Technical Journal Club

UniversitätsSpital Zürich

«*Catch me if you can*»:

methods to visualize newly synthesized proteins in situ

Assunta Senatore

March 31st 2015

The proteome of a cell is highly dynamic in nature and tightly regulated by both protein synthesis and degradation to actively maintain homeostasis.

Many intricate biological processes, such as cell growth, differentiation, diseases, and response to environmental stimuli, require protein synthesis and translational control.
Long-lasting forms of synaptic plasticity, such as those underlying long-term memory, require new protein synthesis in a space- and time-dependent manner.

Therefore, direct visualization and quantification of newly synthesized proteins at a global level are indispensable to unraveling the spatial–temporal characteristics of the proteomes in live cells.

Labeling methods to probe newly synthesized proteins

- Radioisotope or stable isotope labeling to trace and quantify proteome dynamics (SILAC-MS).

- Bioorthogonal non-canonical amino acid tagging (BONCAT) metabolic incorporation of unnatural amino acids.

- Newly synthesized proteins can then be visualized through subsequent conjugation of the reactive amino acids to fluorescent tags via click chemistry (FUNCAT).

- Stimulated Raman scattering (SRS) microscopy, an emerging vibrational imaging technique, for the visualization of nascent proteins in live cells upon metabolic incorporation of deuterium- labeled amino acids

Direct visualization of newly synthesized target proteins *in situ*

Susanne tom Dieck[1,3], Lisa Kochen[1,3], Cyril Hanus[1], Maximilian Heumüller[1], Ina Bartnik[1], Belquis Nassim-Assir[1], Katrin Merk[1], Thorsten Mosler[1], Sakshi Garg[1], Stefanie Bunse[1], David A Tirrell[2] & Erin M Schuman[1]

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ACS chemical biology

Articles

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Imaging Complex Protein Metabolism in Live Organisms by Stimulated Raman Scattering Microscopy with Isotope Labeling

Lu Wei,[†] Yihui Shen,[†] Fang Xu,[†] Fanghao Hu,[†] Jamie K. Harrington,[‡] Kimara L. Targoff,[‡] and Wei Min[*,†,§]

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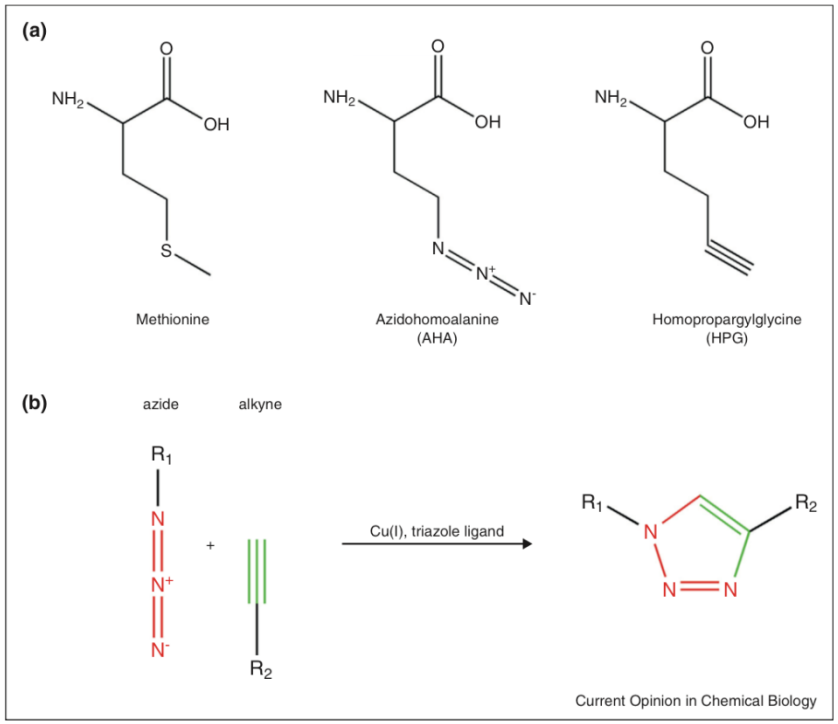
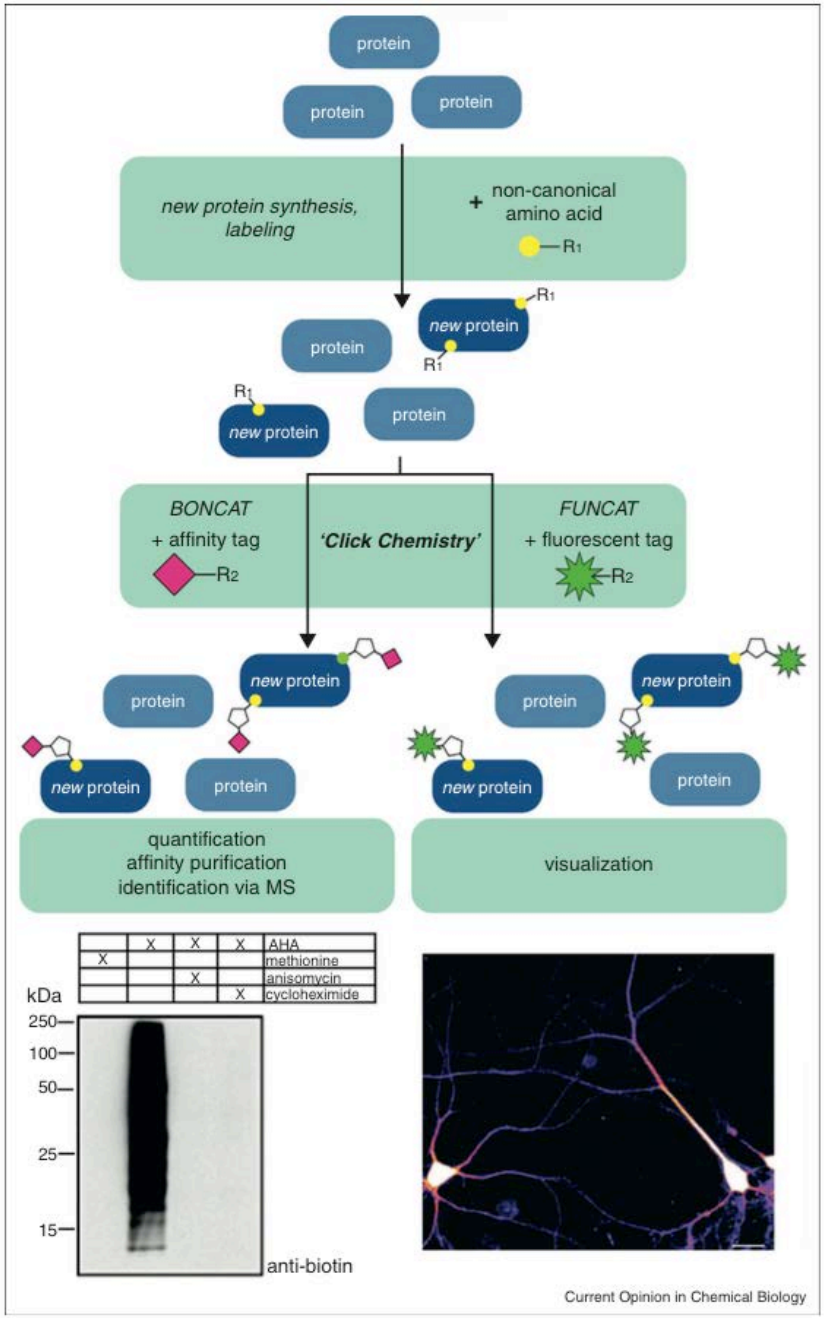
Non-Canonical Amino acid Tagging



Tirrell and coworkers established the use of the azide-bearing non-canonical amino acid azidohomoalanine (AHA) and the alkyne-bearing non- canonical amino acid homopropargylglycine (HPG) as surrogates for methionine which are cotranslationally introduced in newly synthesized proteins.

Azides and alkynes can be covalently linked via selective Cu(I)-catalyzed [3+2] azide-alkyne cycloaddition (termed '**click chemistry**') allowing chemoselective tagging to separate and identify the newly synthesized proteins in mammalian cells.

Incorporation of the azide-bearing amino acid azidohomoalanine is unbiased, not toxic, and does not increase protein degradation


protein
protein
protein
new protein synthesis, labeling
+ non-canonical amino acid
R1
new protein
protein
new protein
protein
BONCAT
+ affinity tag
R2
'Click Chemistry'
FUNCAT
+ fluorescent tag
R2
protein
new protein
new protein
protein
protein
new protein
new protein
protein
quantification
affinity purification
identification via MS
visualization
AHA
methionine
anisomycin
cycloheximide
kDa
250
100
50
25
15
anti-biotin
Current Opinion in Chemical Biology

