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Measuring Diurnal Rhythms in Autophagic and Proteasomal Flux

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TITLE:

Measuring Diurnal Rhythms in Autophagic and Proteasomal Flux

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KEYWORDS:

catabolism, autophagy, circadian rhythm, inflammation, macroautophagy, flux, western, time-point

SUMMARY:

We describe our protocol for measuring biological rhythms in protein catabolism via autophagy and the proteasome in mouse liver.

ABSTRACT:

Cells employ several methods for recycling unwanted proteins and other material, including lysosomal and non-lysosomal pathways. The main lysosome-dependent pathway is called autophagy, while the primary non-lysosomal method for protein catabolism is the ubiquitin-proteasome system. Recent studies in model organisms suggest that the activity of both autophagy and the ubiquitin-proteasome system is not constant across the day but instead varies according to a daily (circadian) rhythm. The ability to measure biological rhythms in protein turnover is important for understanding how cellular quality control is achieved and for understanding the dynamics of specific proteins of interest. Here we present a standardized protocol for quantifying autophagic and proteasomal flux in vivo that captures the circadian component of protein turnover. Our protocol includes details for mouse handling, tissue processing, fractionation, and autophagic flux quantification using mouse liver as the starting material.

INTRODUCTION:

Circadian rhythms refer to daily, predictable variations in biological function that are apparent throughout nature. They exist at every biological scale, from macroscopic behaviors like sleep-wake cycles, to molecular phenomena like the rhythmic abundance of biomolecules. In recent years, research into circadian rhythms has been transformed by the discovery of “clock genes” that are critical for circadian rhythm generation. Studies in clock gene knockout mice have

revealed a central role for circadian rhythms in temporally organizing core cellular processes such as metabolism[1]. Among the ways circadian rhythms make this happen is by imparting a temporal structure to protein catabolism.

Several groups including ours have shown that the two major avenues for cellular protein catabolism, autophagy and the ubiquitin-proteasome system, are subject to diurnal rhythms[2-5]. Autophagy represents the lysosome-dependent arm of protein catabolism in which proteins of interest are delivered to this degradative organelle either through the construction of a novel vesicle (macroautophagy) or through direct translocation though a channel (chaperone mediated autophagy)[6]. The ubiquitin-proteasome system is the main non-lysosomal pathway, where proteins are poly-ubiquitinated and then fed into the proteasome, a macromolecular degradative machine found throughout the cytoplasm and nucleus[7,8]. Rhythms in autophagic and proteasomal activity are important because they likely play a role in cellular housekeeping. As a result, it is valuable to have a standardized procedure that can detect daily oscillations in protein catabolism that is compatible with pre-clinical disease models.

Here, we provide our protocol for quantifying diurnal variations in autophagic flux in mouse liver, which has served as the basis for work in our laboratory[3,9]. Our method is classified as a "turnover assay"[10], an approach used by numerous groups to measure proteolytic activity (or flux). In this approach, protease inhibitors specific to lysosomes or proteasomes are administered to mice and then tissue samples are obtained after a fixed time interval. In parallel, tissue samples are obtained from mice subjected to sham injections. The tissue samples are homogenized and then biochemically separated to obtain the lysosome-enriched and cytoplasmic fractions. These fractions are then analyzed in parallel via western blotting using antibodies specific to macroautophagy markers (LC3b and p62) or proteasomal substrates (poly-ubiquitinated protein). Over time, animals injected with protease inhibitors accumulate proteins that would normally have been recycled. As a result, the rate of turnover is inferred by comparing the abundance of marker proteins in the protease-inhibitor treated samples to the sham-treated samples. By repeating this method at fixed time intervals across the day it is possible to reconstruct circadian variations in proteolysis (**Figure 1A**).

PROTOCOL:

The protocol described here was approved by the Washington University in St. Louis Animal Care and Use Committee (IACUC).

1. Mouse housing and experimental design

1.1. To detect daily rhythms in protein turnover, house mice (male or female C57BL/6J, 4−8 week old, 20−25 g) under standard 12 h light/dark cycles with food provided *ad libitum*. To avoid stressing the animals, acclimate mice for at least one week in the facility prior to use and avoid single housing. For proving that rhythms in protein catabolism are truly circadian (i.e., driven by the animal's internal biological clock), house mice under constant dark conditions, taking care to avoid exposing the mice to exogenous light.

NOTE: For details about mouse housing for circadian rhythm experiments see the following reference[11].

1.2. For each time point, allocate three mice as sham controls and at least three mice for each type of protease inhibitor used (leupeptin for autophagic flux and bortezomib for proteasomal flux measurements). Plan for six time points evenly spaced at 4 h intervals across the day/night cycle to obtain tissue samples.

NOTE: A 24 h, 6 time point experiment with phosphate-buffered saline (PBS), leupeptin, and bortezomib will require a total of 54 mice (3 per treatment x 3 treatments x 6 time points).

2. Preparation of homogenization solution, inhibitor stock solutions, and vials for mouse liver collection

2.1. Prepare fresh homogenization buffer (HB) and keep on ice: 10 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM EDTA pH 8.0, and 1 protease inhibitor tablet per 100 mL.

NOTE: Approximately 10 mL of HB is needed per mouse.

2.2. For downstream processing of biological samples, each sample requires a 15 mL conical tube to hold the crude homogenate, a 15 mL tube to hold the post-nuclear supernatant, two 1.5 mL microcentrifuge tubes to hold the 3,000 x *g* and 20,000 x *g* pelleted material, respectively, and a 1.5 mL microcentrifuge tube to hold the cytoplasmic fraction. Add 7 mL of cold HB to each tube intended for crude homogenate and keep on ice.

2.3. Prepare a 2 mg/mL stock solution of leupeptin hemi-sulfate in sterile PBS. Also prepare a 50 mg/mL solution of bortezomib in dimethyl sulfoxide (DMSO). Aliquot and store the solutions at -80 °C.

3. Protease inhibitor administration

3.1. Thaw leupeptin (2 mg/mL) and bortezomib (50 mg/mL) stock solutions to room temperature 15 min prior to each time point. The leupeptin stock is ready for injection. Dilute the bortezomib in sterile PBS to yield a 50 µg/mL working solution.

NOTE: Bortezomib is light sensitive, so protect the working stock from light until use.

3.2. Weigh mice and perform intraperitoneal injections of "sham" PBS (0.5 mL), leupeptin (40 mg/kg), or bortezomib (1.6 µg/kg). Return mice to appropriately marked cages grouped by the type of injection they received. Record the time the mice are treated or manipulated in a standardized table (see **Supplemental File** "**Sample Data**").

NOTE: A dose calculator is included as an aid in the **Supplemental File "Sample Data"**. It is

important to perform injections expeditiously and in the same order from timepoint to timepoint to minimize variability.

