In Vivo Protein Lifetime Measurements Across Multiple Organs in the Zebrafish

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Abstract

Protein production and degradation are tightly regulated to prevent cellular structures from accumulating damage and to allow their correct functioning. A key aspect of this regulation is the protein half-life, corresponding to the time in which half of a specific protein population is exchanged with respect to its initial state. Proteome-wide techniques to investigate protein half-lives in vivo are emerging. Recently, we have established and thoroughly tested a metabolic labeling approach using ^{13}C lysine (Lys(6)) for measuring protein lifetimes in mice. The approach is based on the fact that different proteins will incorporate a metabolic label at a rate that is dependent on their half-life. Using amino acid pool modeling and mass spectrometry, it is possible to measure the fraction of newly synthesized proteins and determine protein half-lives. In this chapter, we show how to extend this approach to zebrafish (*Danio rerio*), using a commercially available fish diet based on the stable isotope labeling by amino acids in cell culture (SILAC) technology. We describe the methods for labeling animals and subsequently use mass spectrometry to determine the lifetimes of a large number of proteins. In the mass spectrometry workflow proposed here, we have implemented the BoxCar data acquisition approach for increasing sample coverage and optimize machine use. To establish the proteome library used in the BoxCar approach, we recommend performing an in-solution digestion followed by peptide fractionation through basic reversed-phase chromatography. Overall, this chapter extends the use of current proteome technologies for the quantification of protein turnover to zebrafish and similar organisms and permits the study of germline changes following specific manipulations.

Key words Protein lifetime, Protein turnover, Metabolic labeling, Protein mass spectrometry, BoxCar, SILAC

1 Introduction

Proteins are essential macromolecules at the basis of life. To ensure cellular efficiency and health, protein synthesis and degradation are tightly regulated in a process known as protein homeostasis or proteostasis [1]. Aberrant proteostasis can disturb several biochemical pathways and lead to diseases such as premature aging, neurodegeneration, and infertility [2]. Protein turnover studies are relevant for several aspects of biology, including cell differentiation,

organism development, and can be applied to the analysis of several cell types including germlines [1, 3–6]. Mass spectrometry applied to proteomics allows to study changes at the level of the entire proteome and can be used for protein lifetime determinations [3, 5, 7, 8].

Traditionally, protein turnover studies were performed through the metabolic labeling of radioactive isotopes [9–11]. Less hazardous methods have been developed more recently, including the use of deuterated water (^2H_2O) [6], of specific diets containing the stable non-radioactive isotope of nitrogen ^{15}N [12] and other more precise methods based on essential amino acids labeled with a variety of stable non-radioactive isotopes [3, 5, 13–15]. By definition, essential amino acids cannot be synthetized by organisms and need to be absorbed through the diet [11, 16]. Cells and animals can be thus labeled with an approach called stable isotope labeling by amino acids in cell culture (SILAC) [17, 18]. In this approach, the essential amino acid lysine is synthetized substituting all the six carbon atoms (^{12}C) by a stable isotope of carbon (^{13}C), giving rise to a label (Lys(6)) that is incorporated in peptides with the same efficiency of conventional lysine (Lys(0); Fig. 1a). While being functionally identical, the two lysine forms can be distinguished with mass spectrometry, since they differ by 6 Da in molecular weight (Fig. 1b). A typical SILAC pulse labeling approach in vivo requires an animal model that can be fed with a diet where all Lys(0) is substituted with Lys(6). In an optimized workflow, a group of animals is fed for different time periods (Fig. 1c). Following labeling, tissue dissection, and sample preparation, it is possible to reveal the incorporation of Lys(6) by mass spectrometry (Fig. 1d). Proteins that incorporate Lys(6) faster are turned over with shorter times than proteins that incorporate the label slower [3, 5, 13]. The precise determination of the incorporation of Lys(6) allows to study the dynamic of the proteome and define protein lifetimes with precision [5, 13]. Our group has shown that, despite being based on expensive food sources, a well-designed pulse SILAC approaches outperform classical techniques of studying protein turnover in several aspects [5, 13].