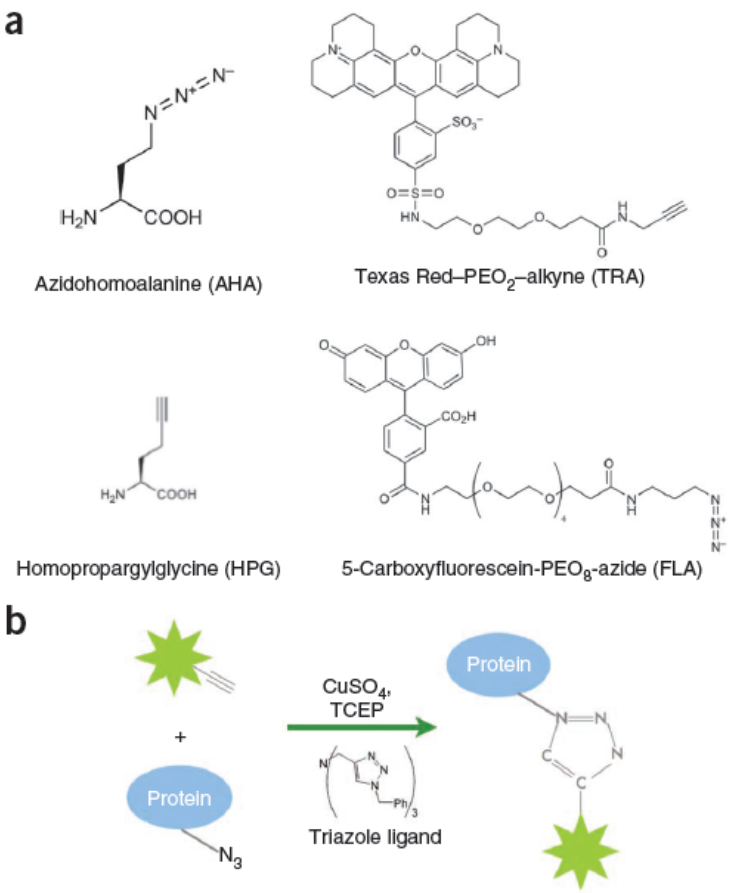
nature neuroscience

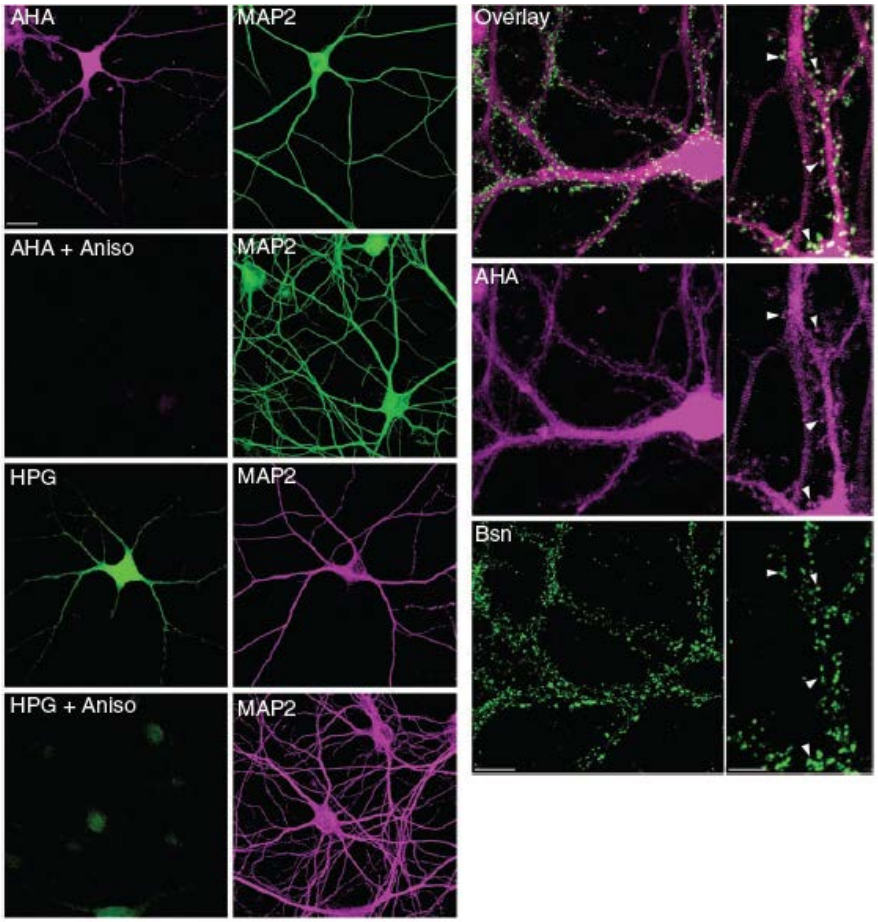
TECHNICAL REPORTS

In situ visualization and dynamics of newly synthesized proteins in rat hippocampal neurons

Daniela C Dieterich[1,2], Jennifer J L Hodas[1], Géraldine Gouzer[3], Ilya Y Shadrin[1], John T Ngo[4], Antoine Triller[3], David A Tirrell[4] & Erin M Schuman[1,5]

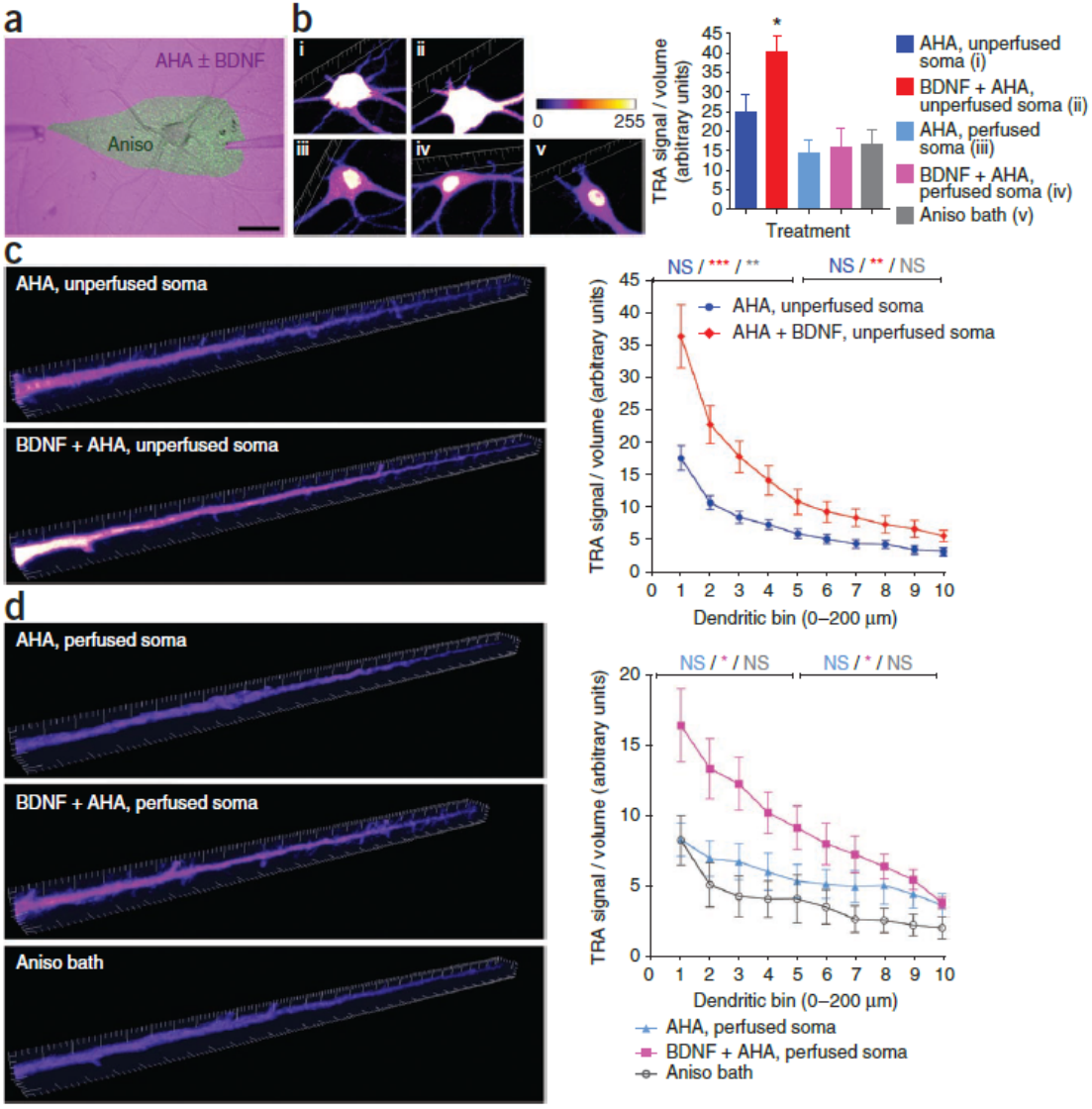
FUNCAT





Exploring the site of translation

BDNF-induced increase of dendritic protein synthesis



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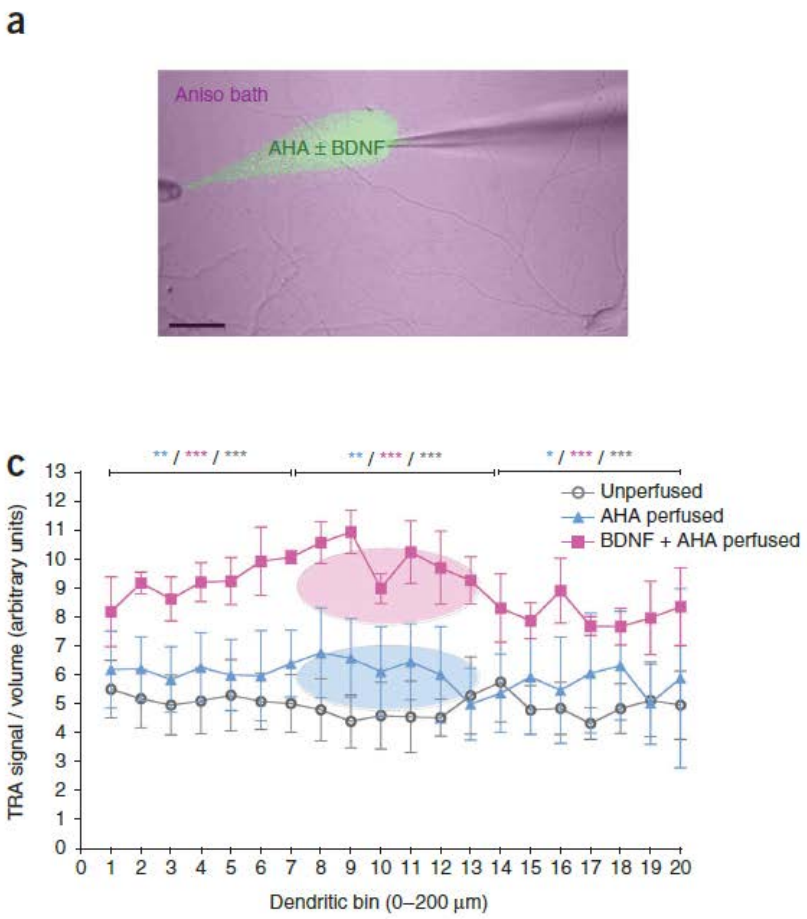

ACS Chemical Neuroscience

Research Article

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Noncanonical Amino Acid Labeling in Vivo to Visualize and Affinity Purify Newly Synthesized Proteins in Larval Zebrafish

