Integration of Fluorescence-based Dosimetry to the IC-FPOP PIXY Platform Increases Quantitative Power for Protein Folding Studies

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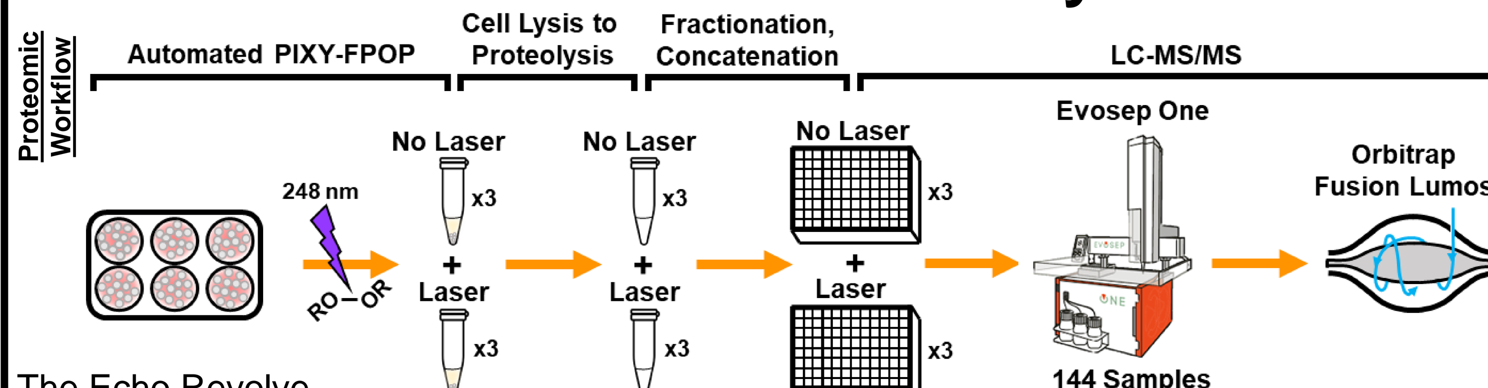
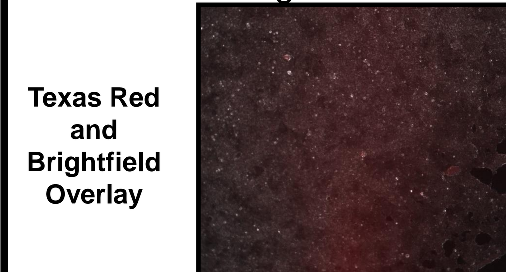
1. Introduction

Platform Incubator with Movable XY stage (PIXY) is an automated system developed to characterize cellular proteins using the hydroxyl radical protein footprinting (HRPF) method In-Cell Fast Photochemical Oxidation of Proteins (IC-FPOP). The capabilities of this high throughput platform permit other cell based experimental applications including fluorescent imaging and time-dependent solution transfer. Owing to this, we have extended the PIXY system for fluorescence-based dosimetry studies as well as for time-dependent protein folding studies that utilize pulse-chase technology. Dosimetry measurements will expand the quantitative nature of IC-FPOP increasing its applicability for studying protein folding.

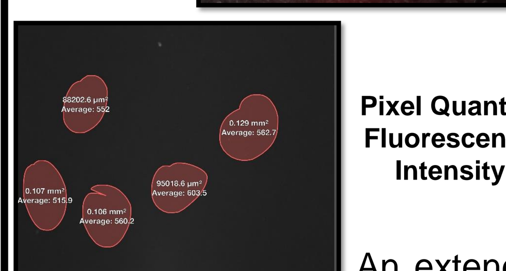
2. Fluorescence-based dosimetry: ECHO Revolve



The Echo Revolve fluorescence microscope has been integrated with the current PIXY system to quantify fluorescence post-IC-FPOP labeling.