4. Tissue acquisition and storage

4.1. Two hours after injection, euthanize mice one at a time in accordance with the institutionally approved IACUC protocol. For example, anesthetize mice in a bell-jar for 1 min with an isoflurane-soaked cotton-ball and then euthanize them by cervical dislocation. Proceed to the dissection step immediately following euthanasia.

NOTE: Ensure complete anesthesia before cervical dislocation by pinching front paws without an observable reflex.

4.2. Remove the left lobe of the liver by making a 1.5 to 2.0 cm incision with Metzenbaum scissors on the mouse's ventral abdomen. Externalize the left lobe of the liver and excise it with surgical scissors. Submerge the liver sample in 7 mL of ice-cold HB in an appropriately labeled 15 mL conical tube. Keep the sample on ice throughout processing.

NOTE: The samples can be kept on ice or at 4 °C overnight before proceeding. If standard markers of macroautophagy and proteasomal activity are the only intended readouts, liver samples can be flash frozen in liquid nitrogen and stored at -80 °C for later processing. To examine other targets of degradation (e.g., via proteomics), process the samples without freezing.

4.3. Proceed to euthanize and dissect the next mouse until all samples are acquired. Process mice in the same group order they were injected and record the duration of this step (see **Supplemental File "Sample Data"**). Each mouse should be processed in under 5 min to limit difference in the exposure times for the injected inhibitors.

5. Biochemical fractionation of liver samples

NOTE: **Figure 1B** shows the fractionation scheme.

5.1. Transfer each liver sample and 7 mL of HB into a 15 mL capacity Dounce homogenizer. Homogenize the sample with 10 strokes of the loose piston and 15 strokes of the tight piston. Return the resultant crude homogenate to the 15 mL conical tube it originated from. Repeat until all liver samples have been homogenized.

5.2. Spin crude homogenate samples at 700 x *g* for 10 min at 4 °C to pellet nuclei and debris. Transfer the top 4 mL of post-nuclear supernatant (**S1**) to a fresh 15 mL conical tube.

5.3. Determine the protein concentration of fraction **S1** using Bradford or bicinchoninic acid (BCA) assays according to the manufacturer's instructions. Next equalize the concentrations of all samples to 2.1 mg/mL or to the desired concentration by adding fresh HB to **S1** where needed. The normalized samples are the "Total Protein" (**Tot**) fraction. For downstream

analytical purposes and quality improvement, aliquot 500 µL of the **Tot** fraction and store at -80 °C.

5.4. Transfer 1.5 mL of the remaining **Tot** fraction into a microcentrifuge tube and spin at 3000 x *g* for 15 min at 4 °C. Transfer 1 mL of the resultant supernatant (**S2**) to a fresh microcentrifuge tube and set on ice.

5.5. Aspirate the remaining supernatant and wash the 3000 x *g* pellet (**3KP**) twice with 1.5 mL of cold HB. The **3KP** pellet can be stored dry at -80 °C if downstream proteomics are anticipated. If only western blotting is anticipated, resuspend the **3KP** pellet in 200 µL of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (**Table of Materials**) and boil at 95 °C for 5 min.

5.6. Spin the **S2** fraction at 20,000 x *g* for 20 min at 4 °C. Transfer the resultant supernatant (**S3**) to a fresh microcentrifuge tube. **S3** is the cytoplasmic (**Cyto**) fraction. For SDS-PAGE, combine 150 µL of **Cyto** fraction with 50 µL of 4x SDS-PAGE sample buffer and boil at 95 °C for 5 min.

5.7. Aspirate the remaining supernatant from the 20,000 x *g* pellet (**20KP**) and wash twice with 1.5 mL of cold HB. The **20KP** pellet can be stored dry at -80 °C if downstream proteomics are anticipated. If only western blotting is anticipated, resuspend the **20KP** pellet in 100 µL of SDS-PAGE sample buffer and boil at 95 °C for 5 min.

6. Western blotting readout

6.1. In order to quantify macroautophagic and proteasomal flux via western blotting, compose a stock solution of purified GST-LC3b (2 µg/mL), p62-His_6 (2 µg/mL), and Lys^{48} linked polyubiquitin (K48-Ub, 50 µg/mL) in 1x SDS-PAGE sample buffer. After boiling at 95 °C for 5 min, aliquot and store at -80 °C for future use.

6.2. At the time of SDS-PAGE, generate a 6-point standard curve of the standard protein mixture by serially diluting 1:3 in 1x SDS-PAGE sample buffer. Load 10 µL of each standard curve sample onto a 26 well 4–12% SDS-PAGE midi-gel, followed by prestained protein standards (**Table of Materials**), a commercial molecular weight standard.

6.3. For measuring autophagic flux, load 12 µL of **3KP** sample into each well.

NOTE: Assuming sham, bortezomib, and leupeptin treatments were done and assuming 3 mice per treatment, each time point should consist of 9 samples. Therefore, each midi-gel can accommodate 2 time points plus the standard curve and a typical time series experiment will require 3 midi-gels for readout.

6.4. For measuring proteasomal flux, load 12 µL of **Cyto** fraction into each well.

6.5. Separate protein samples by SDS-PAGE and transfer to polyvinylidene fluoride (PVDF)

membranes using standard protocols. If western blotting for LC3b is anticipated, transfer gels using 20% methanol-containing transfer buffer. Otherwise, use 10% methanol-containing transfer buffer as this will enable more efficient transfer of high molecular weight protein species.

6.6. Perform western blotting using standard protocols. For analyzing macroautophagic flux, blot membranes containing **3KP** samples with anti-p62 or anti-LC3b antibodies (1:1,000) overnight at 4 °C. For analyzing proteasomal flux, blot membranes containing **Cyto** samples with anti-Lys^{48} specific polyubiquitin (1:1,000) overnight at 4 °C.

6.7. Image western blots using standard secondary antibodies and imaging devices. Image all membranes that constitute a given time series experiment together.

7. Data analysis

NOTE: See **Supplemental File "Sample Data"**.

7.1. To perform densitometry on western blot samples, use standard graphics software Image Studio, or alternatively ImageJ.

7.2. Perform densitometric measurements on bands of interest for both the standard curve and the experimental samples. In Image Studio, using p62 as an example, draw a long rectangle encompassing protein monomer at the bottom and extending to the top of the membrane. Copy, paste, and move the rectangle to the remaining samples. It is important to keep the rectangle used for quantification consistent between samples.

7.3. Save the quantification to a spreadsheet by pressing **Report** and **Launch Spreadsheet** at the bottom right of the analysis window.

7.4. Generate a densitometric standard curve with spreadsheet using the serially diluted standard sample and use either linear or polynomial regression to obtain a best fit standard curve equation. Using this equation, extrapolate the quantity of p62, LC3b-II or Lys^{48}-poly Ub in the experimental samples.

