	200 mL	20%
Total	**1 L**	
The first three ingredients can also be combined at higher concentrations to make a 10x stock solution, and this solution can be stored at 4°C.		

Tris-Buffered Saline with 0.1% Tween-20		
Reagent	Amount	Final concentration
NaCl (3M) in diH_2O	50 mL	150 mM
Tris Base (1 M) pH 7.5 in diH_2O	50 mL	50 mM
Tween-20	1 mL	0.1%
diH_2O	899 mL	
Total	1 L	

Buffer can be stored at 20°C–22°C.

STEP-BY-STEP METHOD DETAILS

Label proteins with low-methionine and AHA-infused chow

Timing: 11–18 days

In this step, animals are put on a low-methionine chow to deplete methionine prior to the AHA "pulse" labeling period. Following the labeling period, regular control chow is reintroduced to eliminate any further incorporation of AHA. Figure 2 is a visual representation of the experimental timeline.

1. Switch chow in cages of experimental animals to low-methionine pellets. Maintain control animals on regular chow.
   a. Transfer mice to a clean cage prior to beginning the low-methionine chow to control for any leftover chow that may be buried in the bedding material.
   b. Provide the low-methionine chow and water ad libitum for 7 days.

   Note: Approximately 3 grams of chow per mouse per day is sufficient. Use this information as a guide to avoid having to discard large quantities of partially eaten chow.

2. After 7 days of low-methionine chow, switch chow in the cages of the experimental animals to the AHA-infused pellets for the "pulse" labeling period. Maintain control animals on regular chow.
   a. Transfer mice to a clean cage prior to beginning AHA chow to control for any leftover chow that may be buried in the bedding material.
   b. Provide the AHA-infused chow and water ad libitum for 4 days.
3. After 4 days of AHA-infused chow, switch chow in cages to regular control pellets for the 3–7 day "chase" period.
   a. Transfer mice to a clean cage prior to beginning regular control chow to control for any leftover chow that may be buried in the bedding material.
   b. Provide the regular control chow and water ad libitum for 3–7 days.

   Note: The rationale for selecting the indicated pulse and chase durations can be found in the parent manuscript, Steinert et al. (2023).[1]

Harvesting muscles for WB-QUAD and IHC-QUAD

Timing: 15–20 min per mouse

Muscles that will be used for WB-QUAD and IHC-QUAD are collected and flash-frozen in liquid nitrogen or liquid nitrogen-chilled isopentane, respectively.

4. Prepare the surgery area for muscle harvesting.
   a. Label 1.5 mL microfuge tubes for all muscles being harvested for WB-QUAD.
   b. Fill a small Dewar with liquid nitrogen to freeze down muscles following harvest.

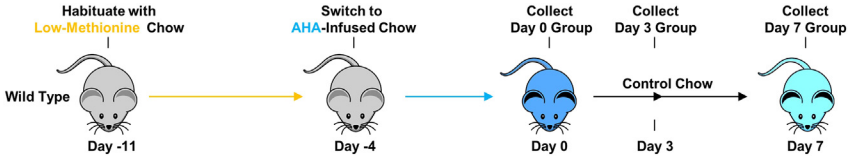

Figure 2. Timeline and experimental design of the pulse-chase labeling strategy for WB-QUAD and IHC-QUAD[1]

c. Prepare the chamber for freezing down IHC-QUAD muscles.
   i. Place a small volume (50–70 mL) of isopentane in a 250–300 mL plastic beaker with a lid. A foam ice bucket can serve as an excellent chamber for this procedure.
   ii. Chill the isopentane by adding liquid nitrogen to the chamber containing the plastic beaker.

Note: Be sure that the liquid nitrogen level is at least half of the level of the isopentane to ensure adequate cooling (Figure 3A).

d. Prepare a surface to measure the length of the muscle. A paper towel wetted with deionized water works well.

5. Anesthetize the mouse.
   a. Prime the induction chamber using an isoflurane anesthesia apparatus; vapor setting 2.5–4% and oxygen flow rate of 1 L/min.
   b. Place the mouse in the induction chamber and wait until the mouse is fully anesthetized and unresponsive to a pedal pinch.
6. Prepare the animal for the surgical procedure.
   a. Transfer the mouse from the induction chamber to the nose cone to maintain anesthesia.
   b. Shave the fur from the lower leg.
7. Harvest the TA muscle for WB-QUAD.
   a. Open the skin on the anterior aspect of the lower limb and expose the TA muscle.
   b. Carefully excise the TA muscle from the mouse.