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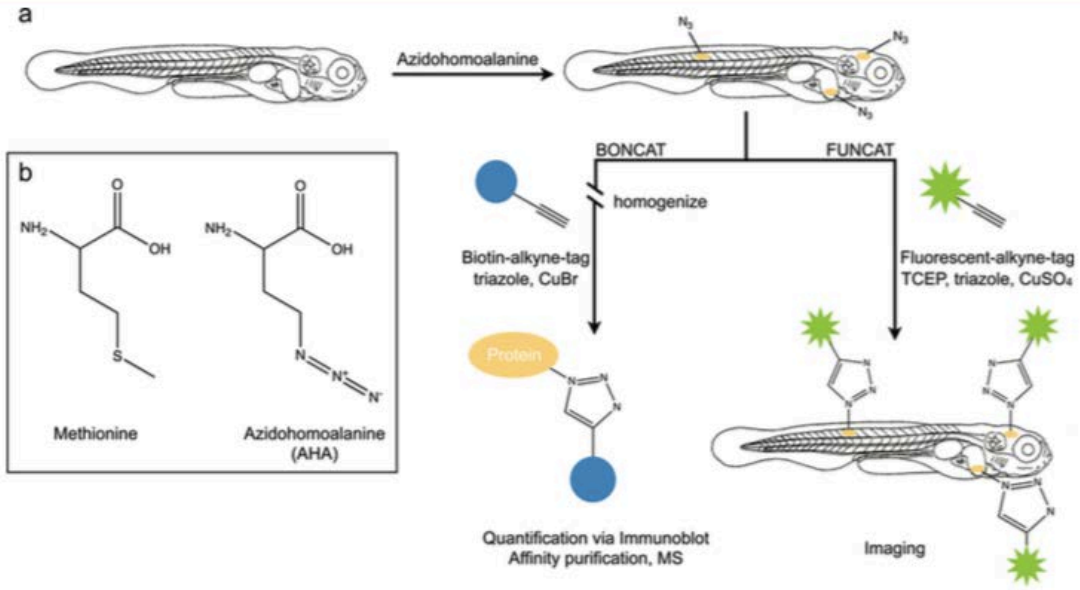
[‡]Max Planck Institute for Brain Research, D-60528 Frankfurt am Main, Germany

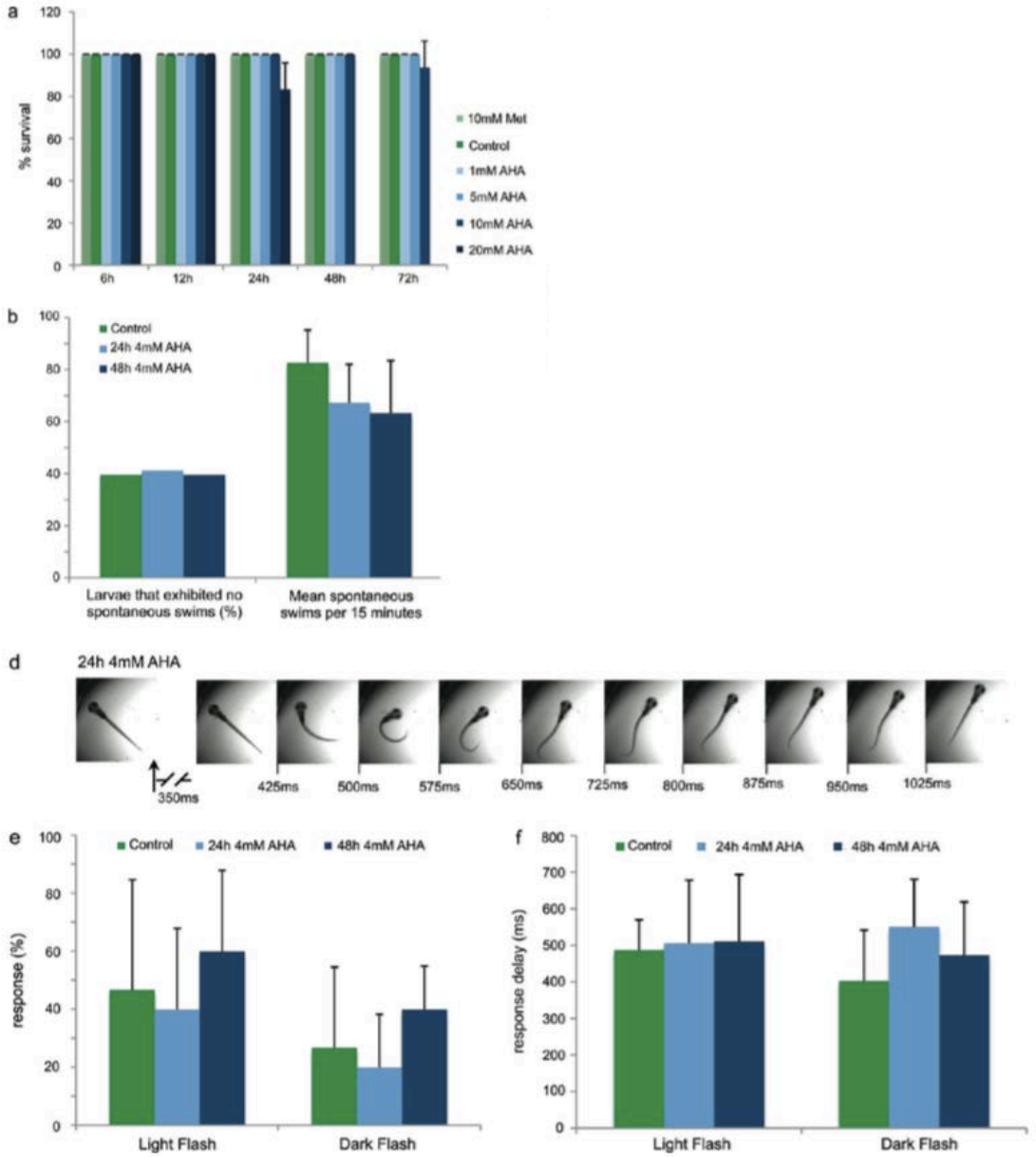
[§]Emmy Noether Research Group Neuralomics, Leibniz Institute for Neurobiology, D-39118 Magdeburg, Germany

[∥]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, United States

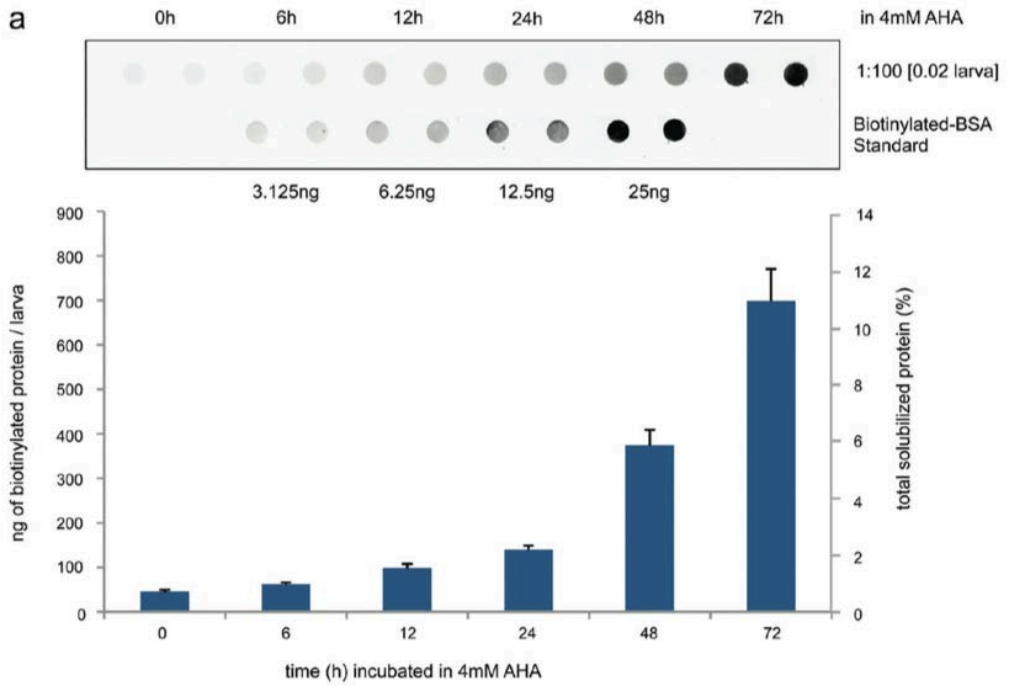


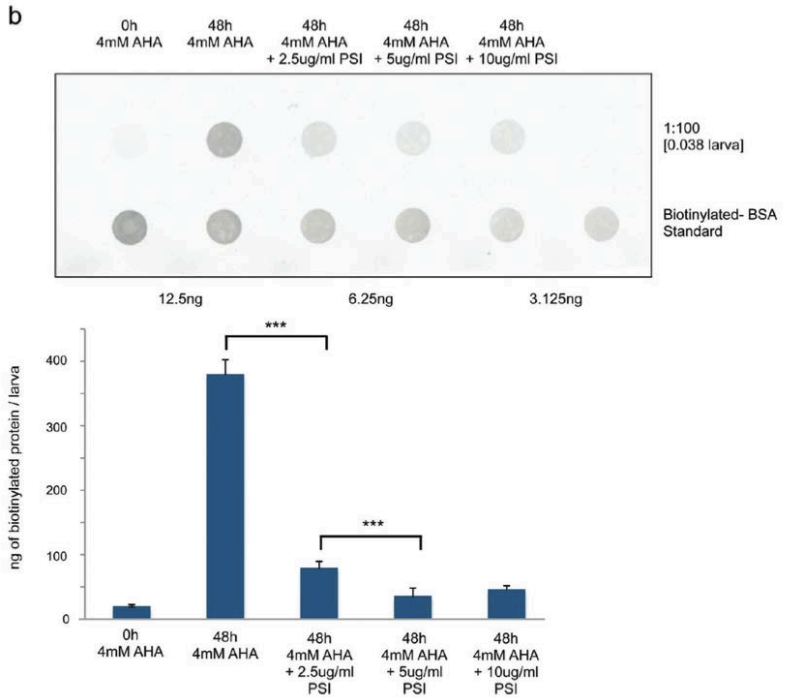
The larval zebrafish: a genetically tractable, simple vertebrate, which is transparent and therefore ideal for imaging. It can absorb small chemical compounds directly from their surrounding medium, all of which make them amendable to chemical screens

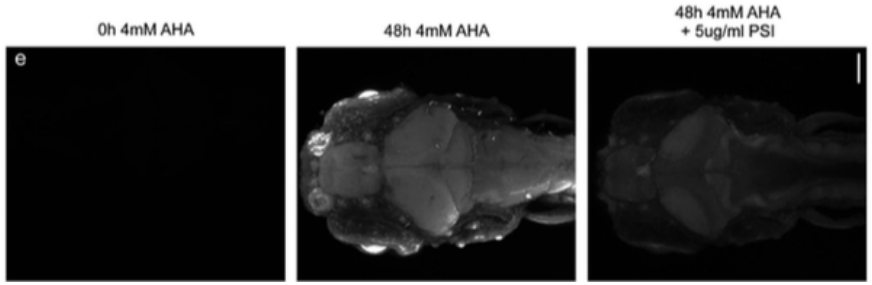
At low concentrations, AHA exposure is not toxic and does not significantly alter simple behaviors



