Aus dem Institut für integrative Neuroanatomie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Establishing bio-orthogonal labeling and click chemistry in *Caenorhabditis elegans* as a tool to identify newly synthesized proteins

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Milena Ullrich
aus Tübingen

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Abstrakt (German)

In diesem Versuchsprotokoll beschreiben wir die Inkorporation von bio-orthogonalen Aminosäuren als eine vielfältig anwendbare Methode um neu synthetisierte Proteine im Rundwurm *Caenorhabditis elegans* zu visualisieren und identifizieren. Unsere Methode ermöglicht die Analyse des *de novo* Proteoms mittels drei verschiedener, sich ergänzender Verfahren: Click Chemistry, gefolgt von Western Blotting, Immunfluoreszenz oder quantitativer Massenspektrometrie durch isobare Markierung für relative und absolute Quantifizierung (iTRAQ). Die Analyse des *de novo* Proteoms war bislang nur in begrenztem Umfang möglich, weil die chemischen Eigenschaften von Proteinen unabhängig vom Zeitpunkt ihrer Synthese sind. Unser Protokoll umgeht dieses Hindernis, indem es die Aufnahme von chemisch modifiziertem Azidohomoalanin anstelle der natürlichen Aminosäure Methionin in neu entstehende Proteine ermöglicht. So kann das markierte *de novo* Proteom identifiziert und *in situ* visualisiert werden. Die Methode ist daher ein äußerst geeignetes Instrument um die *de novo* Proteinbiosynthese in physiologischen und pathologischen Prozessen, wie etwa Lernen und Gedächtnisbildung, zu untersuchen. Es werden 10 Tage für die Anzucht und Synchronisation der Würmer in flüssigem Medium benötigt; 1-2 Tage für die bio-orthogonale Markierung; sowie 3-4 Tage für die Analyse mittels Western Blotting, bzw. 5-6 Tage für Immunfluoreszenz oder ~3 Wochen für Massenspektrometrie.

Abstract (English)

In this protocol we describe the incorporation of bio-orthogonal amino acids as a versatile method for visualizing and identifying *de novo*–synthesized proteins in the roundworm *Caenorhabditis elegans*. This protocol contains directions on implementing three complementary types of analysis: 'click chemistry' followed by western blotting, click chemistry followed by immunofluorescence, and isobaric tags for relative and absolute quantification (iTRAQ) quantitative mass spectrometry. The detailed instructions provided herein enable researchers to investigate the *de novo* proteome, an analysis that is complicated by the fact that protein molecules are chemically identical to each other, regardless of the timing of their synthesis. Our protocol circumvents this limitation by identifying *de novo*–synthesized proteins via the incorporation of the chemically modifiable azidohomoalanine instead of the natural amino acid methionine in the nascent protein, followed by facilitating the visualization of the resulting labeled proteins *in situ*. It will therefore be an ideal tool for studying *de novo* protein synthesis in physiological and pathological processes including learning and memory. The protocol requires 10 d for worm growth, liquid culture and synchronization; 1–2 d for bio-orthogonal labeling; and, with regard to analysis, 3–4 d for western blotting, 5–6 d for immunofluorescence or ~3 weeks for mass spectrometry.

Affidavit

I, Milena Ullrich, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic: **Establishing bio-orthogonal labeling and click chemistry in *Caenorhabditis elegans* as a tool to identify newly synthesized proteins.** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Signature

Detailed Declaration of Contribution

Milena Ullrich had the following share in the following publication:

Milena Ullrich, Vanessa Liang, Yee Lian Chew, Samuel Banister, Xiaomin Song, Thiri Zaw, Hong Lam, Slavica Berber, Michael Kassiou, Hannah R Nicholas & Jürgen Götz
Bio-orthogonal labeling as a tool to visualize and identify newly synthesized proteins in *Caenorhabditis elegans*, Nature Protocols, 2014

Contribution in detail:

Milena Ullrich conducted the experiments for the above-listed publication from October 2011 until July 2012 fulltime for at least 50 hours per week at the *Alzheimer's and Parkinson's Disease Laboratory* at the Brain and Mind Research Institute of the University of Sydney in Australia. From August 2012 until May 2014 she worked from Berlin part-time for approximately 2-10 hours per week with concentrated efforts on editing the publication.

Milena Ullrich conducted her experimental work in Sydney under the theoretical guidance of Prof. Jürgen Götz (now: Director of the Clem Jones Centre for Ageing Dementia Research at the University of Queensland, Australia) and Dr. Hannah Nicholas (Senior Lecturer in Molecular Biology at the University of Sydney, Australia). Prof. Thomas Ohm (Head of Research Group at the Institute of Integrative Neuroanatomy) supervised the progress of her work from Berlin and was informed regularly about the progress.