Optional: Weigh the muscle and record the mass

   c. Quickly transfer the TA muscle to the labeled 1.5 mL microfuge tube and drop the closed microfuge tube into the Dewar of liquid nitrogen to flash freeze the sample.
   d. Upon completion of the muscle harvest, transfer the samples to a storage vessel in a freezer set at −80°C.

Note: Exercise caution during the excision of the TA muscle so that unnecessarily large amounts of force are not placed on the tissue.

8. Harvest the contralateral TA muscle for IHC-QUAD.
   a. Before excising the TA muscle, check the isopentane for the presence of a layer of frozen isopentane with a pool of liquid isopentane in the center of the plastic beaker.
      i. If the isopentane has frozen completely, remove the beaker from the liquid nitrogen and allow some of the frozen isopentane to return to its liquid state.

Note: There must be enough liquid isopentane in the beaker to fully submerge the embedding mold (Figure 3A).

   b. Open the skin on the anterior aspect of the lower limb and expose the TA muscle.
   c. Carefully excise the TA muscle from the mouse.
   d. Place the muscle on a moist paper towel, very slightly stretch the muscle out and then allow it to rebound to its resting length. Measure and record the length of the muscle (Figure 3B).

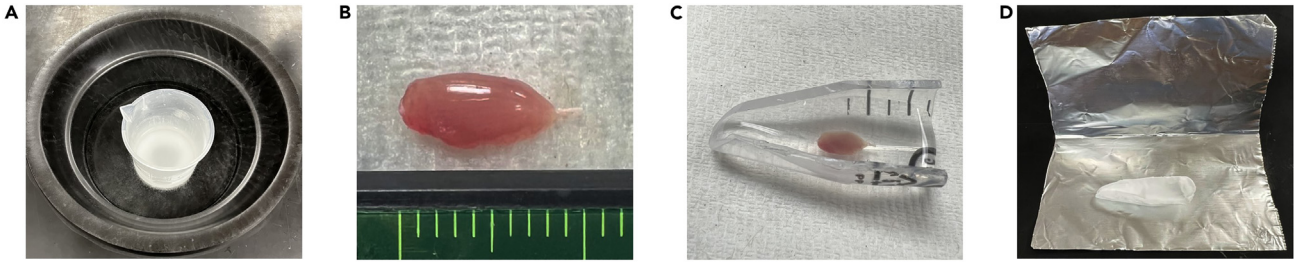

Figure 3. Illustration of the setup and procedure for freezing muscles for IHC-QUAD

(A) Chamber for freezing muscles in OCT for IHC-QUAD. The plastic beaker is filled with a small volume of isopentane. Liquid nitrogen has been added to the bottom of the chamber surrounding the plastic beaker to chill the isopentane. Note the layer of frozen isopentane that is visible on the interior periphery of the beaker whereas the center contains isopentane in a liquid state.

(B) TA muscle that has been excised at resting length.

(C) TA muscle in 20°C–22°C OCT immediately before the embedding mold is placed in the chilled isopentane. Note that the TA is fully submerged in the OCT and it is oriented such that the long axis of the muscle is parallel to the side walls of the embedding mold.

(D) Block of frozen OCT containing the TA muscle that has been removed from the embedding mold, and placed on aluminum foil prior to storage at −80°C.

e. Place a 5–6 mm deep layer of OCT in the embedding mold and drag the muscle through the OCT to fully submerge it.
   i. Maintain the muscle parallel to the sidewalls of the embedding mold.

Note: Dragging the muscle through the OCT will help to keep it on a straight axis and at/near its measured resting length (Figure 3C).

f. Use long forceps to place the embedding mold into the liquid nitrogen-chilled isopentane to freeze the muscle.
   i. Keep the embedding mold submerged in the isopentane for at least 30 s to ensure that the muscle is fully frozen, thereby avoiding freeze damage.

g. Remove the frozen block of OCT from the embedding mold, package it in labeled aluminum foil, and transfer it to a storage vessel in a freezer set at −80°C (Figure 3D).

Pause point: Muscles can be stored at −80°C for at least 6 months.

Homogenize muscles for WB-QUAD

Timing: 30–45 min

Whole muscle homogenates are used to determine the rate of protein degradation with western blotting.

9. Prepare materials for the homogenization process. It is important to work quickly once homogenization has started, thus preparing all necessary materials in advance will aid in a smooth process.
   a. Label one 14 mL snap cap Falcon round bottom test tube for each sample that will be homogenized.
   b. Fill an insulated container with ice to hold the labeled Falcon tubes.
   c. Aliquot the amount of Urea/Tris lysis buffer necessary for the volume of Urea/Tris lysis buffer + inhibitors that will be used to homogenize all of the samples and chill on ice.