Originally zebrafish became popular as a model organism for embryonic development studies, thanks to its handling simplicity and to the possibility of manipulating and observing changes in its phenotype [19, 20]. Over the past decades, a number of genetic studies indicated that ~70% of zebrafish genes are shared with humans [19]. Moreover, large scale forward genetic screenings have shown that ~84% of human disease-associated genes show similar pathological phenotypes in zebrafish [20]. This has rendered the small fish one of the most popular organisms for modeling pathology development and study disease to date. Metabolically labeled diets for protein turnover in zebrafish have been previously introduced and tested, confirming that the metabolic pulsing of this fish is feasible and effective [21, 22].

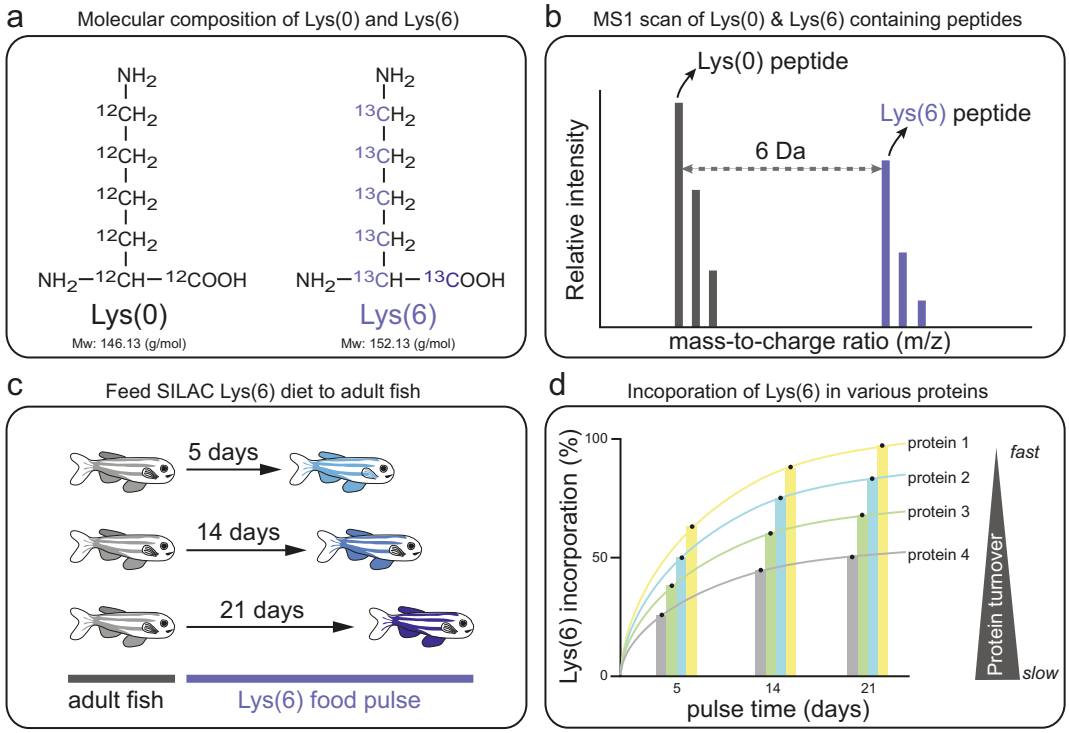

Fig. 1 Overview of the labeling approach. (**a**) Schematic representation of the molecular composition of light lysine Lys(0) containing all the naturally abundant elements (left) and stable isotopic SILAC Lysine Lys (6) where the 6 naturally occurring ^{12}C atoms were replaced with ^{13}C. (**b**) Mass spectrometric scan of peptides containing Lys(0) (left, grey) and Lys(6) (right, blue). The *x*-axis represents the mass-to-charge ratio (m/z). Peptides containing either one Lys(0) or one Lys(6) of a given charge will differ by a m/z interval of 6 Da in the MS scan. (**c**) Timeline for feeding the SILAC diet to animals. (**d**) Incorporation of Lys(6) will proceed faster in proteins that have shorter turnover times and would allow to determine the lifetime of a given protein

Recently, new methods have been developed to increase proteome coverage, including several data acquisition (DA) workflows [23, 24]. BoxCar is one of these methods, which improves proteome coverage with a small amount of starting material and optimizes instrument usage [24]. The most time-consuming aspect of BoxCar is that it requires a large peptide library, which can be created by fractionating some of the samples with a gel-free approach such as high pH chromatography [25]. Once the library is defined, BoxCar can be used to analyze a large number of samples in parallel and is compatible with SILAC measurements.