7.5. To obtain flux measurements, subtract the extrapolated protein quantity of each inhibitor-treated sample from the average value of the PBS samples from the same time point (see **Supplemental File "Sample Data"**).

7.6. Evaluate the statistical significance of temporal variation in protein turnover using 1-way ANOVA. This can be done using standard spreadsheet software.

REPRESENTATIVE RESULTS:

Representative data are presented in **Figure 2A,B**, and the quantification of these data are provided in **Figure 2C,D** (see also **Supplemental File "Sample Data"**). For simplicity, we have not depicted loading controls in **Figure 2** but these should be obtained in parallel. Typically, western

blots against β-actin are used for this purpose, but a total protein stain (such as Ponceau S) will suffice. The primary readout for autophagic flux at any given time point is the difference in the amount of macroautophagy specific markers (p62 or LC3b-II) between the leupeptin-treated versus sham samples in the lysosome enriched 3KP fraction divided by 2 (the number of hours between injection of protease inhibitor and tissue harvest). Similar data can be obtained from 20KP samples, which are also lysosome enriched, but our experience in mouse liver is that most of the signal segregates in the 3KP fraction. Typically, the results are normalized to the mean which simplifies comparison across independent experiments (**Figure 2C,D**). The primary readout for proteasomal turnover is the difference in the amount of Lys^{48}-polyubiquitinated protein between the bortezomib-treated versus sham samples in the cytosolic ("**Cyto**") fraction divided by 2. Because p62 can be a target of both macroautophagy and the proteasome[3], an alternative marker for proteasomal flux is the change in p62 content +/- bortezomib in the 3KP fraction (see **Sample Data**). However, we find this marker is less robust compared to Lys^{48}-polyubiquitinated protein and is best used as supportive data.

FIGURE LEGENDS:

Figure 1: Experiment steps and sample processing. (**A**) Schematic of a typical time series experiment to measure daily rhythms in autophagic and proteasomal activity (flux) in mouse liver. (**B**) Fractionation scheme for obtaining lysosome-enriched and cytosolic liver protein fractions for western blot analysis.

Figure 2: Sample experimental results of flux. Western blots from a representative time series experiment to measure daily rhythms in autophagic flux (**A**), and proteasomal flux (**B**) in mouse liver. Note that under leupeptin-treated conditions, p62 runs as ladder ranging from the monomeric form at about 50 kDa to SDS-stable complexes at about 250 kDa. Times of day are depicted in units of zeitgeber time (ZT), where ZT0 represents lights on and ZT12 represents lights off. Observation times that fall during lights off are shaded black. (**C**,**D**) Quantification of these data. Each data point represents the mean ± SE (n = 3). Statistical significance via one-way ANOVA is depicted. Please see the **Supplemental File "Sample Data"** for a tabular representation of these data.

Supplementary file: Sample data. Laboratory analysis of densitometric data and subsequent statistical analysis.

DISCUSSION:

Our protocol describes a technically straightforward means of measuring biological rhythms in protein turnover in mice using commonly available molecular biology equipment. Because of the length of time series experiments and the number of biological samples involved, it is important to be consistent across the entire experiment regarding how the mice are injected, the timing of tissue acquisition and the biochemical processing of samples. The injection, euthanasia, and cervical dislocation steps may require operator practice prior to initiating a full-scale experiment. It is best for a single operator to perform all tissue dissections since different individuals dissect at different speeds, but if this is infeasible it may be worthwhile to increase the time between

protease inhibitor injection and tissue harvest from 2 hours to 3 or 4 hours (provided this is done consistently across time points).

Common reasons for troubleshooting include variability in flux between biological replicate samples, and poor western blot quality. Regarding sample variability, this can be due to the use of multiple operators with different dissecting speeds or levels of experience. This can be mitigated by executing practice experiments until average dissecting times are less than 5 minutes per mouse. Alternatively, a single operator can be used to carry out dissections. High background on western blots can arise from uneven transfer, inadequate blocking, low quality primary antibody, or inadequate washing steps. Best results are achieved by overnight wet transfer of SDS-PAGE separated protein samples to PVDF in 10−20% methanol containing transfer buffer, using 10% non-fat dry milk for blocking, and extensive washing between steps (3 x 10 minutes minimum on a mechanical rocker).

This technique has several limitations. First, because mice must be killed in order to measure autophagic or proteasomal flux, the protocol described requires significant numbers of animals and is therefore resource intensive. While a minimal time series experiment spans 24 hours, experiments of at least 2 cycles are ideal for confirming that the daily variations being observed in protein turnover are reproducible, and for estimating rhythm characteristics like periodicity and phase[12]. Because time must elapse between when the protease inhibitors are administered to mice and the time samples are harvested (to allow for targets of proteolysis to accumulate), the turnover measurements obtained represent an average activity over a 2 hour interval rather than a point estimate. As a result, there is a limit to the resolution this assay can provide for detecting dynamics changes in protein catabolism. Finally, this protocol is optimized for mouse liver and the biochemical fractionation procedure presented here would likely need to be modified to efficiently obtain lysosomes from other tissue types.

This is the first method to directly measure daily rhythms in autophagic flux that also enables proteome-wide observations of this phenomenon. Future applications of this technique include analysis of proteolytic rhythms in various disease models, including autoimmune disease, cancer, and acute infection. In principal this method can be adapted to other mouse organs and to explanted human samples, which represents an exciting future application with high translational potential.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