Note: Do not chill the buffer for too long or the urea will come out of solution. If you notice that the urea has come out of solution warm the solution slightly and it will go back into solution.

d. Fill a small Dewar with liquid nitrogen.
e. Remove frozen muscle samples from the −80°C freezer and transfer them to the Dewar filled with liquid nitrogen.
f. Make Urea/Tris lysis buffer + inhibitors, and immediately following the addition of PMSF add 0.75 mL of the buffer to each of the labeled Falcon tubes.

⚠ CRITICAL: The Urea/Tris lysis buffer + inhibitors should be used within 15 min of adding the PMSF to the solution.

Note: The volume of buffer needed to homogenize the samples will depend on the size and subsequent protein concentration of the muscle. For example, our lab typically uses 0.75 mL for the TA muscle which will produce a protein concentration of ~11 μg/μL.

10. Homogenize samples.
    a. Remove one 1.5 mL microfuge tube containing a single sample from the liquid nitrogen with long forceps and then quickly place the sample in the corresponding Falcon tube that already contains cold Urea/Tris lysis buffer + inhibitors.
    b. Using a Polytron homogenizer at full speed, homogenize the sample taking care to move the tube both vertically and horizontally to break up the whole sample leaving behind no large chunks.

Note: This step should take no longer than 15–20 s.

    c. Replace the cap and return the Falcon tube to the ice.
    d. Repeat steps 10. a - 10. c with the remaining samples until all samples have been homogenized.

Note: The probe of the polytron should be washed thoroughly with deionized water between samples. Flush the inside of the probe and visually inspect for any unhomogenized sample. Make sure the probe is dry before homogenizing the next sample.

Note: Samples should be homogenized in batches with approximately 6 samples per batch. Fresh Urea/Tris lysis buffer + inhibitors should be made for each batch to restart the 15-min interval during which the PMSF is viable. Samples from all experimental groups should be present in each batch to ensure that any potential batch variance is distributed across all of the groups.

    e. Centrifuge the homogenized samples at 6000 × *g* for 2 min at 20°C–22°C. This will result in a layer of dense foam on the top of the supernatant.
        i. Check for any remaining chunks in the pellet.
        ii. If there are unhomogenized pieces of muscle remaining, repeat step 10. b.
    f. Vortex the homogenized samples to break up the layer of foam.
    g. Centrifuge at 6000 × *g* again for 2 min.
        i. Repeat steps 10. f and 10.g until the foam layer is gone and has become incorporated into the homogenate. This may take as many as 6 repetitions.

Measure and standardize the sample protein concentrations

Timing: 30–45 min

When preparing samples for the *'click'* reaction it is important to standardize the protein concentration for all of the samples so that each *'click'* reaction for each sample contains the same amount of Urea/Tris lysis buffer + inhibitors.

11. Use 1.5 μL of the homogenate to measure the protein concentration of each sample using the BioRad DC protein assay with BSA standards.
12. Add 1.5 μL Urea/Tris stock solution to each of the BSA standards and also use this solution as the blank on the plate.
    a. While the protein assay is incubating, label two additional microfuge tubes for each sample.

Note: The accuracy of the protein concentration measurements is critical when calculating the total amount of AHA-labeled proteins per sample. Thus, to minimize variance, all samples should be measured in triplicate and, whenever possible, all samples for a given experiment should be measured within a single assay.

13. Once the protein concentrations have been determined for each sample, transfer 100 μL of each sample to one of the newly labeled microfuge tubes.
14. Dilute each sample to a final concentration of 8 μg/μL using the leftover Urea/Tris lysis buffer + inhibitors.
15. In the other labeled tube, take 80 μL of the 8 μg/μL stock prepared in step 14 and dilute it to a final concentration of 1.15 μg/μL by adding 476.5 μL of DPBS.

Perform the *'click'* reaction on the sample homogenates

Timing: 75–90 min

The *'click'* reaction will covalently bind a biotin tag to the AHA on the AHA-labeled proteins as explained in Figure 4.

16. Perform the *'click'* reaction.
    a. Label a new microfuge tube for each sample that will be subjected to the WB-QUAD 'click' reaction.
    b. Make fresh WB-QUAD *'click'* reaction master mix in a volume suitable for the number of samples being used.
    c. For each sample, combine 27 μL of the WB-QUAD *'click'* reaction master mix with 173 μL of the diluted (1.15 μg/μL) sample.
    d. Vortex each sample thoroughly.