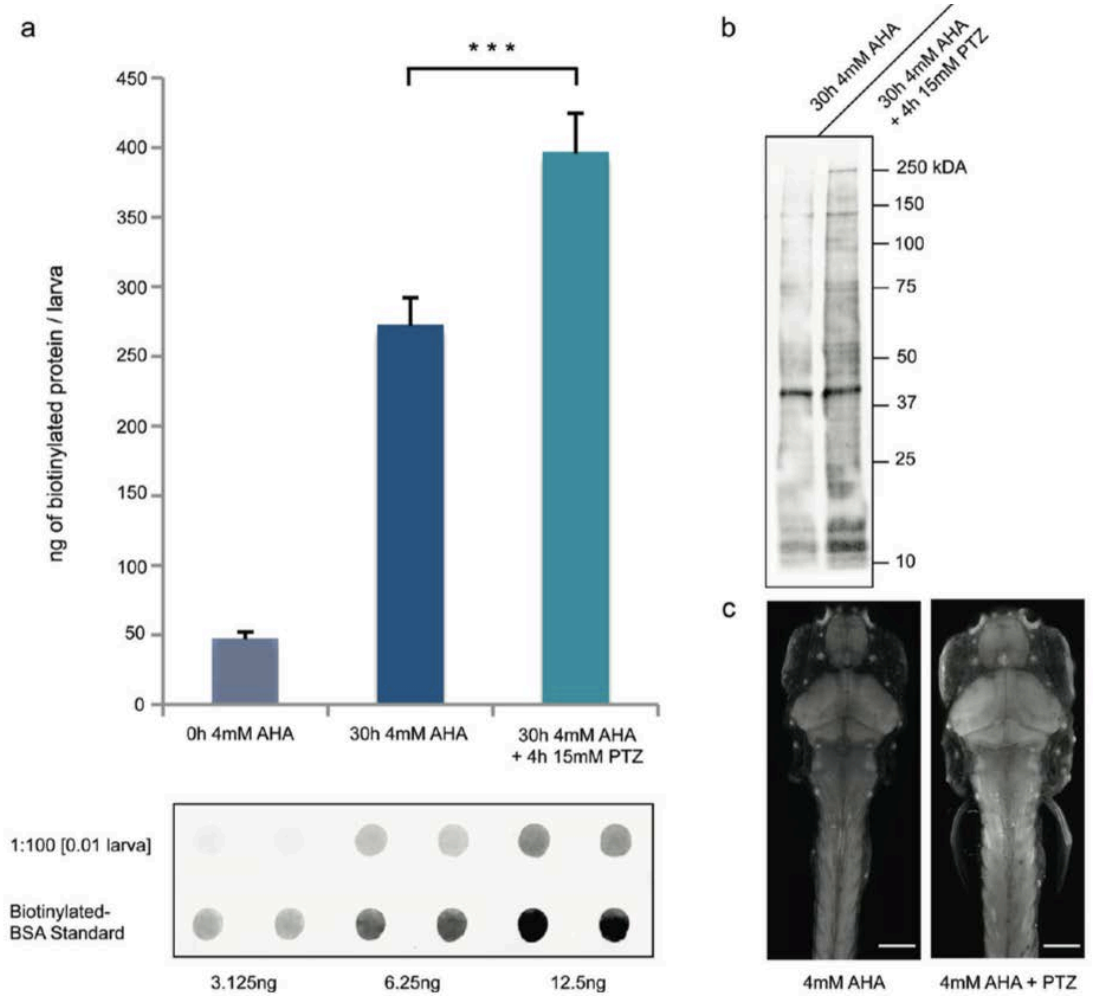
AHA is metabolically incorporated into larval zebrafish proteins in vivo.







GABA antagonist PTZ induces increased protein synthesis in larval zebrafish.



How can we visualize a specific endogenous protein as newly synthesized in situ?

Direct visualization of newly synthesized target proteins *in situ*

Susanne tom Dieck[1,3], Lisa Kochen[1,3], Cyril Hanus[1], Maximilian Heumüller[1], Ina Bartnik[1], Belquis Nassim-Assir[1], Katrin Merk[1], Thorsten Mosler[1], Sakshi Garg[1], Stefanie Bunse[1], David A Tirrell[2] & Erin M Schuman[1]

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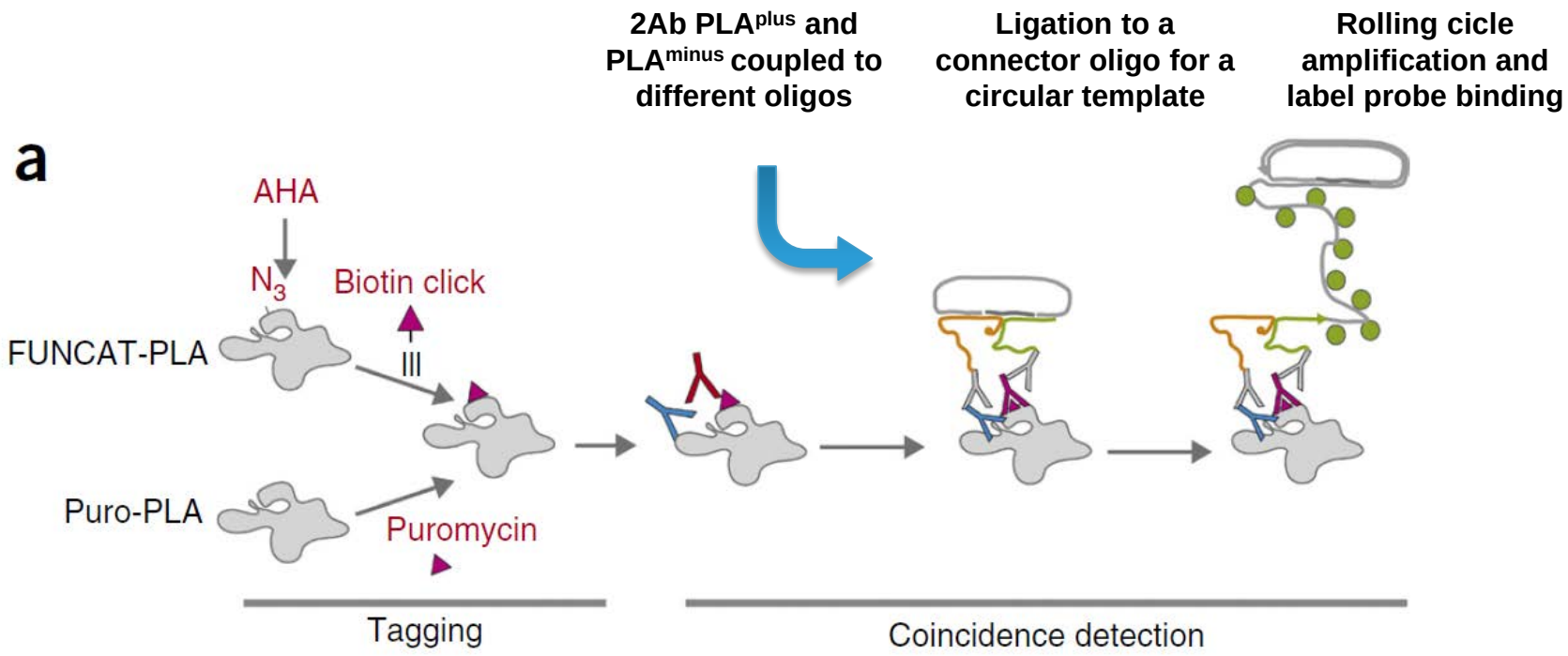
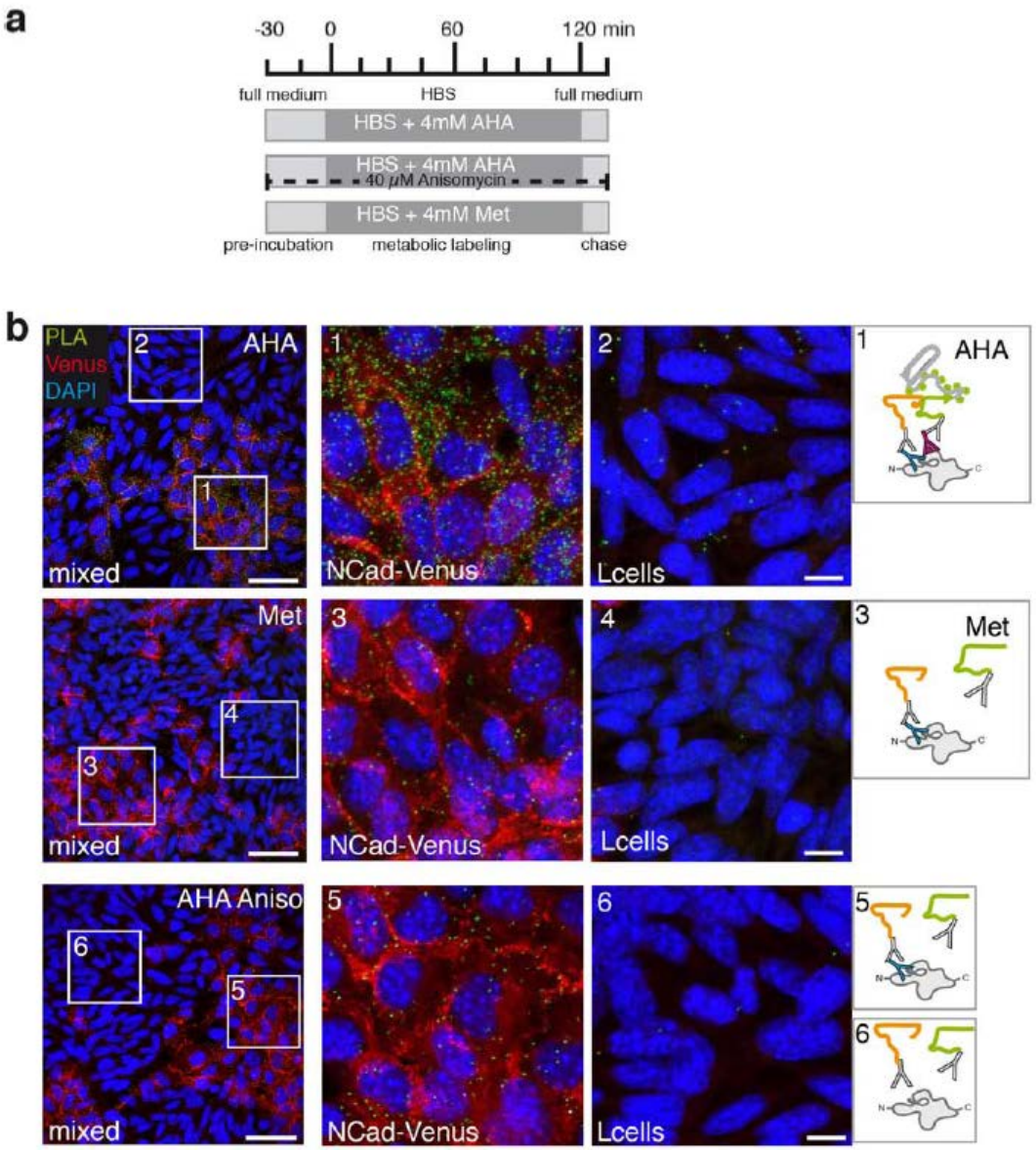
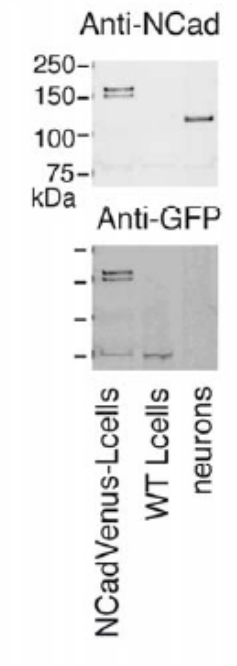
Proximity ligation assay (PLA)-based strategy