Texas Red and Brightfield Overlay

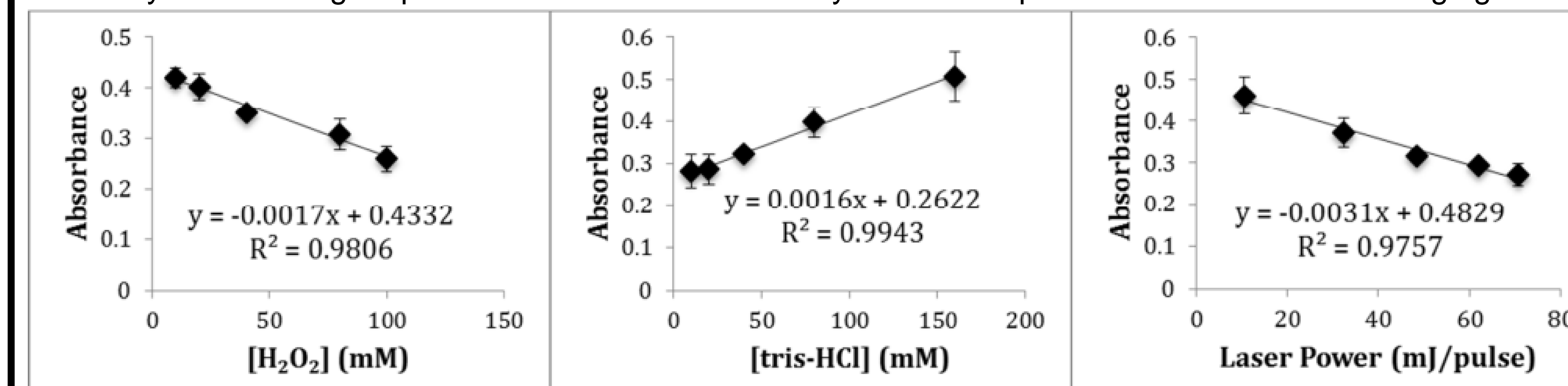


Pixel Quant of Fluorescence Intensity

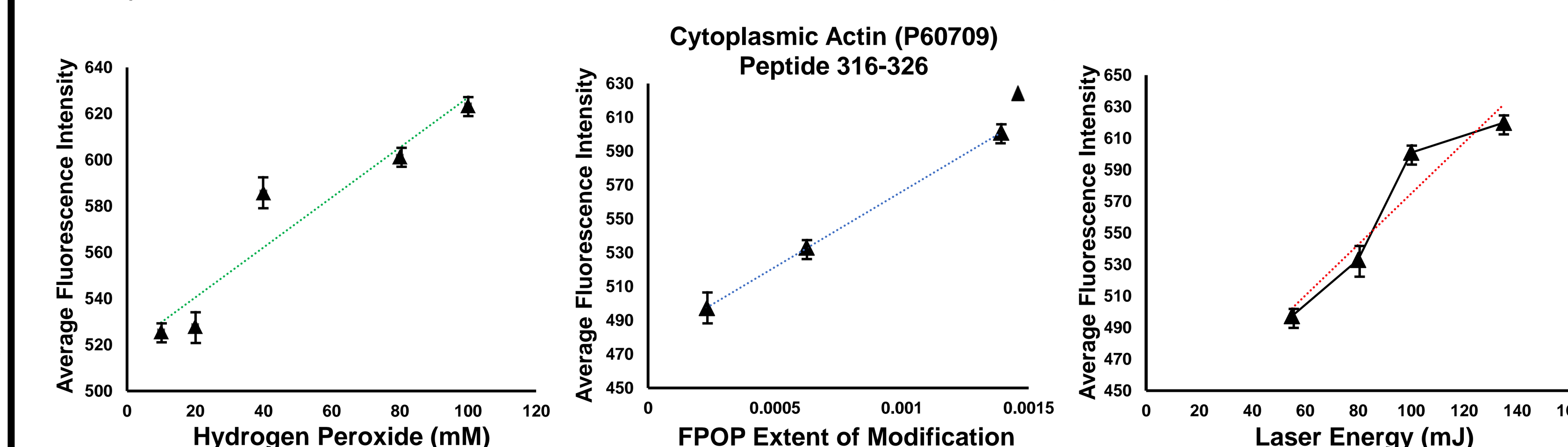
An extended rail system shuttles the PIXY incubator out of alignment with the laser, and into alignment with the imaging microscope of the Echo system. The CellROX Deep Red fluorophore is used to detect radical generation upon laser photolysis.