Milena Ullrich conducted the major part of the experimental work that enabled the implementation of bioorthogonal labeling in the roundworm *C. elegans*. From October 2011 until April 2012 she performed all fundamental experiments fully independently under theoretical guidance of Professor Jürgen Götz and Dr. Hannah Nicholas. During that time she established the protocol of AHA-labeling, click chemistry and the detection of newly synthesized proteins via Western blot analysis in the roundworm *C. elegans*. After Milena Ullrich had established the method in *C. elegans* and found the concentration of AHA that allows for non-toxic labeling in the worm, Honours student Vanessa Liang and PhD candidate

Yee Lian Chew adapted Milena Ullrich's protocol for the detection via immune fluorescence and Dr. Xiaomin Song for mass spectrometry. However, Milena Ullrich was at all times the driving force behind all experiments and she performed not only the majority of the experimental work, but provided also the foundation for all adaptations of the protocol. Without her effort the data and the method would never have been published in *Nature Protocols*. It was at all times out of question that the first authorship of the publication was due to Milena Ullrich.

The detailed contribution of Milena Ullrich's work in the publication is shown in the enclosed table that display the respective work steps and expenditure of time of her work.

Work steps of the protocol that were developed *alone or for the most part* by Milena Ullrich:

Time	**Work step**	**Experimental and practical work performed by (approximate share in %)**	**Hereby developed steps in the protocol and parts of the paper**
September 2011	Literature research for the protocol	Milena Ullrich (100%)	Foundation of the whole paper
October 2011	Literature research for the protocol	Milena Ullrich (100%)	Foundation of the whole paper
	Proof of principle and replication of the protocol for bio-orthogonal labeling in cell culture as described by Dieterich et al. in *Nature Protocols*, 2007	Milena Ullrich (100%)	Foundation of the whole paper
	Revision and adaption of the protocol described by Dieterich et al. in cell culture to our laboratory conditions	Milena Ullrich (100%)	Foundation of the whole paper
	Draft of a first test protocol for bio-orthogonal labeling in *C. elegans*	Milena Ullrich (100%)	Foundation of the whole paper
November 2011 - February 2012	Several (initially failed) attempts to implement bio-orthogonal labeling in *C. elegans*. Hereby step by step adaption and modification of the first test protocol	Milena Ullrich (100%)	Foundation of the whole paper, esp. **TROUBLESHOOTING**, p.2251-2252
	End of february: Proof of principle of a first working protocol in *C. elegans* developed by Milena Ullrich that enables bio-orthogonal labeling, click chemistry and protein detection via Western blot	Milena Ullrich (100%)	Foundation of the whole paper, esp. **Bio-orthogonal labeling** (Steps 16-24, p.2245-2247) and **Visualization and identification of de novo-synthesized proteins by western blotting** (A), p.2247-2249)
	Replication of the results and application of the protocol with different strains of worms and bacteria (N2, bus-17 C. elegans worms and HB101, OP 50, CAG18491 E. coli bacteria)	Milena Ullrich (100%)	Experimental work for **Box 2 - Which strains of worms and bacteria to use for labeling?**, p. 2245

March - April 2012	Development of a supplementary protocol for generating large amounts of synchronized worms: investigation and analysis of worm culture under different conditions (age/number of worms/incubation periods/bleaching time/liquid worm culture)	Milena Ullrich (70%), Vanessa Liang (30%)	Experimental work for **Figure 2 Protocol workflow**, p.2240 and **Worm culture** (Steps 1-15), p.2244-2245 and **TIMING, p**. 2252
	Improvement of the protein detection via Western blotting by modifying different parameters (length and number of sonification cycles/wet vs. semi-dry transfer/different antibodies/ modification of 'stripping'/protein concentration)	Milena Ullrich (100%)	Experimental work for **Visualization and identification of de novo-synthesized proteins by western blotting** (A), p.2247-2249
May 2012	Establishing the concentration of azidohomoalanine (AHA), that allows a non-toxic labeling in the worm. Testing the toxicity of bio-orthogonal labeling for *C. elegans* by application of different concentrations of azidohomoalanine and toxicity screening via thrashing assay	Milena Ullrich (ca. 70%), Vanessa Liang (ca. 30%)	Experimental work for **Box 3 - Thrashing assay for toxicity screening**, p. 2246 and **Figure 4 - Testing for AHA toxicity - the thrashing assay**, p.2248
	Analysing the uptake of AHA in *C. elegans* by applying the protocol with dead vs. living E. coli bacteria. Quantification of the bacterial uptake during the bio-orthogonal labeling process	Milena Ullrich (100%)	Experimental work for **ANTICIPATED RESULTS, Figure 7c**, p.2252
June - July 2012	Bio-orthogonal labeling of samples for iTRAQ analysis. (Controle via Western blotting by Milena Ullrich; Implementation of iTRAQ Mass Spec analysis by Xiaomin Song in the Australian Proteome Facility, see table below)	Milena Ullrich (60%), Vanessa Liang (40%)	Samples for further development of step **(C) Identification of *de novo*-synthesized proteins by iTRAQ quantitative proteomics**, p.2250-2251
	Collection of data, statistical analysis of Western blot results	Milena Ullrich (100%)	Foundation of the paper
	Replication of experiments for the paper, photo documentation	Milena Ullrich (100%)	Western blots for **ANTICIPATED RESULTS, Figure 7a**, S.2252 and photos for **Figure 3**, p.2245 and **Figure 6**, p.2249
August - November 2012	Composing a draft for the following parts of the paper: Introduction, Figure 1, Figure 2, Figure 3, Figure 4, Figure 6, Figure 7, Box 2, Box 3, Materials, Protocol Step 1-24 (A), Troubleshooting, Anticipated Results	Milena Ullrich (100%)	The draft was the basis for the final version of **Abstract**, **Introduction,** p.2237-2241, **Materials,** p.2241-2244, **Protocol Step 1-24 (A),** p.2244-2249, **Troubleshooting,** p. 2251-2252, **Anticipated Results,** p. 2252-2254, **Figure 1,** p. 2239 **Figure 2,** p.2240, **Figure 3,** p. 2245, **Figure 4,** p.2248, **Figure 6,** p.2249, **Figure 7,** p.2252, **Box 2,** p.2245, **Box 3,** p.2246