In this chapter, we detail a method to metabolically label zebrafish in combination with mass spectrometry which enables to determine lifetimes at the proteome level in several tissues (*see also* **Notes 1** and **2**). The comparative study of protein turnover in several tissues of zebrafish and eventually of various disease models will be instrumental for the understanding of basic biological processes

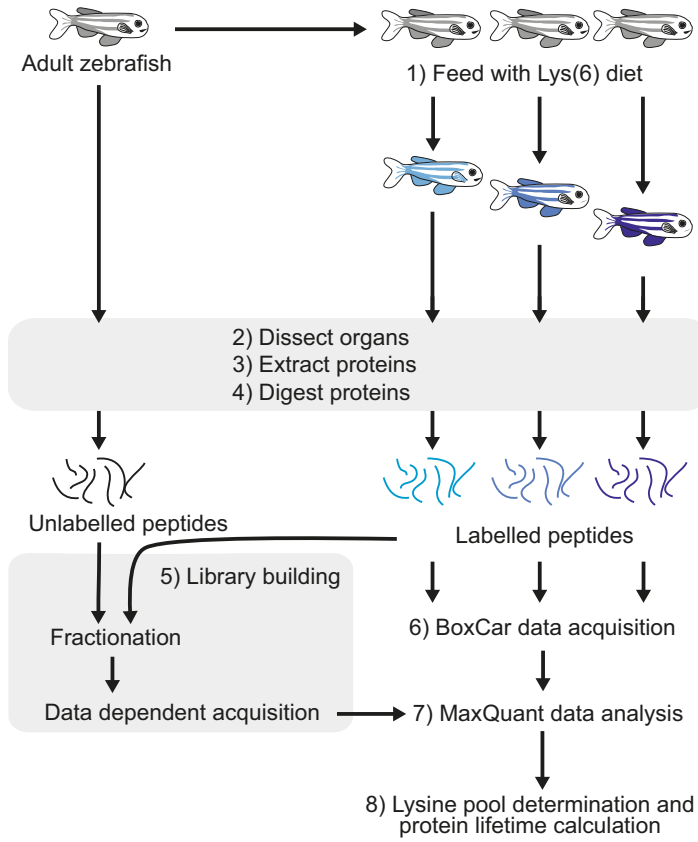

Fig. 2 Schematic representation of the workflow used for performing protein turnover measurements in zebrafish. For details see main text

and for the development of new therapies. The major steps of the workflow presented here are summarized in Fig. 2 and include: (1) feeding zebrafish with the labeled diet, (2) sacrificing the animals and dissecting the organ of interest, (3) lysing and solubilizing proteins from tissues, (4) digesting proteins, (5) fractionating the peptides for library preparation, (6) acquiring the data with the BoxCar method, (7) analyzing the data, and (8) determining the protein lifetimes.

2 Materials

To ensure high-quality results, prepare all solutions right before use and follow the indications for the storage of samples and materials.

2.1 Feeding Zebrafish

1. Zebrafish (*D. rerio*) strain AB maintained under standard conditions.

2. Diet: SILAC diet (Silantes, adult fish feed; ZFAd), granules size 4 (Aqua Schwarz).
3. Water parameters: pH 7.2–8.0, conductivity 500–600 μS, housing temperature 24–25 °C.
4. Weighing: analytical balance.
5. Dissection: forceps Inox Dumont No. 5 (biological tip), scissors, 10 cm Petri dish, 1.5 ml vial, liquid Nitrogen.

2.2 Tissue Preparation and Protein Extraction

1. 1% Rapigest (Waters).
2. Lysis buffer: 100 mM HEPES, 0.1% Rapigest, 0.1% NP-40, and protease inhibitor cocktail, pH 8.
3. Acetonitrile (ACN): (LiChrosolv grade; Merck).
4. Water (LiChrosolv grade; Merck).
5. Bioruptor (Diagenode).
6. BCA Protein Assay Kit.