3. Dosimetry: Preliminary Data

In FPOP, hydroxyl radicals are generated via laser photolysis of hydrogen peroxide. The quantitative nature of the method is improved by using dosimetry to quantify radical production. In vitro FPOP studies have been routinely performed using adenine as a dosimeter. Adenine is not an appropriate dosimeter for in-cell FPOP owing to the high number of molecules in the cell that absorb at the same wavelength. CellROX Deep Red is a fluorophore that is only fluorescent in the presence of reactive oxygen species making it suitable for IC-FPOP dosimetry and the stage top incubator used in the PIXY system is compatible with fluorescence imaging.



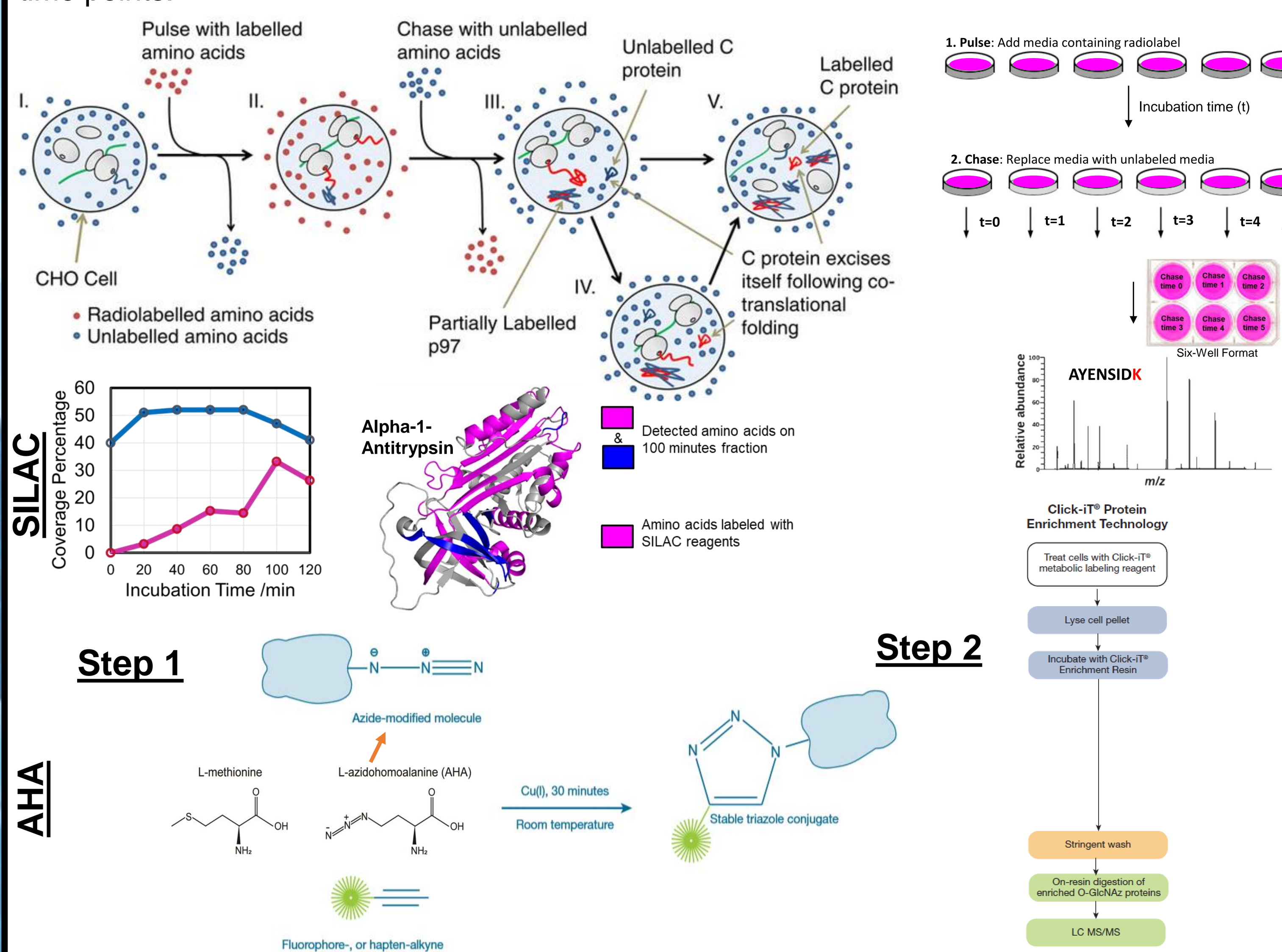
Dose response curve of adenine in condition of different concentrations of variables. *Boer Xie, and Joshua S Sharp. Analytical Chemistry. 2015*