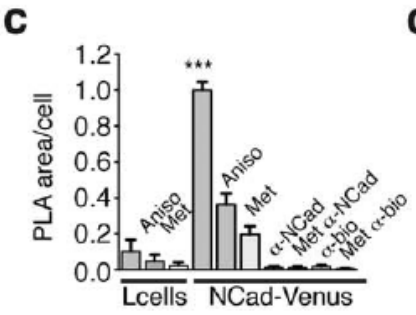


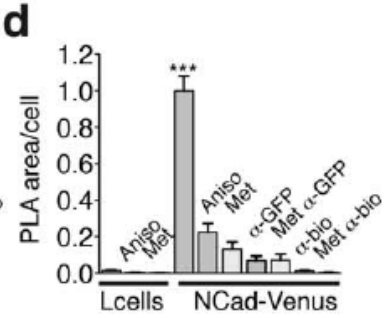
Proximity ligation assay **(PLA)-based strategy detects the spatial coincidence of two antibodies**: one that identifies a newly synthesized protein tagged with either FUNCAT or puromycylation and another that identifies a specific epitope in a protein of interest (POI)

Selectivity and specificity of labeling newly synthesized proteins

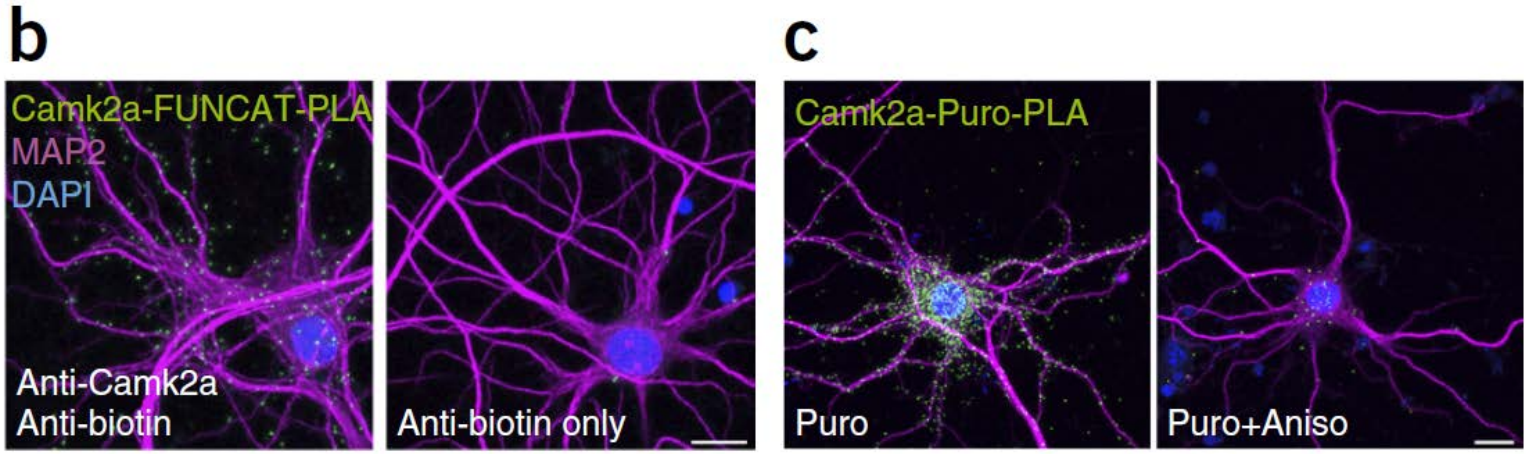


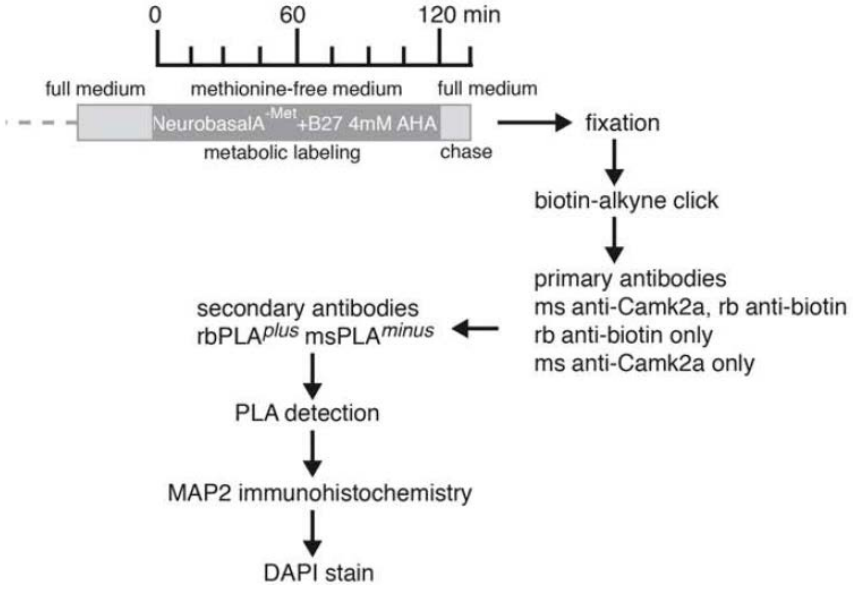


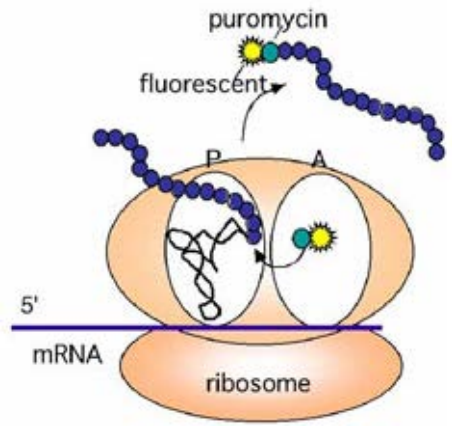




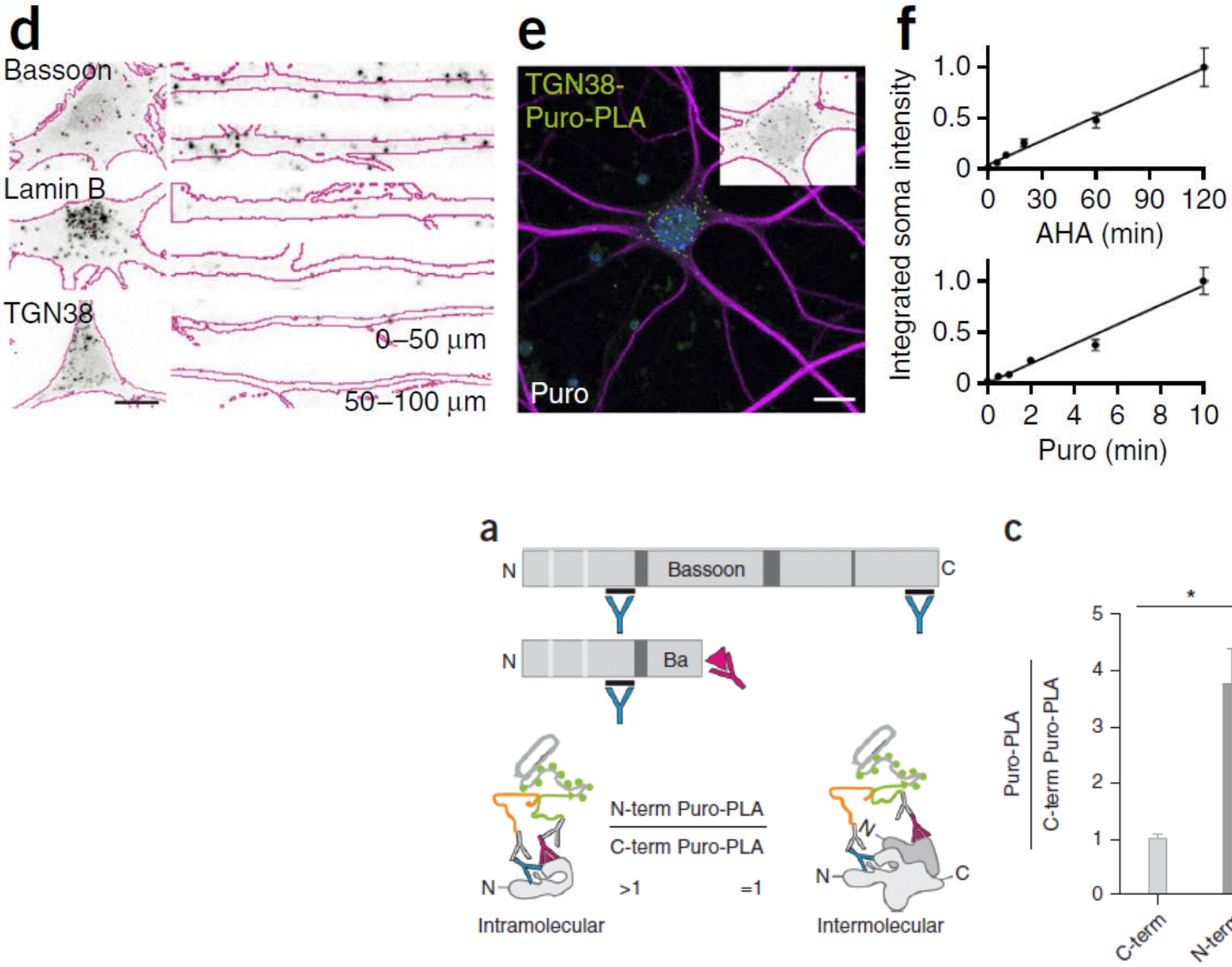
Labeling specific newly synthesized proteins with FUNCAT-PLA and Puro-PLA.