2.3 Protein Digestion and Desalting

1. Digestion buffer: 100 mM ammonium bicarbonate, pH 8. Prepare freshly prior to use.
2. 10 mM dithiothreitol in digestion buffer. Prepare freshly prior to use.
3. 55 mM chloracetamide in digestion buffer. Prepare freshly prior to use.
4. Sequence Grade Modified Trypsin (Promega): 0.1 μg/μl in digestion buffer. Always prepare freshly prior to use.
5. C18 tip column (Empore, Fisher Scientific).
6. Trifluoroacetic acid.
7. SpeedVac Vacuum Concentrator.
8. Tabletop centrifuge.
9. Waterbath sonicator.
10. Thermomixer.

2.4 Basic Reversed-Phase Chromatography

1. Fractionation mobile phase A: 10 mM ammonia, pH 10.
2. Fractionation mobile phase B: 10 mM ammonia in 80% ACN (LiChrosolv grade) pH 10.
3. HPLC system coupled with fraction collector.
4. Basic reverse C18 XBridge column (3.5 μm; 1 × 150 mm; Waters).

2.5 Mass Spectrometry

1. Glass LC vials.
2. Sample suspension buffer: 5% ACN containing 0.1% TFA.
3. Self-packed fused silica capillary (Polymicro Technologies) columns filled with 75 μm inner diameter, 30 cm length packed

with Reprosil-Pur Basic C18-AQ 1.9 μm pore size beads (Dr. Maisch GmbH). Alternatively, use the commercially available Acclaim PepMap 100 C18 LC columns (Thermo Fisher Scientific), following the instrument setup provided by the supplier.

4. Trapping C18 column AcclaimPepMap100 C18–300 μm i.d. × 5 mm, 5 μm, 100 Å.
5. Buffer A: Water (LiChrosolv Grade) containing 0.01% TFA.
6. Buffer B: 95% ACN (LiChrosolv Grade) containing 0.01% TFA.
7. Q Exactive HF- Mass spectrometer.

2.6 Online Tools and Tutorials

1. MaxQuant: https://www.maxquant.org/.
2. MaxQuantLive: http://www.maxquant.live/.

3 Methods

3.1 Feeding and Dissecting Zebrafish

1. For simplicity, aliquot and store the tubes containing the amount of food for one meal (10 mg of fish diet, either SILAC or regular).
2. Select four AB of the same age and size.
3. Determine their weight in accordance with animal handling regulations.
4. Separate each fish in a single container.
5. Feed the fish for either 7, 14, or 21 days with 10 mg of food daily.
6. Also include one fish that will be housed separately, kept on a conventional diet, and used as an unlabeled control.
7. At the end of pulse determine and record the weight again.
8. Sacrifice the fish in accordance with regulations.
9. Dissect the various organs of fish:
   (a) Cut out a piece of muscle with scissors beginning at the anal fin along the belly to the gills along the gills to the spine, along the side and down to the anal fin again. Store the sample in 1.5 ml vial, label correctly and freeze immediately in liquid Nitrogen.
   (b) Now the organs are visible. With forceps, take out organs of interest such as testis, stomach, liver, kidney, heart, and gills. Store the samples in 1.5 ml vial, label correctly and freeze immediately in liquid Nitrogen.
   (c) Decapitate the fish, open the head with forceps and take out the brain. Store the sample in 1.5 ml vial, label correctly and freeze immediately in liquid Nitrogen.

(d) Open the spine with forceps and take out the central nervous system. Store the sample in 1.5 ml vial, label correctly and freeze immediately in liquid Nitrogen.

10. Transfer samples from liquid Nitrogen into a − 80 °C freezer. Store them until ready for sample preparation.

3.2 Tissue Preparation and Protein Extraction

1. Add 100 µl of lysis buffer. Sonicate in a bioruptor using the following parameters: total time 30 min, 10 duty cycle, 30 s ON/OFF. Repeat until the particulates solubilize completely making the sample a homogeneous mixture (for denser tissues, such as muscle, *see* **Note 3**).
2. Determine the protein concentration using a BCA assay kit following the instructions provided by the company.