Note: The final concentration for the components of the WB-QUAD *'click'* reaction when added to the sample (total volume = 200 μL) are as follows: TBTA (127.5 μM), Copper Sulfate (1 mM), Biotin PEG4-alkyne (40 μM), and TCEP (1 mM).

Note: The *'click'* reactions should be processed in batches with approximately 6 samples per batch. Samples from all experimental groups should be present in each batch to ensure that any potential batch variance is distributed across all of the groups.

17. Incubate samples for 1 h on a 25 RPM nutating rotator at 20°C–22°C protected from light.

Pause point: At the end of the incubation period the samples can be flash frozen, returned to the freezer at −80°C, and stored for a short time (~1 week).

Resolve the AHA-labeled proteins with western blotting

Timing: 2 days

The Western blot protocol allows for quantification of the amount of total protein and the amount of AHA-labeled proteins in each lane. With these values, the AHA-labeled protein to total protein ratio

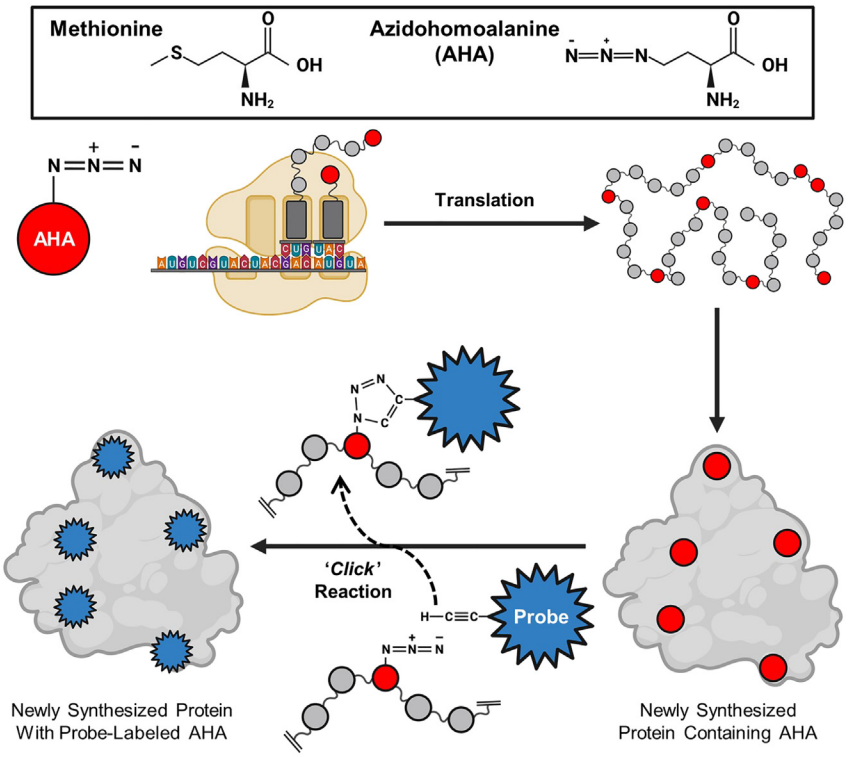

Figure 4. Visualization of AHA incorporation using a *'click'* reaction

AHA is a methionine analog that possesses a reactive azide group. During translation, AHA can be incorporated into newly synthesized proteins. The newly synthesized proteins can contain multiple AHAs and each AHA can be covalently bound to an alkyne-bearing probe via a copper-catalyzed azide-alkyne cycloaddition reaction (i.e., *'click'* reaction). In WB-QUAD, an alkyne-bearing probe that is conjugated to biotin is used to tag the AHA, and then streptavidin conjugated to horseradish peroxidase along with enhanced chemiluminescence is used to visualize and quantify the amount of AHA-labeled proteins in tissue lysates. Similarly, with IHC-QUAD, an alkyne-bearing probe that is conjugated to a fluorophore (e.g., AZDye 594) is used to tag the AHA-labeled proteins and then a fluorescence microscope is used to visualize and quantify the amount of AHA-labeled proteins in histological sections.

can be calculated for each sample. With samples that were collected immediately after the AHA "pulse" period (i.e., Day 0), as well as samples that were collected at 3 and 7 days into the chase period, one can calculate the time-dependent loss of AHA-labeled proteins (i.e., the rate of protein degradation).

18. Make or purchase 10% polyacrylamide gels.

Pause point: A separating gel can be prepared and stored at 4°C the day before the western blot is run. Be sure to bring the gel to 20°C–22°C before adding the stacking gel.