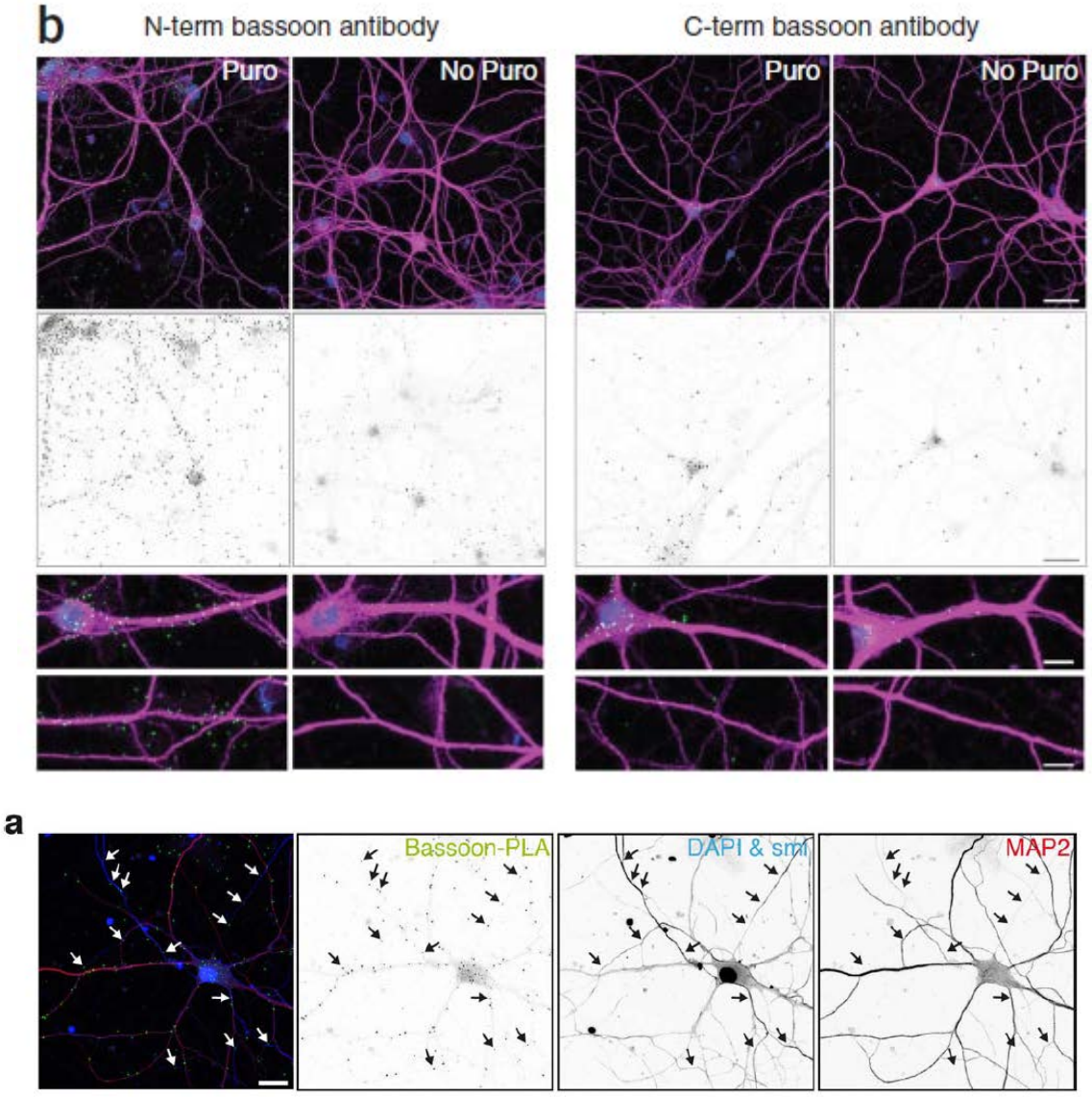




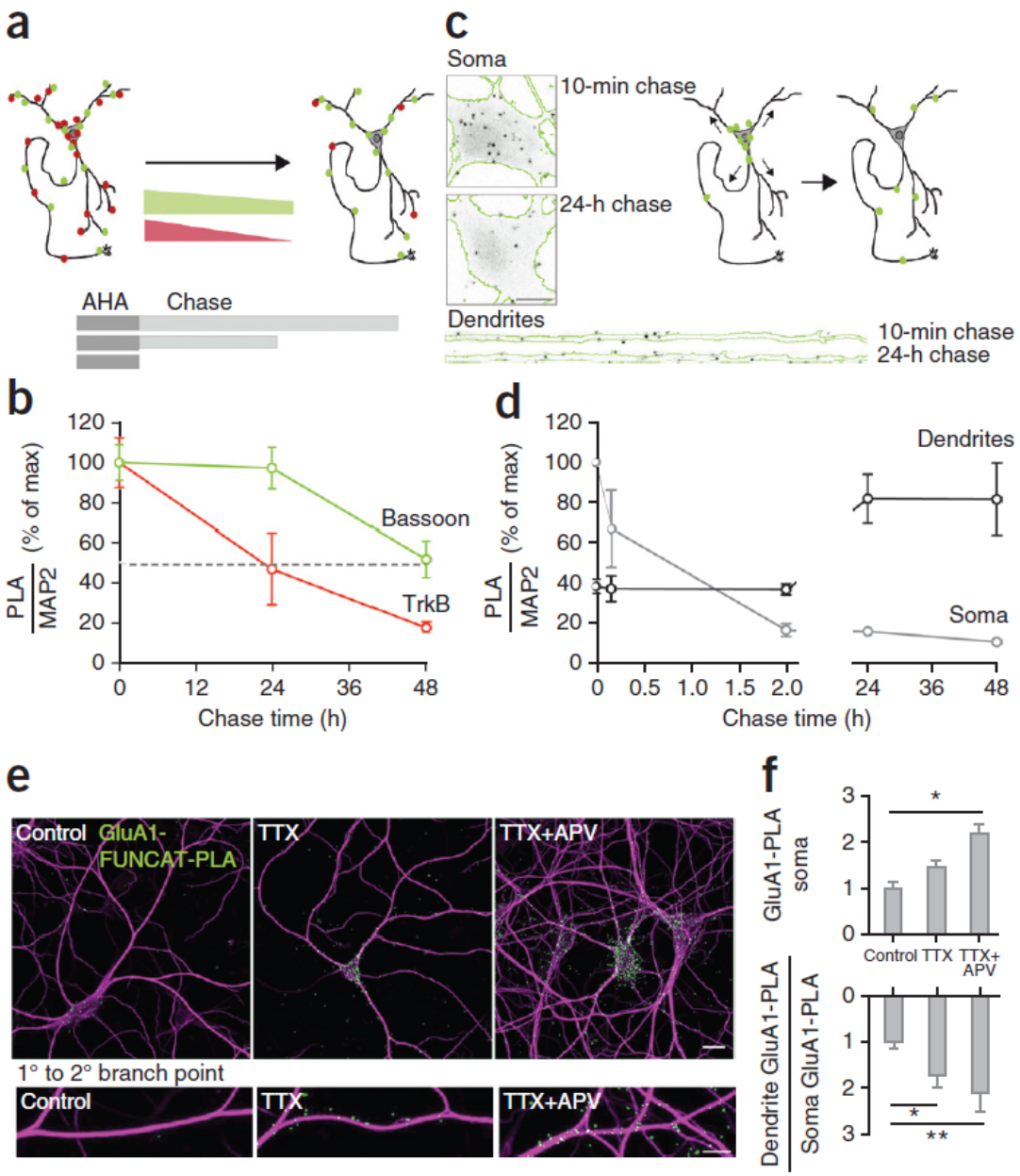
Labeling specific newly synthesized proteins with FUNCAT-PLA and Puro-PLA.



Assessing the site of synthesis of Basson with PURO-PLA and Puro-PLA.



Following protein lifetime, distribution changes and synthesis rate changes with FUNCAT-PLA



Supplementary Table 1

	Puro-PLA	**FUNCAT-PLA**
Incorporation	Fast	Slow
	No activation required	Uptake by amino acid transporter and activation by MetRS needed
	Competition with all activated tRNAs	Competition with methionine
	Labeling in full conditioned medium	Labeling in methionine-free medium
	No starvation necessary	Methionine starvation conditions promote labeling (either with pre-starvation or at least during incubation)
	C-terminal incorporation, possible at any site but limited to one Puromycin per protein	Only replacement of methionine residues possible, more than one methionine replacement possible per protein
Protein	Full labeling (= one puromycin per protein) would lead to protein synthesis block	Full labeling in theory possible
	Truncated, premature termination of labeled protein	Full length protein with small bio-orthogonal groups
	Enhanced degradation/ turnover of truncated proteins expected, non-physiological protein fate	Physiological fate
Method characteristics	Fast, sensitive	Lag phase, especially when used without methionine starvation, less sensitive than Puro-PLA
	Short labeling, unlikely to influence short term physiology	Labeling conditions might impact short term physiology, but also see Supplementary Figure 7
	Puro antibodies needed N-terminal POI antibodies are predicted to work better than C-terminal	Biotin antibodies needed
		Additional step required (biotin click)
	Estimating intra- vs intermolecular detection with N-/C-term antibody against POI possible	Modification by direct click of a PLA oligo possible

Vibrational imaging of newly synthesized proteins in live cells by stimulated Raman scattering microscopy

Lu Wei[a], Yong Yu[b,c], Yihui Shen[a], Meng C. Wang[b,c,1], and Wei Min[a,d,1]

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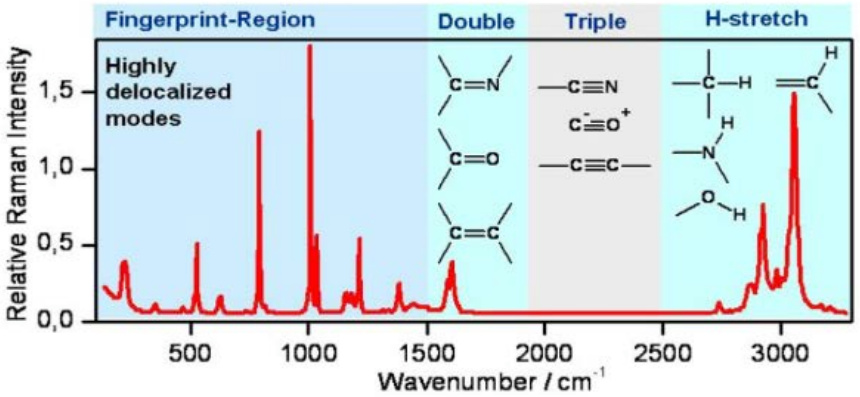
Edited by David A. Tirrell, California Institute of Technology, Pasadena, CA, and approved May 31, 2013 (received for review February 27, 2013)

Raman microscopy

It enables chemical imaging. It is based on the Raman scattering effect of molecules that was discovered by C.V. Raman in the early 1930s.