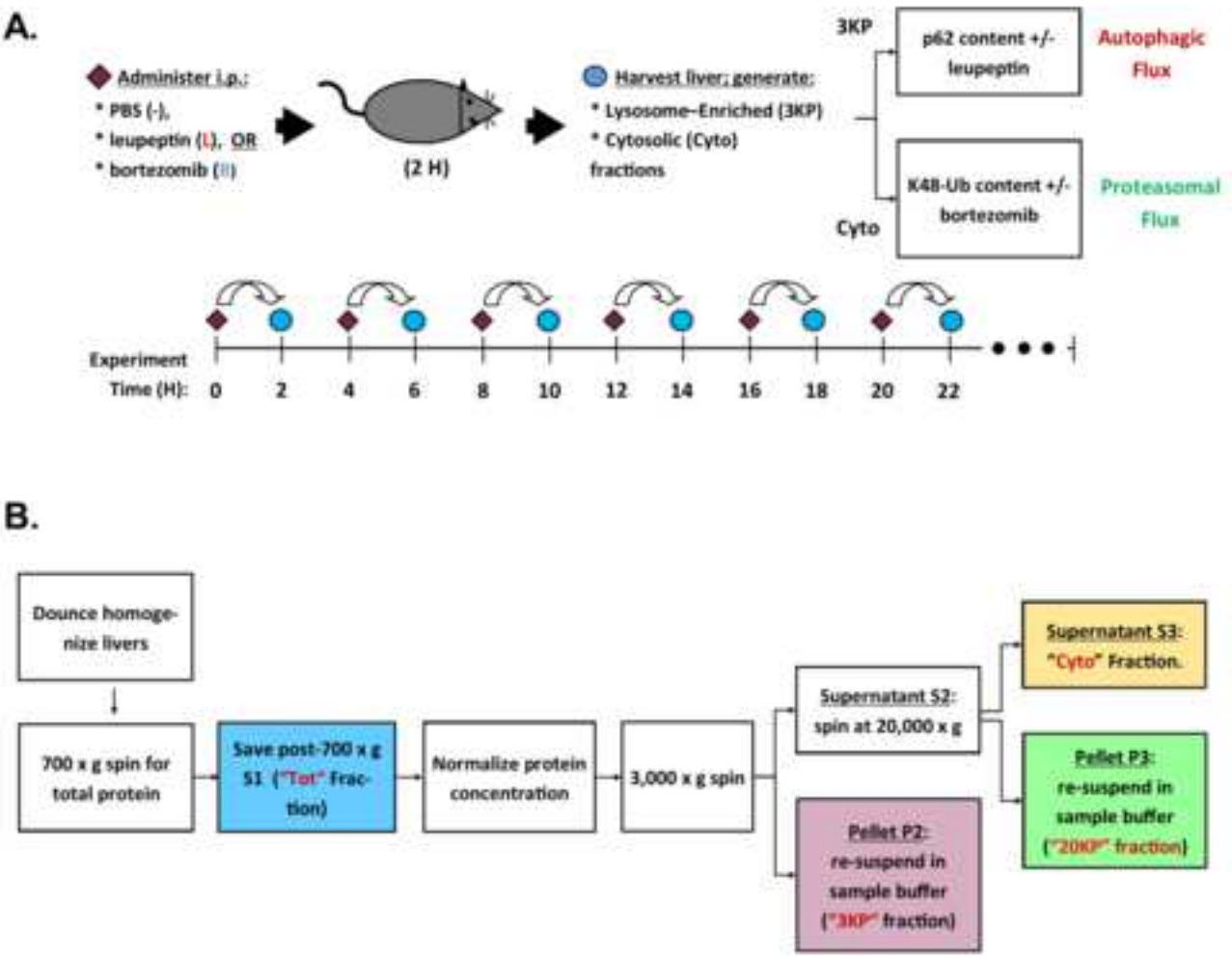

Figure 2

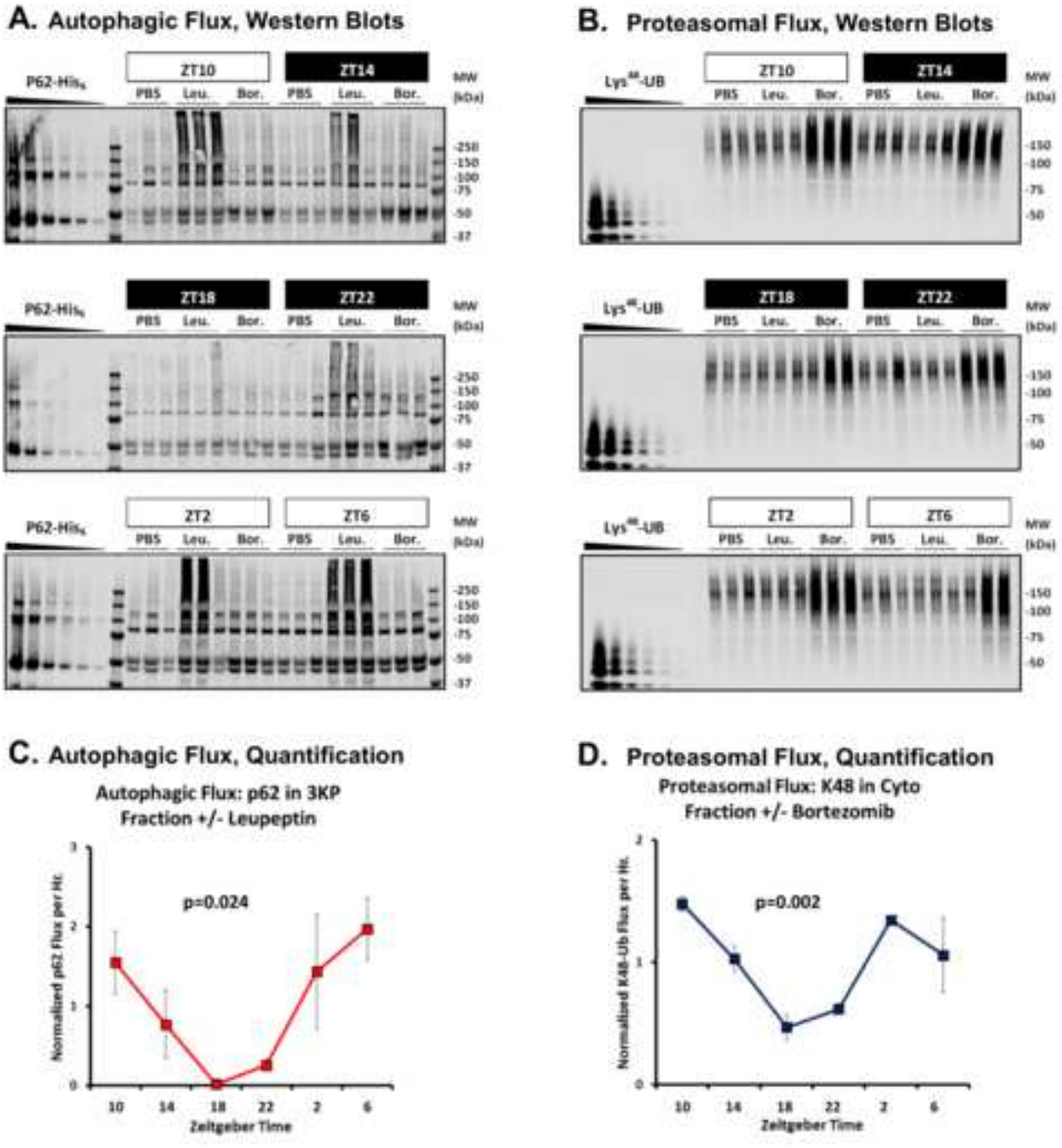

Table of Materials

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Material	Manufacturer	Catalog Number
4x SDS PAGE Sample Buffer	Invitrogen	Cat# NP0008
Bortezomib	EMD Millipore	Cat# 5.04314.0001; CAS: 179324-69-7
Image Studio	LICOR	N/A
Immobilon-FL PVDF membrane 0.45 micron	Merck Millipore Ltd	Cat# IPFL00010
K48-linkage Specific Polyubiquitin (D9D5) Rabbit mAb	Cell Signaling Technology	Cat#8081S; RRID:AB_10859893
LC3a	Boston Biochem	Cat# UL-430
LC3b antibody	Novus	Cat#NB100-2220; RRID:AB_10003146
LC3b antibody	Cell Signaling Technology	Cat#2775; RRID:AB_915950
Leupeptin	Sigma	Cat# L2884; CAS: 103476-89-7
NuPAGE 4-12% Bis-Tris Midi Protein Gels	Thermo Fisher Scientific	Cat# WG1403BOX
NuPAGE LDS Sample Buffer (4x)	Thermo Fisher Scientific	Cat# NP0007
P62-his	Novus	Cat# NBP1-44490
Precision Plus Protein All Blue Prestained Protein Standards	Bio-Rad	Cat# 1610373
Rabbit Anti-p62/SQSTM1	Millipore-Sigma	Cat#P0067; RRID:AB_1841064
rhPoly-Ub WT (2-7) (K48)	Boston Biochem	Cat# UC-230
SDS-PAGE Midi-size Gels	Invitrogen	Cat# WG1403
SIGMAFAST Protease Inhibitor Tablets	Millipore-Sigma	Cat# S8830

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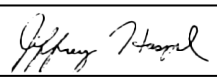
Signature:	
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Washington University in St. Louis
SCHOOL OF MEDICINE

John T. Milliken Department of Medicine
Division of Pulmonary and Critical Care Medicine

Jeff Haspel, M.D., Ph.D.
Assistant Professor in Medicine

May 24, 2019

Dear Dr. Weldon:

We are pleased to submit our revised protocol for your consideration, which is entitled "*Measuring Diurnal Rhythms in Autophagic and Proteasomal Flux*". Please also convey our thanks to our three Reviewers who have helped us to improve this work. We are attaching revised files as well as a point-by-point response the Reviewer's comments (below). We look forward to your feedback and appreciate your consideration.

Sincerely,

Jeffrey Haspel, M.D.-Ph.D.

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Response to Reviewers' comments:
Reviewer #1:
**Please, consult the statistician and confirm that the sample number and data normality support the use of a parametric test. Give also detailed instructions about the sample numbers with information when to apply a parametric or a non-parametric test.*
Response: Parametric tests have traditionally been used for determining the statistical significance of autophagic flux and represents the accepted standard. To our knowledge, non-parametric tests are never employed in this type of readout. Additional information on statistical treatment can be found in two of our prior publications[1,2]. Regardless of the statistical test used, the protocol for executing our technique is the same.

**Please, indicate in western blots, where the right band should be. The antibodies appear rather unspecific and also the positive controls show numerous bands.*
Response: We thank the Reviewer and have emended the figure legend to Figure 2a to clarify this as follows: "Note that under leupeptin-treated conditions, p62 runs as ladder ranging from the monomeric form at about 50 kDa to SDS-stable complexes at about 250 kDa". Regarding the positive controls, p62 tends to naturally form SDS-stable complexes. The purified Lys48-Ub protein used as a standard in Fig. 2B comes from the manufactures as a heteromeric mixture, so a ladder is expected.

**Strictly speaking, the levels of p62(/SQSTM1?) do not indicate autophagy flux. This must be discussed in more detail.*
Response: Macroautophagic flux is defined as the difference in p62 content between leupeptin treated and sham treated samples as described at length in our prior publication, which focuses on the logic of our measurement[1]. This protocol is focused on how to use a turnover assay to observe circadian rhythms in both autophagic and proteasomal activity.

Minor Concerns:
**It remains obscure why some text is highlighted with yellow color.*
Response: This was do at the request of the Journal editor so they can estimate how much video recording will be needed to document the protocol.

**An Excel sheet may not get properly opened with all program versions and it contains links to some other files.*
Response: We thank the Reviewer and have corrected this.