19. Prepare the samples.
    a. Combine 26.25 μL of a thawed *'click'* reaction aliquot with 8.75 μL of 4x Laemmli buffer that contains the freshly added DTT.
    b. Heat samples in boiling water for 5 min.
20. Separate proteins using SDS-PAGE.
    a. Load 30 μL of each of the prepared samples to designated wells on the gel.
    b. Place the loaded gel into the electrophoresis apparatus and fill it with Tris-Glycine running buffer as instructed by the manufacturer.
    c. Separate samples by running at 100 V for 1.5 h or until the dye front has reached the bottom of the gel.

21. Transfer proteins to a PVDF membrane.
    a. Wet the PVDF membrane in a chamber containing methanol for 5 min with continuous agitation on an orbital shaker at 60 RPM.

    Note: All washes and blocking should be performed at 20°C–22°C.

    b. After 5 min, replace the methanol in the chamber with transfer buffer and then return the chamber to the orbital shaker at 60 RPM.
    c. Wet the transfer filter paper and the sponges with transfer buffer.
    d. Gently remove the gel from the running apparatus and build the sandwich of materials for transfer as follows: sponge, filter paper, gel, PVDF membrane, filter paper, sponge.
    e. Place the transfer sandwich into the transfer apparatus with the gel oriented closest to the cathode and the membrane oriented closest to the anode.
    f. Perform a wet transfer at 300 milliamps for 1 h and 45 min in transfer buffer.
    g. Once the transfer is complete, wash the PVDF membrane for 3 × 5 min in diH_2O at 200 RPM on an orbital shaker.
22. Visualize the total protein content in each lane using the "no-stain" labeling protocol.
    a. Follow the manufacturer's instructions to complete the "no stain" labeling procedure.
    b. Image the labeled membrane with an appropriate imaging system.

    Note: The "no-stain" fluorescent signal has an emission maximum at ~ 590 nm. The fluorophore can be excited using a UV light transilluminator or a blue or green light source.

    c. Capture the fluorescent signal with an appropriate emission filter while ensuring that the captured image(s) are not overexposed.
23. Continue running the western blot to visualize the AHA-labeled proteins.
    a. Wash the membrane for 3 × 5 min in diH_2O at 200 RPM on an orbital shaker.
    b. Block the membrane with 5% milk in TBST for 1 h at 60 RPM on an orbital shaker.
    c. Wash the membrane 3 × 10 min with TBST at 200 RPM on an orbital shaker.
    d. Incubate the membrane with peroxidase-conjugated streptavidin diluted at 1:100000 in 1% a BSA-TBST for 12–16 h on a 25 RPM nutating rotator at 4°C.
    e. Wash the membrane for 2 × 5 min then 2 × 10 min in TBST at 200 RPM on an orbital shaker.
    f. Use enhanced chemiluminescence along with an appropriate imaging system to capture image(s) of the membrane and ensure that the captured image(s) are not overexposed.

IHC-QUAD

Timing: 6–8 h

Using IHC analysis will allow for the quantification of AHA at the whole muscle section and myofiber-specific levels. By having samples collected immediately after the AHA "pulse" period, as well as samples that were collected at 3 and 7 days into the chase period, one can calculate the time-dependent loss of AHA-labeled proteins (i.e., the rate of protein degradation). The samples should be processed in batches with approximately 6 samples per batch. Samples from all experimental groups should be present in each batch to ensure that any potential batch variance is distributed across all of the groups.

24. Prepare the slides.
    a. Using a cryostat set to −20°C, cut 10 μm thick sections from the mid-belly (determined using the recorded muscle length) of the muscles that were frozen in OCT.
    b. Mount the sections on microscope slides with one section per slide.
25. Complete the immunohistochemical staining procedure.
    a. Fix sections in acetone for 10 min.
        i. Prior to fixing the sections, chill the acetone to −20°C in a Coplin staining jar.

ii. When acetone is chilled, add the slides to the vessel and fix for 10 min.
iii. After fixation, allow the sections to dry and warm to 20°C–22°C for 5 min.
iv. Use a PAP pen to draw a hydrophobic circle around the sample.

Alternatives: Paraformaldehyde (PFA) fixation can also be used and is compatible with the 'click' reactions outlined in these methods.

b. Wash the sections for 3 × 5 min with DPBS at 20°C–22°C on an orbital shaker at 50 RPM.

Note: All subsequent washes and incubations should be performed at 20°C–22°C on an orbital shaker at 50 RPM.

c. Incubate the sections with Buffer A for 30 min
d. Wash the sections 3× 10 min with DPBS. Prepare the IHC-QUAD *'click'* reaction master mix during the last wash.