When monochromatic light is shined on a molecule, it can be inelastically scattered and gives off light at lower energy. All molecules have specific Raman signatures typically spanning from 100 cm^{-1} to 3500 cm^{-1}.

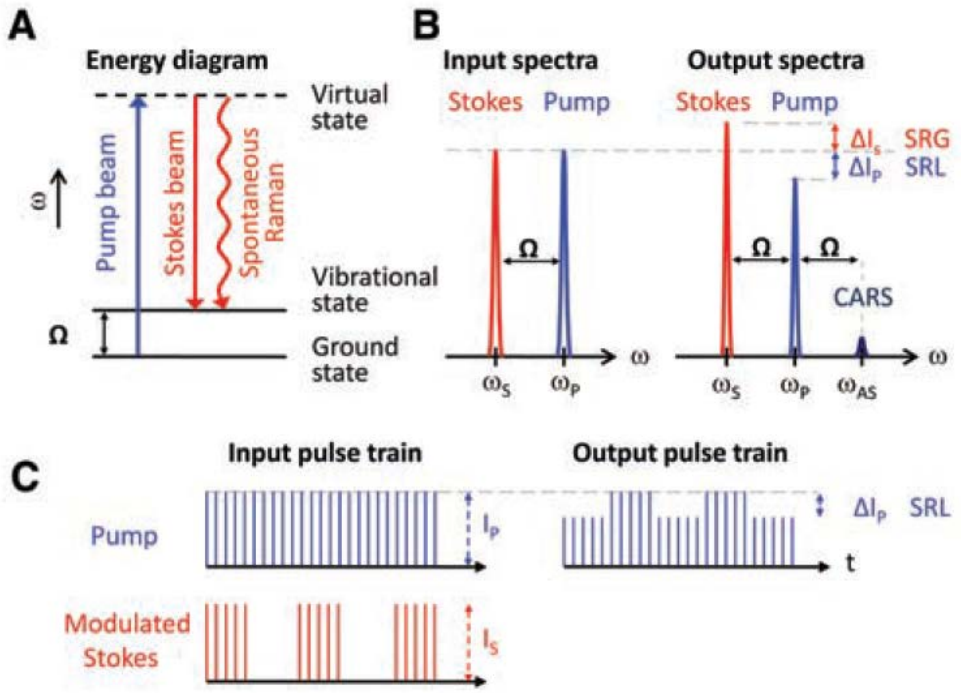


Because different chemical functional groups scatter light at different frequencies, Raman spectroscopy can be used as a tool for chemical structure analysis, chemical fingerprinting and chemical imaging.

The Raman spectrum is highly dependent on the chemical structure, but almost unaffected by the local environment, of the molecule. Therefore, it is not only specific, but also quantitative.
Spontaneous Raman microscopy provides specific vibrational signatures of chemical bonds, but is often hindered by low sensitivity.

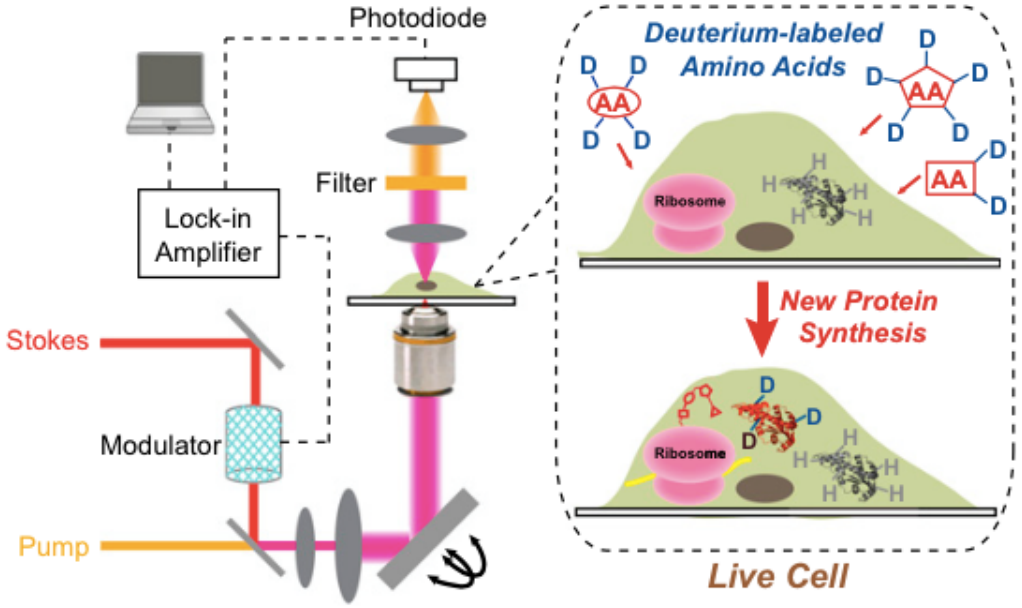
Stimulated Raman scattering (SRS)

The sensitivity of SRS imaging is significantly greater than that of spontaneous Raman microscopy, which is achieved by implementing high-frequency (megahertz) phase-sensitive detection.



Stimulated Raman scattering (SRS) microscopy set up

Lock-in amplifiers are used to detect very small signals even when the small signal is obscured by noise sources many thousands of times larger. Lock-in amplifiers use a technique by which noise signals, at frequencies other than the reference frequency, are rejected and do not affect the measurement.



Inverted laser scanning microscope optimized for IR throughtput

For the visualization of nascent proteins in live cells, it is coupled through metabolic incorporation of **deuterium- labeled amino acids**. Newly synthesized proteins are imaged via their **unique vibrational signature of carbon–deuterium bonds (C–D)**.

SRS microscopy advantages

- in comparison with fluorescence microscopy it is label-free, i.e. it does not require fluorophores, allowing the study of unaltered cells and tissues;

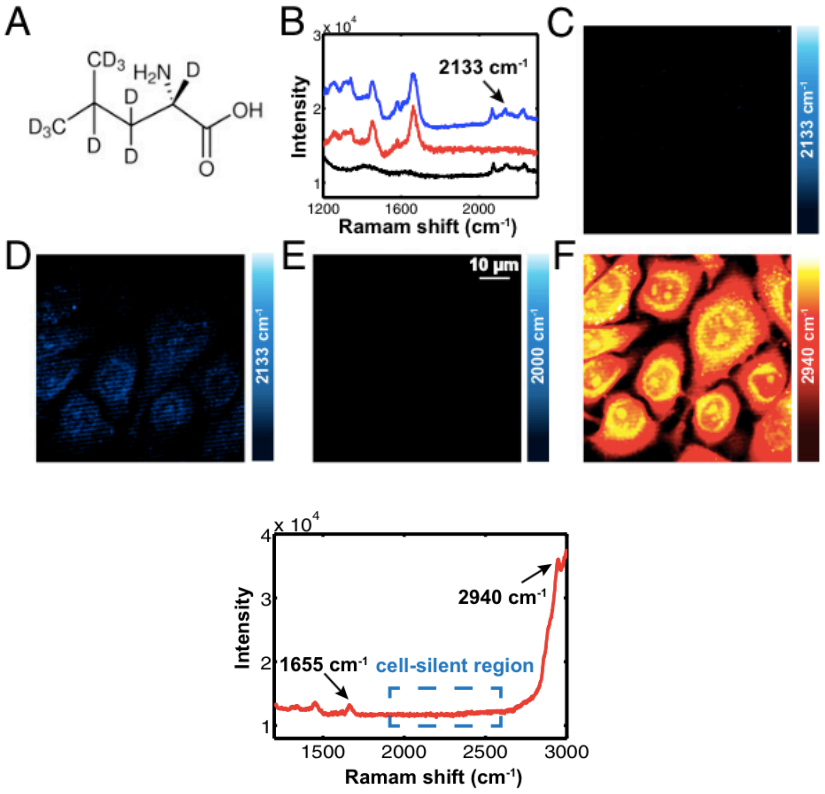
- it typically works out of resonance, i.e. without population transfer into electronic excited molecular states, thus minimizing photobleaching and damage to biological samples;