Average fluorescence intensity of CellROX Deep Red compared to different variables. Each data point represents the average of five fluorescent measurements replicates, with error bars representing average standard deviation.

4. Pulse Chase IC-FPOP for Protein Folding: Pulse Approaches

For these tests, we are using Huh-7 cells and detection by pcIC-FPOP coupled to mass spectrometry. We are first optimizing the pulse time points where we can identify the newly synthesized proteins. Our original approach was using Stable Isotope Labeling by Amino Acids (SILAC) reagents to incorporate isotopically labeled lysines and arginines into proteins. However, the time for incorporation for these reagents was too long for protein folding studies. We have since altered our strategy are testing L-azidohomoalanine (AHA) for pulse-chase time points.

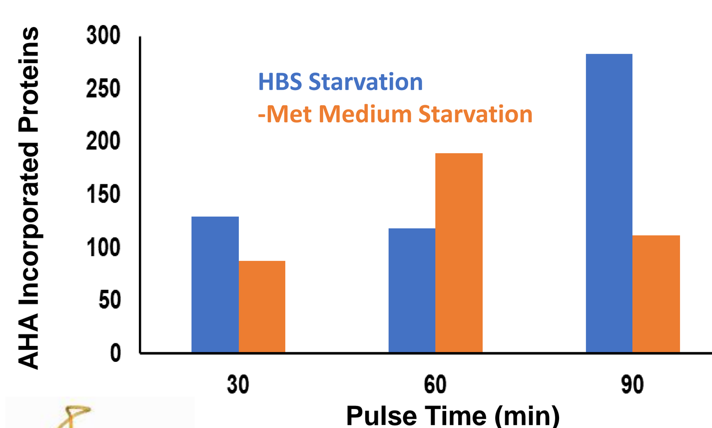


5. Pulse Experiment : Long Pulse Length

Conditions:

- **Starvation length:** 30 min
- **Starvation solution:** HBS or -Met medium
- **Pulse length:** 30 – 90 min
- **Pulse medium:** -Met medium with 4 mM AHA
- **Chase length:** None
- **Chase medium:** None
- **Click chemistry / enrichment:** None

Nine proteins with AHA in all six conditions	
P02787	Serotransferrin
P02760	Protein AMBP
P02768	Albumin
P05787	Keratin
P02649	**Apolipoprotein E**
P02771	Alpha-fetoprotein
P14314	Glucosidase 2 subunit beta (80K-H protein)
P0DP25	Calmodulin-3
P07237	Protein disulfide-isomerase