Reviewer #2:
1. Did the authors investigate taking blood samples from the mice in order to correlate the "in vivo" flux with secreted protein levels? This would certainly increase the usability of the protocol for many researchers for whom clinical relevance is a major concern. This point is nothing more than something to think about rather than a requirement for acceptance of the protocol in the journal.

Response: We thank the Reviewer and agree with their assessment. We have indeed used this technique on blood samples but have not yet published this. As such we confined ourselves to outlining published applications of our technique.

2. It would be appropriate for the authors to move the table of Reagents and Materials from its current location on line 271 to before section 2. Also, this will eliminate the need to incorporate this information in the description of the protocol as done for e.g. in Section 2.2 (Line 101) or as an appendix.
Response: We thank the Reviewer and have moved the table accordingly.

3. On Line 146, the use of decant is ambiguous. It may suggest a removal of the 7 mL of HB added in 4.2 (Line 131) with a subsequent addition of another 7 mL aliquot before homogenization. Alternatively, it appears that the authors mean to transfer the liver sample and the 7 mL of HB in which it is immersed into the Dounce homogenizer. If the latter is what the authors mean, a replacement of "decant" with "transfer".
Response: The term “decant” was replaced with “transfer” to provide a more straightforward description.

4.On Line 281, the authors state that the "It is best for a single operator to perform all tissue dissections since different individuals dissect at different speeds, but if this is infeasible it may be worthwhile to increase the time between protease inhibitor injection and tissue harvest from 2 hours to 3 or 4 hours (provided this is done consistently across time points). I find it somewhat impractical to recommend a single operator for a 24-hour experiment especially one that requires the operator to be present every 2 hours for the entire of the study. Did the authors evaluate the variation between single and multiple-operator to determine the impact of having multiple people work on the study?
Response: The Reviewer is quite correct that these experiments are arduous and are easier to execute with more than one operator. We do tend to employ single operators for most experiments but recognize this is a tall order. We therefore described the use of multiple operators in further detail in the discussion section. The main thing is to perform training experiments to ensure all operators use consistent dissecting techniques prior to attempting a full time series experiment, and to carefully record any run-time issues for future troubleshooting.

5. The discussion, while appropriate, lacks a description of common problems the authors encountered and which other scientists will likely encounter in the conduction of an study of this scale.
Response: We thank the Reviewer and have emended the discussion section accordingly.

Reviewer #3:

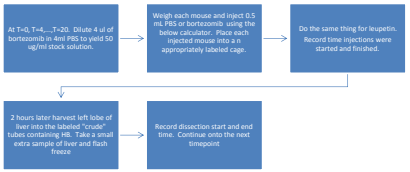
The protocol described by Ryzhikov et al. is aimed at measuring biological rhythms in protein catabolism via autophagy and the proteasome in the liver with an in vivo mouse model. The described methodology is quite straightforward and well explained although a big number of mice is required (one mouse per time point for each condition) to run such an experimental protocol which makes it less practical. The figure legends should be expanded to be more self explanatory, especially for Figure 2 for which it would be also important to explain why the time points reported in figures 2C & 2D (Zeitgeber Time axis), are not the same as those shown for the western blots presented in figures 2A & 2B, respectively.
Response: We thank the Reviewer and apologize for this typo. We have corrected the X-axes for Figures 2C and 2D. We have also included sample lab notes to help readers become better acquainted with this technique. Regarding the numbers of mice needed to perform this kind of experiment, this is indeed a limitation of our technique which we have now highlighted in the discussion section.