Note: When preparing the IHC-QUAD *'click'* reaction master mix, vortex the solution after the addition of each component (i.e., add TBTA then vortex, add copper then vortex, etc…).

e. Immediately after the final vortexing, add 50 μL of the IHC-QUAD *'click'* reaction master mix to each sample and incubate for 1 h protected from light.

Note: At this point, look at the sample(s) with a light microscope. Fine precipitates should be visible.

f. Wash the sections for 6 × 5 min alternating between DPBS (3x) and Buffer A (3x).
g. Wash the sections for 1 × 5 min then 1 × 20 min with Buffer A containing 10% DMSO and 5 mM EDTA pH 8.8.
h. Wash the sections for 3 × 5 min with DPBS.

Note: At this point, look at the sample(s) with a light microscope. The precipitates that were previously visible should be gone.

i. Incubate the sections with 50 μL of rabbit anti-laminin primary antibody diluted at 1:500 in Buffer A for 1 h.
j. Wash the sections for 3 × 5 min with Buffer A.
k. Incubate the sections with 50 μL of Alexa Fluor 488 goat anti-rabbit IgG secondary antibody diluted at 1:5000 in Buffer A for 1 h.
l. Wash the sections for 3 × 5 min with Buffer A.
m. Capture images of the signals for the Alexa Fluor 488 and AZDye 594 on an epifluorescence microscope and ensure that the captured images are not overexposed.

⚠ CRITICAL: Images for all of the samples from all of the batches should be captured with identical settings on the microscope (e.g., magnification, exposure time, excitation light intensity, etc…).

EXPECTED OUTCOMES

The purpose of the methodologies outlined in this manuscript is to enable visualization and quantification of the *in vivo* rate of protein degradation in whole tissue lysates with WB-QUAD or at the single myofiber level with IHC-QUAD. As shown in Figure 5A, the 'no stain' procedure allows for the quantification of total protein from the same Western blot membranes that are used to quantify the amount of AHA-labeled proteins. These values can be used to determine the AHA : protein

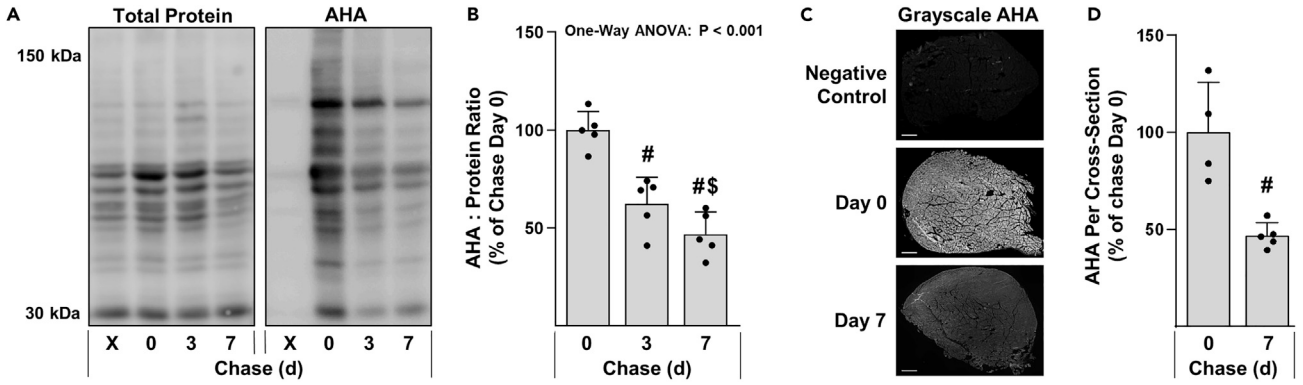

Figure 5. Illustration of the expected outcomes

(A) Representative Western blot of the AHA-labeled proteins from muscles collected at each time point indicated in A (note: the X condition indicates the negative control sample).

(B) Quantitative analysis of the AHA : Protein ratio at each time point.

(C and D) Representative images and quantification of the AHA-labeled proteins from muscle cross-sections collected at the indicated time points. The values in B and D are reported as the group mean + SEM and are expressed relative to the chase day 0, n = 4–5 muscles/group. Statistical significance was determined using a one-way ANOVA (B) or an unpaired t-test (D). # indicates a significant difference from chase day 0, $ from chase day 3, $P < 0.05$. Scale bars = 500 μm. Adapted from Steinert et al. (2023).[1]

ratio (Figure 5B), and from this value that total amount of AHA-labeled proteins per muscle can be determined. Insight into the rate of protein degradation can then be gained by assessing how the total amount of AHA-labeled proteins per muscle changes during the chase period. The same principle applies to IHC-QUAD (Figures 5C and 5D), but in this case, the total amount of AHA-labeled protein per cross-section or per myofiber is used to assess the rate of protein degradation.