3.3 Protein Digestion and Desalting

1. Aliquot 10 µg of SILAC-labeled protein lysate. For library building, aliquot 100 µg of lysate in a separate vial.
2. Add 10 µl of 1% Rapigest and heat at 70 °C for 10 min to solubilize the proteins. Spin the vial.
3. Add 10 µl of digestion buffer. Mix well by vortexing and spin.
4. Add 10 µl of 10 mM dithiothreitol solution. Incubate at 37 °C for 30 min on the thermomixer at 750 rpm to reduce the disulfide bonds.
5. Spin the vials. Add 10 µl of 55 mM chloracetamide solution to the vial (mix well).
6. Spin and incubate the vials on a thermomixer at 750 rpm in dark vial to alkylate the reduced cysteines. Cover the thermomixer lid with aluminum foil to avoid light.
7. Add 200 µl of digestion buffer to dilute the Rapigest detergent concentration from 1% to 0.1%. Vortex and spin.
8. Add 1:50 protein-to-trypsin amount to the vial to trypsinize the proteins into peptides. Briefly add 2 µl and 20 µl of trypsin (0.1 µg/µl) to 10 µg labeled and 100 µg unlabeled protein sample respectively.
9. Mix well and spin. Incubate the vials on thermomixer at 37 °C, 750 rpm overnight. About 16–18 h of lysate incubation are sufficient for efficient trypsinization of proteins.
10. Next day, spin the vials. Add 20 µl of 10% TFA to each vial. Mix well and spin. Check the pH using litmus paper. The acidified peptide solution has a pH of about 2. Incubate the vials on thermomixer at 37 °C, 750 rpm for 2 h. This (a) stops the trypsin activity and (b) degrades and precipitates the Rapigest detergent.
11. Spin the vials at maximum speed on tabletop centrifuge for 5 min. Carefully pipette out the supernatant in fresh vials. Discard the pellet.

12. Wash C18 columns tips using 50 µl of methanol by centrifuging at 1000 × *g* for 5 min. Subsequently, equilibrate the column tips by passing 50 µl of water containing 0.1% TFA. Note: for 100 µg of lysate use two C18 column tips.
13. Load the peptide solution from **step 11** on the washed, equilibrated C18 column tips.
14. Wash four times with 50 µl of water containing 0.1% TFA.
15. Finally, elute the peptides in fresh vials by passing 20 µl of 80% of ACN containing 0.1% TFA through the column twice.
16. Dry the eluted peptides in SpeedVac.

3.4 Fractionation of Unlabeled Lysate Peptides for Setting the Library

1. Add 100 µl of fractionation mobile phase A to the dried 100 µg of digested, desalted lysate (Subheading 3.3, **step 16**).
2. Sonicate for 3 min in a waterbath to resuspend the peptide in the buffer properly.
3. Spin for 2 min on the maximum speed of tabletop centrifuge to pellet any particulates.
4. Inject the solubilized ~50 µg peptides on a basic reversed-phase XBridge C18 column (Waters) connected to the Agilent LC system.
5. To fractionate the loaded peptides, use a 2-step linear gradient from 0% to 50% and 50% in 45 min and to 100% in next 10 min. Continue the isocratic separation at 100% for 10 min and switch back to 100% of buffer A for 5 min in the end.
6. Monitor the peptides at wavelength 214, 260, and 280 nm to observe peptides, RNA/DNA and aromatic amino acid peptides respectively.
7. Collect the fractions at every 1 min.
8. Dry the eluted peptides in SpeedVac.

3.5 DDA Runs of Unlabeled to Set Up the Library

1. Resuspend the dried peptides of fractions from basic reversed-phase chromatography in 20 µl of sample suspension buffer *see also* **Notes 4–6** for suggestions).
2. Sonicate in a waterbath for 3 min. Centrifuge to max speed. Discard pellet.
3. Transfer the supernatant in clean glass LC vials.
4. Inject 8 µl of resuspended peptide solution on a trap column followed by 30 cm C18 column coupled to Q-Exactive HF-X instrument for 88 min for Top30 method. Briefly, load the peptides in 5% buffer B. Fractionate the peptides using a 2-step linear gradient, i.e., for 3 min to 10% of buffer B and then until 75.9 min to 42% of buffer B. Finally, wash the column isocratically in 90% of buffer B for 5 min and switch back 5% of buffer B. For MS1, set the resolution at 60,000 with AGC target of 1e6 and max. IT of 50 ms. Set the scan range

from 350 to 1600 m/z. For MS2, set the resolution at 15,000 with AGC target as 1e5 and max. IT of 54 ms. Set the loop count parameter to 30.