REFERENCES

1. Haspel, J. *et al.* Characterization of macroautophagic flux in vivo using a leupeptin-based assay. *Autophagy.* **7** (6), 629-642, (2011).
2. Ryzhikov, M. *et al.* Diurnal Rhythms Spatially and Temporally Organize Autophagy. *Cell Rep.* **26** 1-13, (2019).

SAMPLE TIMESERIES EXPERIMENT WITH LEUPEPTIN AND BORTEZOMIB DOSE CALCULATOR

Procedure for Conducting the Timeseries Experiment



Bortezomib calulator (stock 50 mg/ml)	
Mouse Weight [g]	25
Amount to inject [ml]	0.8

Leupeptin calulator (stock=2 mg/ml)	
Mouse Weight [g]	25
Amount to inject [ml]	0.5

Experiment Start 11/13/17 2:00 PM

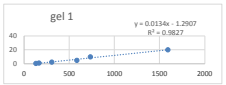
Timepoint	Time for protease inhibitor injection	Time For Dissection Start	Time of Dissectiopn End	Comments
T=0	11/13/17 2:00 PM	11/13/17 4:00 PM	4:10:00 PM	
T=4	11/13/17 6:00 PM	11/13/17 8:00 PM	8:10:00 PM	
T=8	11/13/17 10:00 PM	11/13/17 12:00 PM	12:15:00 PM	
T=12	11/14/17 2:00 AM	11/14/17 4:00 AM	4:15:00 AM	
T=16	11/14/17 6:00 AM	11/14/17 8:00 AM	8:30:00 AM	
T=20	11/14/17 10:00 AM	11/14/17 12:00 PM	12:30:00 PM	

p62 Quantification in 3KP Fractions

Gel1

Sample Name	Vol loaded	Signal 800	Extrapolated p62 content	Average For Sham Controls	p62 Difference	p62 Difference Per Hour	Mean Flux	SE Flux
1x Standard (STD)	10	1592.134277	20.04389932					
1:2 STD	10	733.1665039	8.533731152					
1:4x STD	10	584.1923828	6.53747793					
1:8x STD	10	305.7993164	2.80701084					
1:16x STD	10	164.2299805	0.909981738					
1:32x STD	10	130.1044922	0.452700195					
Ladder	5							
T=0 3KP PBS V1	12	384.8681641	3.866533398	15.4300922				
T=0 3KP PBS V2	12	1830.537109	23.23849727					
T=0 3KP PBS V3	12	1528.055664	19.1852459					
T=0 3KP Leup V1	12	2866.416504	37.11928115		21.689189	10.84459	13.22256	3.374662679
T=0 3KP Leup V2	12	2582.336914	33.31261465		17.882522	8.941261		
T=0 3KP Leup V3	12	4215.257324	55.19374814		39.763656	19.88183		
T=0 3KP MG341 V1	12	1067.331055	13.01153613		0	0	6.017764	3.544854026
T=0 3KP MG341 V2	12	2110.572266	26.99096836		11.560876	5.780438		
T=0 3KP MG341 V3	12	3079.589355	39.97579736		24.545705	12.27285		
T=4 3KP PBS V1	12	1157.180664	14.2155209	15.0228862				
T=4 3KP PBS V2	12	795.0844727	9.363431934					
T=4 3KP PBS V3	12	1700.030273	21.48970566					
T=4 3KP Leup V1	12	1716.171387	21.70599658		6.6831104	3.341555	6.564551	3.643103044
T=4 3KP Leup V2	12	3282.384766	42.69325596		27.67037	13.83518		
T=4 3KP Leup V3	12	1593.090332	20.05671045		5.0338243	2.516912		
T=4 3KP MG341 V1	12	2476.229004	31.89076865		16.867882	8.433941	8.302003	2.541507777
T=4 3KP MG341 V2	12	3103.487305	40.29602988		25.273144	12.63657		
T=4 3KP MG341 V3	12	1789.894043	22.69388018		7.670994	3.835497		
Ladder	5							

p62 STD curve	
20	1592.134277
10	733.1665039
5	584.1923828
2.5	305.7993164
1.25	164.2299805
0.625	130.1044922



Experiment Time	ZT	p62 flux, Leupeptin	SE	Normalized Flux	SE	p62 flux, MG341	SE	Normalized Flux	SE
0	10	13.22256123	3.374662679	1.54997056	0.3955836	6.017764	3.544854	1.325302489	0.780689341
4	14	6.564550732	3.643103044	0.76950753	0.4270506	8.302003	2.541508	1.828364579	0.559720659
7	18	0.151199685	0.107112512	0.01772388	0.0125559	0	0	0	0
12	22	2.192153841	0.592673808	0.2569791	0.0694742	1.093607	0.06239	0.24084692	0.013740276
16	2	12.23506199	6.134692372	1.43421427	0.7191188	4.512845	1.890952	0.993871769	0.416447539
20	6	16.81955253	3.357279466	1.97161585	0.3935459	7.317811	2.487004	1.611614243	0.547717133

ANOVA: Autophagic Flux

0	4	8	12	16	20
10.84459448	3.341555208	0.095371745	2.563549349	19.1468191	13.152812
8.94126123	13.83518485	0	2.981307747	17.5583669	13.781554
19.88182798	2.516912142	0.358227311	1.031604427	0	23.524292

Anova: Single Factor

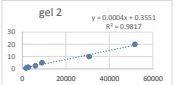
SUMMARY

Groups	Count	Sum	Average	Variance
ANOVA: Autophagic	4	39.66768369	9.916920923	66.4857278
	4	23.6936522	5.923413049	28.1886297