Conclusions:

- **HBS starvation in future experiments**
- **30 min pulse = ~100 proteins with AHA**
- **Poor overlap in detected proteins with AHA**
- **Enrichment not critical for longer pulse times**

APO-E

- Preliminary experiment testing AHA incorporation times in standard vs Hepes buffer.
- Hepes was used as a methionine depleted media works better than normal cell culture media void of methionine at least for the 30 and 90 min incubations.
- There we 9 proteins that showed incorporation in all experimental groups.
- APO-E protein due to its biological importance as a risk factor to Alzheimer's and has MD simulations to help study the protein intermediates makes it a new model protein of interest.

6. Pulse-Chase Experiment : Short Pulse Length

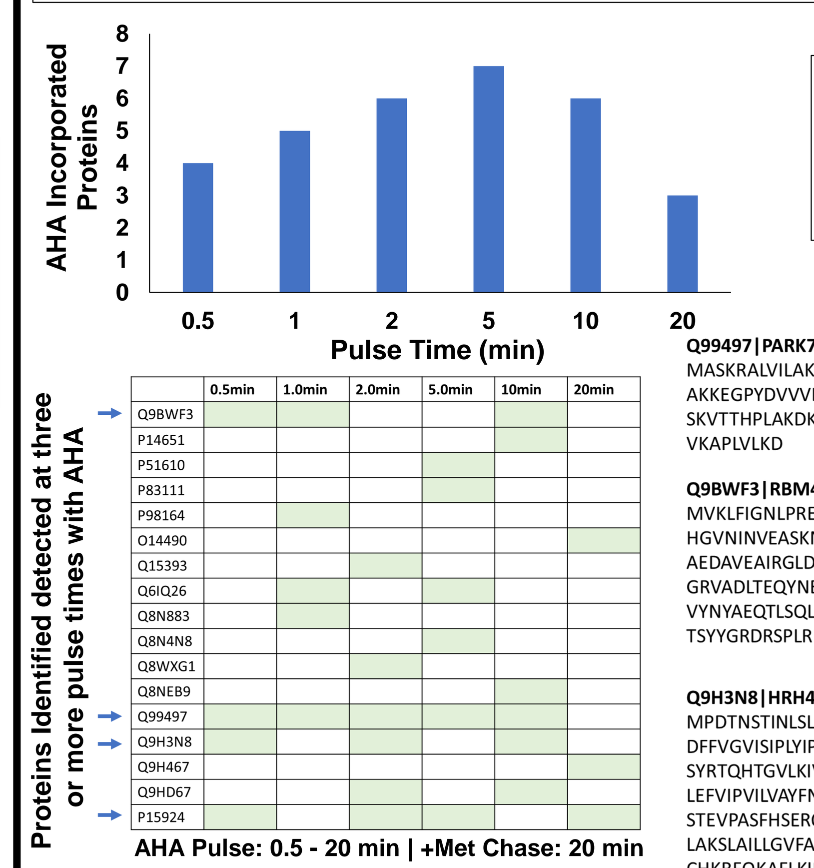
Conditions:

- **Starvation length:** 30 min
- **Starvation solution:** HBS
- **Pulse length:** 0.5 – 20 min
- **Pulse medium:** -Met medium with 4 mM AHA
- **Chase length:** 20 min
- **Chase medium:** +Met medium
- **Click chemistry / enrichment:** Yes

Four proteins with AHA in at least three pulse conditions (none detected in all six)	
Q9BWF3	RNA-binding protein 4
Q99497	Parkinson disease protein 7
Q9H3N8	Histamine H4 receptor
P15924	Desmoplakin

Conclusions:

- **AHA incorporation by 0.5 min confirmed**
- **Poor overlap in detected proteins with AHA**
- **Few proteins with AHA detected (enrichment?)**