3.6 BoxCar Mass Spectrometry Runs for the Labeled Lysate Digest

1. Resuspend 10 μg digested lysate in 50 μl of sample suspension buffer.
2. Sonicate for 3 min to resuspend peptides.
3. Centrifuge at maximum speed on tabletop centrifuge for 2 min to pellet the particulates. Pipette the supernatant in clean LC glass vials.
4. Inject 5 μl of resuspended peptide solution on trap column followed by 30 cm C18 column coupled to Q-Exactive HF-X with BoxCar method. For MS1 and MS2, use BoxCar settings mentioned in MQLive tutorial available. Briefly, set the loop count to 1. Set two Full MS-SIM scans for Scan Group tab at 120,000 resolution. Set the first Full MS SIM scan from 909 to 1001 m/z range, while the second Full MS SIM scan from 908 to 1088 m/z. For MQLive, save 1001 as BoxCar "magic number."

3.7 Data Analysis and Protein Turnover Interpretation

1. The MaxQuant software coupled to Andromeda search engine can be used to determine the protein turnover. Submit library and BoxCar RAW files as separate groups. Follow rest of the instruction as given in the BoxCar publication [24].
2. For protein lifetime determinations refer to our published set of tools [26]. Upload the H/L ratios of proteins. As a result, the scripts will give an output file containing the lifetimes of the input proteins (*see* **Notes 7** and **8**).

4 Notes

1. The described protocol for studying protein turnover in zebrafish is also applicable to other model organisms. The detailed step-wise protocol for performing protein turnover in mouse has been tested and is available online [26].
2. Our original mass spectrometric approach for pulsed SILAC quantification was based on the fractionation of the tissue lysate with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This approach required cutting each gel lane into 23 slices followed by in-gel digestion and mass spectrometric analysis of each individual gel slice. In general, while being reliable and precise, this approach was tedious and time-consuming. If the protein lysate is directly processed for in-solution digestion (without gel fractionation), the number of proteins identified is limited, due to the complexity of

Table 1
Estimate of protein amounts obtained by the dissection of one fish for several organs/tissues

Organ	Protein amount (μg)
Brain	400
Gills	40
Heart	70
Kidney	10
Liver	10
Muscle (~2 mm^3)	300
Stomach	300
Testis	70
Ovary	200

peptide spectra which overlap and render the quantification inefficient [7, 8, 27, 28].

3. Dense tissues like muscles are difficult to lyse, therefore, it is recommended to sonicate the tissue in a bioruptor for longer times (up to 1 h). It is recommended to perform the sonication at 4 °C so that the samples do not heat up during long sonication cycles of bioruptor. If the sample is not solubilized, we recommend to add 10 μl of 8 M urea to the 100 μl of lysis buffer ad sonicate for an additional 10 min.
4. For setting up the library, it is recommended to start with relatively high sample amounts (~100 μg). Table 1 reports a rough estimate of protein amounts obtained by the dissection of one fish for several organs/tissues. Depending on the final protein amount required, a higher number of fishes can be sacrificed and pooled. Note that the exact protein amounts may vary from lab to lab due to dissection practices.
5. Gel-based fractionation techniques through SDS-PAGE or gel-free fractionation approaches such as strong cation exchange and size-exclusion chromatography can be applied as alternative methods to basic reversed-phase chromatography. Nevertheless, in our hands, basic reversed-phase chromatography outperforms the aforementioned fractionation methods.
6. For setting up the library, it is recommended to load at least 50 μg of digested protein lysate. Lowering the loading amounts might decrease the number of identifications in the experiment. Increasing excessively the amounts loaded might block the column or saturate the MS.

7. Protein turnover studies on various genetically modified zebrafish lines could be aimed at testing the causes of alterations at the basis of pathologies such as aging or germline exhaustion. For instance, the swimming ability of zebrafish slows down over aging, thus protein turnover study at various ages of zebrafish may pinpoint the molecular causes of this impairment [29].
8. Zebrafish show high dimorphism between males and females, and the time course of protein production/stabilization during gonad development could be analyzed in detail with metabolic labeling. The technique would be also able to pinpoint differences between models of infertility, clarifying which of the essential proteins are either stabilized or turned over more rapidly.

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