Gel2

Sample Name	Vol loaded	Signal 800	Extrapolated p62 content	Average For Sham Controls	p62 Difference	p62 Difference Per Hour	Mean Flux	SE Flux
1x Standa	10	51702.14	21.01596					
1:2 STD	10	30703.65	12.61656					
1:4x STD	10	8977.06	3.925924					
1:8x STD	10	6005.015	2.737106					
1:16x STD	10	2747.038	1.433915					
1:32x STD	10	1667.761	1.002204					
Ladder	5							
T=8 3KP F	12	9831.92	4.267868	3.501034				
T=8 3KP F	12	6410.84	2.899436					
T=8 3KP F	12	7501.745	3.335798					
T=8 3KP L	12	8391.694	3.691778		0.190743	0.095372	0.1512	0.107113
T=8 3KP L	12	5214.402	2.420861		0	0		
T=8 3KP L	12	9705.972	4.217489		0.716455	0.358227		
T=8 3KP M	12	3500.111	1.735144		0	0	0	0
T=8 3KP M	12	6422.733	2.904193		0	0		
T=8 3KP M	12	5178.418	2.406467		0	0		
T=12 3KP	12	5669.329	2.602831	2.94739				
T=12 3KP	12	4188.598	2.010539					
T=12 3KP	12	9734.251	4.228801					
T=12 3KP	12	19348.47	8.074489		5.127099	2.563549	2.192154	0.592674
T=12 3KP	12	21437.26	8.910006		5.962615	2.981308		
T=12 3KP	12	11688.75	5.010599		2.063209	1.031604		
T=12 3KP	12	12310.59	5.259336		2.311946	1.155973	1.093607	0.06239
T=12 3KP	12	12310.83	5.259432		2.312042	1.156021		
T=12 3KP	12	11374.86	4.885044		1.937653	0.968827		
Ladder	5							

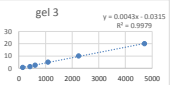
p62 STD curve	
20	51702.14
10	30703.65
5	8977.06
2.5	6005.015
1.25	2747.038
0.625	1667.761



Gel3

Sample Name	Vol loaded	Signal 800	Extrapolated p62 content	Average For Sham Controls	p62 Difference	p62 Difference Per Hour	Mean Flux	SE Flux
1x Standa	10	4711.077	20.22613					
1:2 STD	10	2241.649	9.607592					
1:4x STD	10	1091.179	4.660568					
1:8x STD	10	607.2827	2.579816					
1:16x STD	10	413.312	1.745742					
1:32x STD	10	141	0.5748					
Ladder	5							
T=16 3KP	12	3701.434	15.88466	16.38055				
T=16 3KP	12	5575.813	23.9445					
T=16 3KP	12	2173.02	9.312486					
T=16 3KP	12	12722.25	54.67419		38.29364	19.14682	12.23506	6.134692
T=16 3KP	12	11983.44	51.49728		35.11673	17.55837		
T=16 3KP	12	3025.211	12.97691		0	0		
T=16 3KP	12	4198.273	18.02107		1.640524	0.820262	4.512845	1.890952
T=16 3KP	12	6445.372	27.6836		11.30305	5.651525		
T=16 3KP	12	7103.616	30.51405		14.1335	7.066749		
T=20 3KP	12	3910.052	16.78172	17.96886				
T=20 3KP	12	3480.376	14.93412					
T=20 3KP	12	5167.965	22.19075					
T=20 3KP	12	10303.72	44.27449		26.30562	13.15281	16.81955	3.357279
T=20 3KP	12	10596.16	45.53197		27.56311	13.78155		
T=20 3KP	12	15127.66	65.01745		47.04858	23.52429		
T=20 3KP	12	5759.651	24.735		6.766135	3.383068	7.317811	2.487004
T=20 3KP	12	7279.179	31.26897		13.30011	6.650054		
T=20 3KP	12	9730.462	41.80949		23.84062	11.92031		
Ladder	5							

p62 STD curve	
20	4711.077
10	2241.649
5	1091.179
2.5	607.2827
1.25	413.312
0.625	141



	4	8.453599056	2.113399764	15.4238628	
	4	18.57646152	4.644115381	24.7509861	
	4	52.70518598	13.17629649	78.8125906	
	4	70.45865759	17.6146644	25.0714623	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	673.1870444	5	134.6374089	3.38379519	0.0249496	2.772853
Within Groups	716.1997776	18	39.78887653			
Total	1389.386822	23				

ANOVA: Proteasomal Flux

0	4	8	12	16	20
0	8.433941243	0	1.155972933	0.82026209	3.3830677
5.780438086	12.63657186	0	1.156020833	5.6515252	6.6500536
12.27285259	3.835497005	0	0.968826644	7.06674906	11.920312

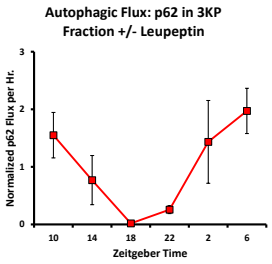
Anova: Single Factor

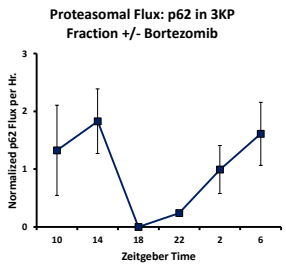
SUMMARY

Groups	Count	Sum	Average	Variance
0	3	18.05329067	6.017763558	37.6979702
4	3	24.90601011	8.302003369	19.3777853
8	3	0	0	0
12	3	3.28082041	1.093606803	0.01167757
16	3	13.53853635	4.512845451	10.7270935
20	3	21.95343374	7.317811247	18.5555623

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	169.6279516	5	33.92559032	2.35675967	0.1038813	3.105875
Within Groups	172.7401779	12	14.39501483			
Total	342.3681295	17				



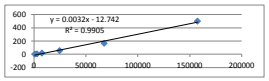


Lys^{48}-Ub Quantification in Cyto Fractions

Gel1

Sample Name	Vol loaded		Average For Sham Controls	K48 Difference	K48 Difference Per Hour	Mean Flux	SE Flux
1x Standard (STD)	10	157470	491.1628266				
1:2 STD	10	67627	203.6657109				
1:4x STD	10	24764	66.50346563				
1:8x STD	10	7730	11.99481719				
1:16x STD	10	2974	-3.225970313				
1:32x STD	10	893	-9.884796875				
Ladder	5						
T=0 Cyto PBS V1	12	13394	30.11922344	63.6473797			
T=0 Cyto PBS V2	12	30771	85.72607344				
T=0 Cyto PBS V3	12	27450	75.09684219				
T=0 Cyto Leup V1	12	30554	85.03071719		69.600625	34.80031	32.65722	1.101271579
T=0 Cyto Leup V2	12	28270	77.72108438		62.290992	31.1455		
T=0 Cyto Leup V3	12	28820	79.48177344		64.051681	32.02584		
T=0 Cyto MG341 V1	12	87009	265.6876313		250.25754	125.1288	136.719	5.813874624
T=0 Cyto MG341 V2	12	97370	298.8410344		283.41094	141.7055		
T=0 Cyto MG341 V3	12	98380	302.0754375		286.64535	143.3227		
T=4 Cyto PBS V1	12	28808	79.44486406	89.5908891				
T=4 Cyto PBS V2	12	32359	90.80664375					
T=4 Cyto PBS V3	12	34770	98.52115938					
T=4 Cyto Leup V1	12	20224	51.97503281		36.952147	18.47607	32.46816	8.455602299
T=4 Cyto Leup V2	12	28201	77.50044688		62.477561	31.23878		
T=4 Cyto Leup V3	12	38483	110.4021625		95.379276	47.68964		
T=4 Cyto MG341 V1	12	73222	221.5696766		206.54679	103.2734	94.95293	9.783498236
T=4 Cyto MG341 V2	12	75008	227.2825563		212.25967	106.1298		
T=4 Cyto MG341 V3	12	55836	165.9339938		150.91111	75.45555		
Ladder	5						