As shown in Figure 5, it is important to include a negative control sample when running WB-QUAD and IHC-QUAD. The negative control sample should be from the same tissue used in the experimental samples and obtained from an animal placed on the regular control chow during the pulse period. The negative control sample is important because it will provide assurance that the bulk of the signal being observed / quantified is coming from AHA-labeled proteins rather than non-specific background and it is needed for an appropriate correction of the background signal.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 10–20 min per sample

1. Images obtained from WB-QUAD can be analyzed with ImageJ software.
   a. Load the raw image into ImageJ.
   b. Use the rectangle tool to draw a rectangle that captures one full lane. Check to be sure that the size of the rectangle is large enough to capture each of the individual lanes on the blot as this will ensure that the same total area is quantified for each lane.

   Note: If all of the lanes in the blot do not fit well within the rectangle as initially drawn, the rotation tool can be used to manipulate the shape of the rectangle to better accommodate the lane while preserving the total area.

   c. Use the measurement tool to record the mean intensity of each lane.
   d. Measure the amount of total protein.
      i. Take at least three measurements of the background (i.e., regions on the membrane that do not contain samples).
      ii. Average values obtained from the three background measurements.

iii. Subtract the average background intensity from the mean intensity value that was measured in each individual lane.

Note: This is the background corrected value for total protein in each lane.

e. Measure the amount of AHA-labeled protein.
   i. Subtract the mean intensity of the negative control lane from the mean intensity observed in each lane containing the experimental samples.

Note: This is the background corrected value for the amount of AHA-labeled proteins in each lane.

f. Calculate the AHA : Protein ratio for each sample.
   i. Divide the background corrected value for the amount of AHA-labeled proteins by the background corrected value for the total protein.
g. Calculate the total amount of protein per muscle by multiplying the protein concentration in the original muscle lysate (from step 11) by the volume of the lysate.
h. Multiply the total amount of protein per muscle by the AHA : Protein ratio (step f). This value is the total amount of AHA-labeled proteins per muscle.
i. The relative rate of protein degradation can be determined by assessing how the total amount of AHA-labeled proteins per muscle changes during the chase period.

2. Images obtained from IHC-QUAD can be analyzed with ImageJ.
   a. Determine the total AHA signal for each muscle cross-section.
      i. Trace the periphery of the whole muscle cross-section for each sample.
      ii. Use the measurement tool to obtain the mean AHA intensity within the muscle cross-section.
      iii. Calculate the background corrected AHA intensity by subtracting the mean AHA intensity of the negative control sample, from the mean intensity observed in each of the experimental samples.
      iv. Calculate the total AHA per muscle cross-section by multiplying the cross-sectional area of each sample by its background corrected AHA intensity.
   b. Determine the total AHA signal for the individual myofibers within each cross-section.
      i. Trace the periphery of at least 60 randomly selected myofibers per sample and use the measurement tool to obtain the mean AHA intensity within each myofiber.
      ii. Subtract the mean AHA intensity of the myofibers in the negative control sample from the mean intensity observed in each of the experimental myofibers.

Note: This is the background corrected AHA intensity for each myofiber.

      iii. Multiply the cross-sectional area of each myofiber by its background corrected AHA intensity to calculate the total AHA per myofiber.

⚠ CRITICAL: To eliminate any bias, the investigator performing all quantitative analyses should be blinded to the sample identifications.

LIMITATIONS

The WB-QUAD and IHC-QUAD methods for visualizing and quantifying protein degradation *in vivo* have many advantages, but they are not without limitations. For instance, the animals are fed an AHA-infused chow for a 4-day "pulse" labeling period, and then they are given the control chow for the chase period of 3–7 days. The difference in the amount of AHA measured between these three time points is used to determine the rate of protein degradation. However, different proteins can have different rates of turnover with some proteins remaining stable for weeks to months while others turn over every few hours.[4–6] Due to the differences in turnover rate, it is likely that the bulk of

the proteins that are labeled with AHA during the 4-day pulse are proteins that have a high turnover rate. To overcome this limitation, the duration of the AHA-labeling period could be increased. Alternatively, it may also be possible to overcome this limitation by measuring protein-specific turnover rates. For example, immunoprecipitation of myosin followed by WB-QUAD would enable one to obtain degradation rates that are specific to myosin.