Q99497|PARK7_HUMAN Parkinson disease protein 7
MASKRALVILAKGAEEMETVIPVDVMRRAGIKVTVAGLAGKDPVQCSRDVVICPDASLED
AKKEGPYDVVVLPGGNLGAQNLSESAAVKEILKEQENRKGLIAAICAGPTALLAHEIGFG
SKVTTHPLAKDKMMNGGHYTYSENRVEKDGLILTSRGPGTSFEFALAIVEALNGKEVAAQ
VKAPLVLKD

Q9BWF3|RBM4_HUMAN RNA-binding protein 4
MVKLFIGNLPREATEQEIRSLFEQYGKVLECDIIKNYGFVHIEDKTAAEDAIRNLHHYKL
HGVNINVEASKNKSKTSTKLHVGNISPTCTNKELRAKFEEYGPVIECDIVKDYAFVHMER
AEDAVEAIRGLDNTEFQGKRMHVQLSTSRLRTAPGMGDQSGCYRCGKEGHWSKECPIDRS
GRVADLTEQYNEQYGAVRTPYTMSYGDSLYYNNAYGALDAYYKRCRAARSYEAVAAAAAS
VYNYAEQTLSQLPQVQNTAMASHLTSTSLDPYDRHLLPTSGAAATAAAAAAAAAAVTAAS
TSYYGRDRSPLRRATAPVPTVGEGYGYGHESELSQASAAARNSLYDMARYEREQYADRAR YSAF

Q9H3N8|HRH4_HUMAN Histamine H4 receptor
MPDTNSTINLSLSTRVTLAFFMSLVAFAIMLGNALVILAFVVDKNLRHRSSYFFLNLAIS
DFFVGVISIPLYIPHTLFEWDFGKEICVFWLTTDYLLCTASVYNIVLISYDRYLSVSNAV
SYRTQHTGVLKIVTLMVAVWVLAFLVNGPMILVSESWKDEGSECEPGFFSEWYILAITSF
LEFVIPVILVAYFNMNIYWSLWKRDHLSRCQSHPGLTAVSSNICGHSFRGRLSSRRSLSA
STEVPASFHSERQRRKSSLMFSSRTKMNSNTIASKMGSFSQSDSVALHQREHVELLRARR
LAKSLAILLGVFAVCWAPYSLFTIVLSFYSSATGPKSVWYRIAFWLQWFNSFVNPLLYPL
CHKRFQKAFLKIFCIKKQPLPSQHSRSVSS

Peptide regions with AHA incorporated

Other Observations:

- Peptides detected with AHA were not located near the protein N-termini
- No Evidence for "chase" peptides

Conclusions:

- Starvation length >30 min is necessary
- Chase length >20 min?

Overall Conclusions: Preliminary pulse-chase experiments to optimize pulse time points showed that 30 min pulse times lead to sufficient incorporation of AHA in several proteins in the proteome. Preliminary dosimetry results supports a positive correlation of fluorescence intensity to increasing hydrogen peroxide concentration and laser energy. Coupling fluorescence-based dosimetry with PIXY for greater quantitative power is new approach for IC-FPOP and requires further investigation to become an integral step to our proteomic workflow.

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References: 1)Johnson, D., Punshon-Smith, B., Espino, J.A., Gershenson, A., Jones, L.M. Platform Incubator with Movable XY Stage: A New Platform for Implementing In-Cell Fast Photochemical Oxidation of Proteins. J. Vis. Exp. (171), e62153, doi:10.3791/62153 (2021)

2) Dieterich, D. C.; Lee, J. J.; Link, A. J.; Graumann, J.; Tirrell, D. A.; Schuman, E. M., Labeling, detection and identification of newly synthesized proteomes with bioorthogonal non-canonical amino-acid tagging. *Nat Protoc* **2007,** *2* (3), 532-40.