K48 (ng)	Signal
500	157470.2583
166.6666667	67627.40967
55.55555556	24764.20801
18.51851852	7730.255371
6.172839506	2973.759277
2.057613169	892.8759766



Experiment Time	ZT	p62 flux, MG341	SE		Normalized Fl	SE
0	10	136.7189711	5.813874624		1.48096313	0.0629769
4	14	94.95292801	9.783498236		1.02854625	0.1059765
7	17	43.16389479	9.7348033		0.46755864	0.105449
12	22	56.91236678	4.037854641		0.61648443	0.0437387
16	0	124.3049791	3.025353822		1.34649266	0.0327711
20	4	97.85249905	28.20110463		1.0599549	0.3054792

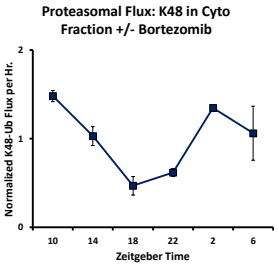
ANOVA: Proteasomal Flux

0	4	8	12	16	20
125.1287695	103.2733952	23.71620627	58.48894354	127.525659	41.517083
141.7054711	106.129835	53.68757912	49.26487913	118.25857	123.64374
143.3226727	75.45555379	52.08789899	62.98327767	127.130709	128.39667

Anova: Single Factor

SUMMARY

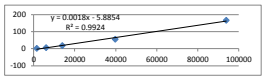
Groups	Count	Sum	Average	Variance
0	3	410.1569133	136.7189711	101.403414
4	3	284.8587784	94.95292801	287.150513
8	3	129.4916844	43.16389479	284.299186
12	3	170.7371003	56.91236678	48.9128103
16	3	372.9149374	124.3049791	27.4582972



Gel2

Sample Name	Vol loaded	Signal 800	Extrapolated K48 content	Average For Sham Controls	K48 Difference	K48 Difference Per Hour	Mean Flux	SE Flux
1x Standa	10	194791.4	344.7391					
1:2 STD	10	93868.47	163.0778					
1:4x STD	10	39732.84	65.63371					
1:8x STD	10	13957.36	19.23785					
1:16x STD	10	6053.095	5.010171					
1:32x STD	10	1529.413	-3.13246					
Ladder	5							
T=8 Cyto I	12	29456.79	47.13682	40.54055				
T=8 Cyto I	12	24087.38	37.47189					
T=8 Cyto I	12	23832.4	37.01292					
T=8 Cyto I	12	28351.63	45.14753		41.64649	20.82325	21.1121	0.380523
T=8 Cyto I	12	28155.54	44.79457		41.29353	20.64677		
T=8 Cyto I	12	29510.57	47.23363		43.7326	21.8663		
T=8 Cyto I	12	31566.03	50.93345		47.43241	23.71621	43.16389	9.734803
T=8 Cyto I	12	64867.55	110.8762		107.3752	53.68758		
T=8 Cyto I	12	63090.13	107.6768		104.1758	52.0879		
T=12 Cyto	12	27352.86	43.34975	51.74067				
T=12 Cyto	12	23341.78	36.1298					
T=12 Cyto	12	45348.8	75.74244					
T=12 Cyto	12	24562.7	38.32745		35.38006	17.69003	17.5792	0.807021
T=12 Cyto	12	25927.42	40.78396		37.83657	18.91829		
T=12 Cyto	12	22828.53	35.20596		32.25857	16.12928		
T=12 Cyto	12	69894.82	119.9253		116.9779	58.48894	56.91237	4.037855
T=12 Cyto	12	59645.86	101.4771		98.52976	49.26488		
T=12 Cyto	12	74888.53	128.9139		125.9666	62.98328		
Ladder	5							

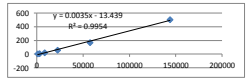
K48 (ng)	Signal
500	194791.4
166.6667	93868.47
55.55556	39732.84
18.51852	13957.36
6.17284	6053.095
2.057613	1529.413



Gel3

Sample Name	Vol loaded	Signal 800	Extrapolated K48 content	Average For Sham Controls	K48 Difference	K48 Difference Per Hour	Mean Flux	SE Flux
1x Standa	10	143750.7	489.6883					
1:2 STD	10	57531.53	187.9214					
1:4x STD	10	22506.1	65.33236					
1:8x STD	10	8649.719	16.83502					
1:16x STD	10	3145.933	-2.42823					
1:32x STD	10	1185.583	-9.28946					
Ladder	5							
T=16 Cyto	12	25314.37	75.16131	87.83821				
T=16 Cyto	12	24414.91	72.01318					
T=16 Cyto	12	37079.76	116.3402					
T=16 Cyto	12	29044.81	88.21782		71.83728	35.91864	39.05325	1.982999
T=16 Cyto	12	30529.24	93.41333		77.03278	38.51639		
T=16 Cyto	12	32934	101.83		85.44944	42.72472		
T=16 Cyto	12	81391.68	271.4319		255.0513	127.5257	124.305	3.025354
T=16 Cyto	12	76096.2	252.8977		236.5171	118.2586		
T=16 Cyto	12	81165.99	270.642		254.2614	127.1307		
T=20 Cyto	12	27786.62	83.81418	84.01381				
T=20 Cyto	12	33273.16	103.0171					
T=20 Cyto	12	22471.2	65.21021					
T=20 Cyto	12	26672.62	79.91517		61.94631	30.97316	32.22026	3.256119
T=20 Cyto	12	30904.65	94.72726		76.7584	38.3792		
T=20 Cyto	12	24578.49	72.58573		54.61686	27.30843		
T=20 Cyto	12	32697.72	101.003		83.03417	41.51708	97.8525	28.2011
T=20 Cyto	12	79627.24	265.2564		247.2875	123.6437		
T=20 Cyto	12	82343.2	274.7622		256.7933	128.3967		
Ladder	5							

K48 (ng)	Signal
500	143750.7
166.6667	57531.53
55.55556	22506.1
18.51852	8649.719
6.17284	3145.933
2.057613	1185.583



20	3	293.5574971	97.85249905	2385.90691

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20105.61454	5	4021.122908	7.69560712	0.0018814	3.105875
Within Groups	6270.262258	12	522.5218548			
Total	26375.8768	17				