November 2012 - May 2014	Revision of the manuscript in the *Nature Protocols* peer-review process	Milena Ullrich, Prof. Jürgen Götz and Dr. Hannah Nicholas did the main part of the work with input of all authors	Final version of the paper

Work steps of the protocol that were *not* developed alone or for the most part by Milena Ullrich (Contribution of Co-Authors)

Time	**Work step**	**Experimental and practical work performed by (approximate share in %)**	**Hereby developed parts of the paper**
August - September 2011	Chemical synthesis of the biotin-PEG3-propargylamide (biotin-alkyne tag)	Dr. Samuel Banister (100%) School of Chemistry at the University of Sydney	Production of the biotin-alkyne tag that was used for all the experiments and **Box 1 - Synthesis of biotin-PEG3-propargylamide, the biotin-alkyne tag**, p. 2242
May - September 2012	Adaption of Milena Ullrich's protocol for bio-orthogonal labeling via western blotting for the supplementary analysis via immunofluorescence	Vanessa Liang (90%), Yee-Lian Chew (10%)	Experimental work for **Visualization of *de novo*-synthesized proteins by fluorescence microscopy, (B)**, p. 2249-2250 and **Figure 8**, p.2253
June - September 2012	Adaption of Milena Ullrich's protocol for bio-orthogonal labeling via western blotting for the supplementary analysis via iTRAQ quantitative proteomic analysis. Application of samples that were bio-orthogonally labeled by Milena Ullrich and Vanessa Liang (see above)	Dr. Xiaomin Song and Dr. Thiri Zaw (100%)	Experimental work for **Identification of *de novo*-synthesized proteins by iTRAQ quantitative proteomics, (C),** p. 2250-2251 and **Figure 9**, p.2253 and the following paper: *Liang V, Ullrich M, Lam H, Chew YL, Banister S, Song X, Zaw T, Kassiou M, Gotz J, Nicholas HR. Altered proteostasis in aging and heat shock response in C. elegans revealed by analysis of the global and de novo synthesized proteome. Cell Mol Life Sci, 2014. 71(17): p. 3339-61.*
September - October 2012	UPR assay as a supplementary toxicity screening to the by Milena Ullrich and Vanessa Liang performed thrashing assay	Yee-Lian Chew (50%), Hong Lam (30%) (PhD candidate), Slavica Berber (20%, PhD candidate)	Experimental work for **Box 4 - UPR assay for toxicity screening,** p. 2247 and **Figure 5**, p. 2248

Signature of the doctoral candidate

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☐	10	J PROTEOME RES	1535-3893	17943	5.056	5.223	0.882	544	3.9	0.06668	1.453
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Ullrich M, Liang V, Chew YL, Banister S, Song X, Zaw T, Lam H, Berber S, Kassiou M, Nicholas HR, Gotz J. Bio-orthogonal labeling as a tool to visualize and identify newly synthesized proteins in Caenorhabditis elegans. Nat Protoc, 2014. 9(9): p. 2237-55

http://dx.doi.org/10.1038/nprot.2014.150

Curriculum Vitae

My curriculum vitae is not published in the electronical version of my dissertation for reasons of data protection.

Complete list of publications

Ullrich M, Liang V, Chew YL, Banister S, Song X, Zaw T, Lam H, Berber S, Kassiou M, Nicholas HR, Gotz J. Bio-orthogonal labeling as a tool to visualize and identify newly synthesized proteins in Caenorhabditis elegans. Nat Protoc, 2014. **9**(9): p. 2237-55.

Impact Factor 2013: 7, 960

Liang V, Ullrich M, Lam H, Chew YL, Banister S, Song X, Zaw T, Kassiou M, Gotz J, Nicholas HR. Altered proteostasis in aging and heat shock response in C. elegans revealed by analysis of the global and de novo synthesized proteome. Cell Mol Life Sci, 2014. **71**(17): p. 3339-61.

Impact Factor 2013: 5, 856

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