Another limitation of this methodology is that there is no differentiation between the types of protein degradation involved in this process (e.g., ubiquitin-proteasome system (UPS) vs. autophagy). To overcome this limitation, animals could be treated with UPS- or autophagy-specific inhibitors during the chase period.

Lastly, the cost of the two custom rodent chows could be a limitation, especially if the technique is being used in larger animals such as rats. Specifically, 1 kg of each of the custom chows used in this protocol was purchased from Cambridge Isotope Laboratories at a combined cost of over $3000. A total of 1 kg of each chow is enough for the habituation and subsequent "pulse" labeling for approximately 70 mice. However, since the time of the initial experiments, we have discovered alternative distributors of AHA (e.g., Iris Biotech) and companies (e.g., Envigo Teklad) that can produce the two custom chows at approximately half the cost. It is our hope that, with the emergence of alternative sources for producing the custom chow and AHA, the WB-QUAD and IHC-QUAD methods of measuring protein degradation will stand as a simple and cost-effective means for visualizing and quantifying the rate of protein degradation.

TROUBLESHOOTING

Problem 1: Low/no AHA signal following WB-QUAD and/or IHC-QUAD

If, upon the completion of steps 22 and 23 (WB-QUAD) or step 24 (IHC-QUAD), there is little or no signal, this may be an indication that there was a problem running the Western blot or a problem with the *'click'* reaction.

Potential solution

- The no-stain image should provide validation that there was protein run in each lane and that it was effectively transferred to the membrane. If there is a poor signal from the no-stain, use Coomassie blue to stain the membrane. If no signal is observed with either method then there was likely a problem with the transfer step.
- The use of a negative control sample is necessary to verify that even a low signal is, indeed, from the AHA-labeled proteins and not simply the result of non-specific background. If the Day 0 samples and the negative control sample have the same signal intensity, then it can be inferred that there was either a problem with the *'click'* reaction or a problem with the AHA pulse labeling.
- The AHA-infused chow contains a blue dye and, therefore, blue-coloration of the feces can be used to verify that the animals are consuming the AHA-infused chow.
- A robust signal-to-noise ratio (e.g., difference in the signal from a Day 0 sample versus the negative control) with IHC-QUAD can be used to verify that there was effective AHA-labeling and therefore provide insight into whether a low western blot signal is due to a problem with the WB-QUAD *'click'* conditions and/or the detection of the biotin tag (i.e., the peroxidase-conjugated Streptavidin). The inverse logic applies if there is an issue with the signal in IHC-QUAD.
- As mentioned in the materials and equipment section, it has been recommended that fresh TCEP be used to make the WB-QUAD *'click'* reaction master mix to maximize the efficiency of the *'click'* reaction. Our lab has had success using aliquots frozen at −20°C, but if little or no AHA signal is detected and fresh TCEP was not used it might be beneficial to repeat the experiment using fresh TCEP.

Problem 2: High AHA signal following WB-QUAD and/or IHC-QUAD, but a low signal-to-noise ratio

If upon the completion of steps 22 and 23 (WB-QUAD) or step 24 (IHC-QUAD), there is a high AHA signal but a low signal-to-noise ratio this may be an indication that there was a problem with the *'click'* reaction and further optimization is needed. The most likely problem is a sub-optimal concentration of the alkyne probe.

Potential solution

To optimize the WB-QUAD *'click'* reaction, design a titration experiment in which both a negative control sample and a Day 0 sample are subjected to *'click'* reactions containing various amounts of Biotin PEG4-Alkyne probe (e.g., 100, 75, 50, 25, 10, or 3.5 μM). Use the results of this blot to make a decision regarding the amount of the alkyne probe that produces the greatest signal-to-noise ratio (i.e., the Day 0 intensity divided by the negative control sample intensity for a given concentration of the probe). To optimize the IHC-QUAD *'click'* reaction, design a titration experiment in which both a negative control sample and a Day 0 sample are subjected to *'click'* reactions containing various amounts of the AZDye 594 Alkyne probe (e.g., 30, 10, 3, 1, or 0.3 μM). Again, use the results to determine the amount of the probe that produces the greatest signal-to-noise ratio.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Troy A. Hornberger (troy.hornberger@wisc.edu).

Materials availability

This study did not generate new unique reagents. All materials used in this protocol are commercially available.

Data and code availability

This study did not generate or analyze code.

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AUTHOR CONTRIBUTIONS

J.E.H. contributed to manuscript writing, visualization, and funding acquisition. K.W.J. contributed to the methodology and visualization. W.G.Z. contributed to the methodology. N.D.S. contributed to conceptualization, methodology, and visualization. T.A.H. contributed to supervision and funding acquisition. All authors contributed to the review and editing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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