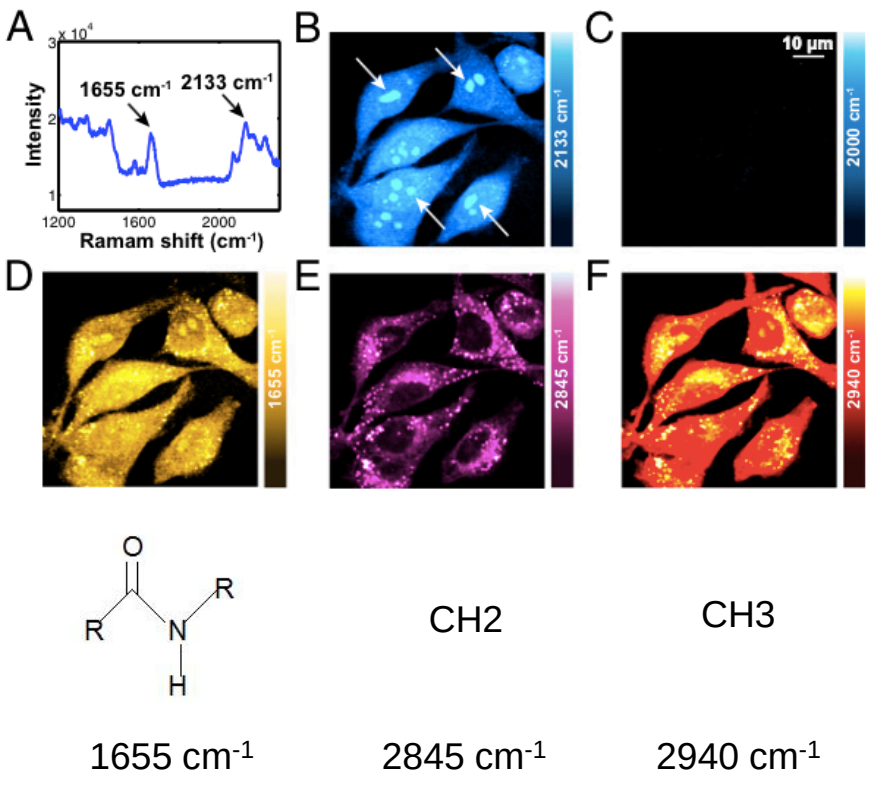
- since CRS exploits a coherent superposition of the vibrational responses from the excited oscillators, it is considerably more sensitive than spontaneous Raman microscopy, allowing extremely higher imaging speeds, up to the video rate;

- being a nonlinear microscopy techniques, with the signal generation confined to the focal volume, it exhibits a three-dimensional sectioning capability similar to that of multiphoton fluorescence microscopy;

- the use of near-infrared excitation (700-1200 nm) has the advantage of a high penetration depth, which allows imaging through thick tissues, and a low phototoxicity, minimizing multi-photon absorption induced damage.

SRS Imaging of Newly Synthesized Proteins by Metabolic Incorporation of Leucine-d_{10} in Live HeLa Cells





CH2

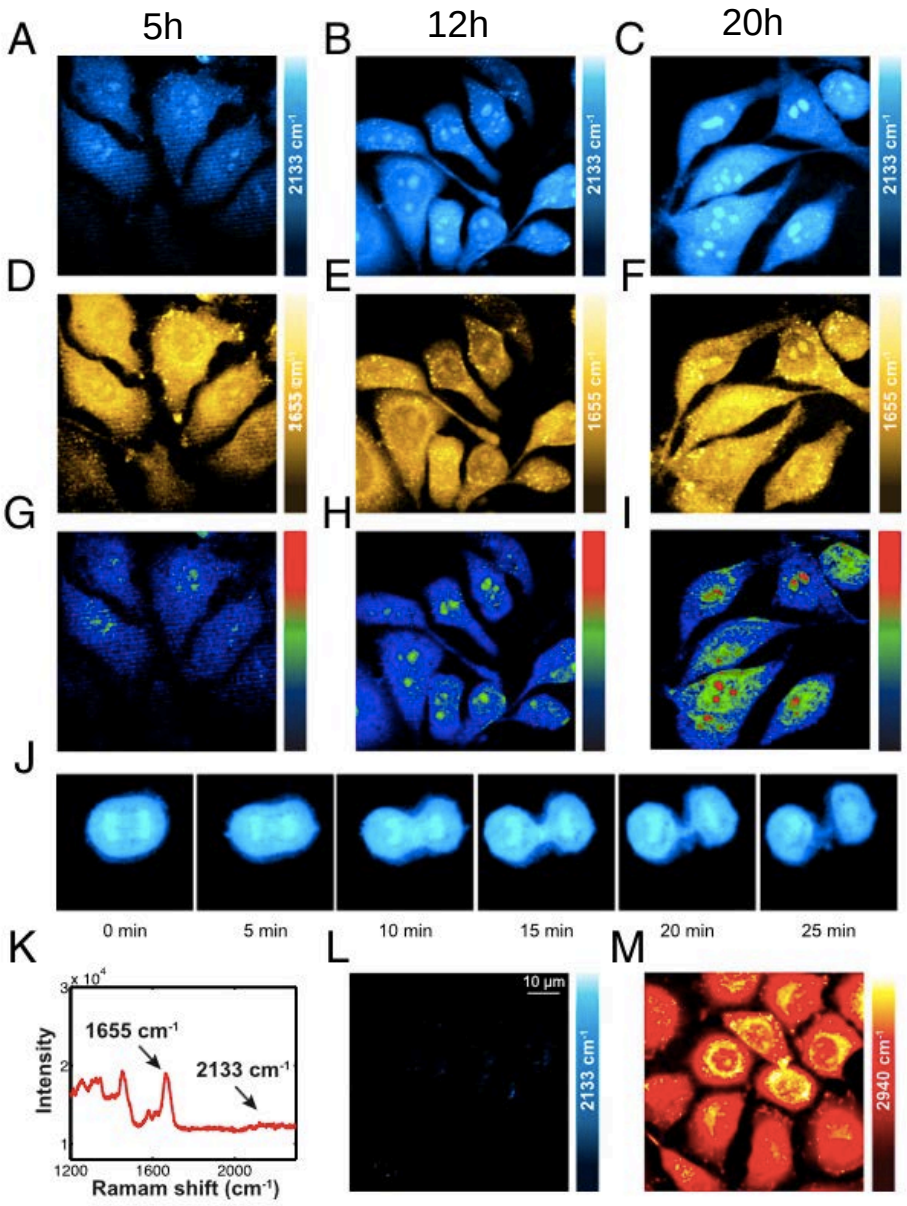
CH3

1655 cm^{-1}

2845 cm^{-1}

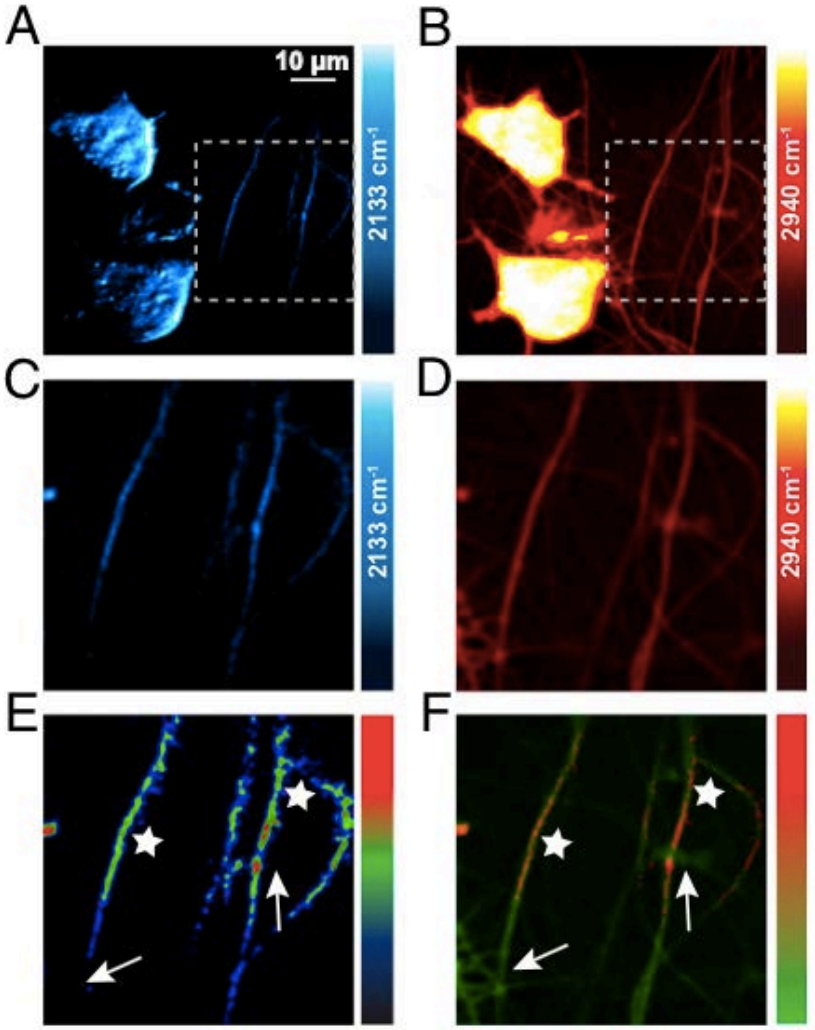
2940 cm^{-1}

Time-Dependent de Novo Protein Synthesis and Protein Synthesis Inhibition.



12h, DAA medium
Plus 5uM anisomycin

SRS imaging of newly synthesized proteins in both cell bodies and newly grown neurites of differentiable mouse neuroblastoma (N2A) cells



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Imaging Complex Protein Metabolism in Live Organisms by Stimulated Raman Scattering Microscopy with Isotope Labeling

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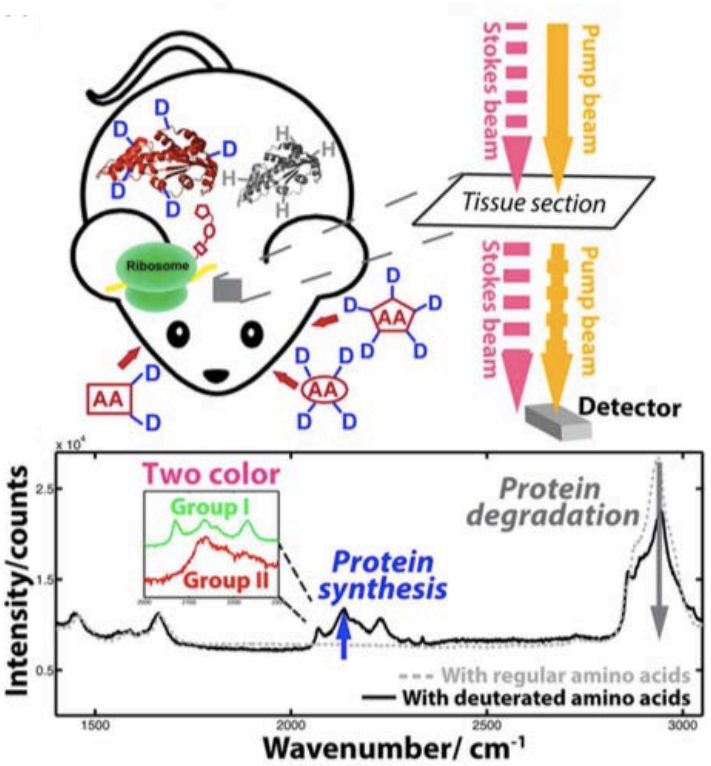
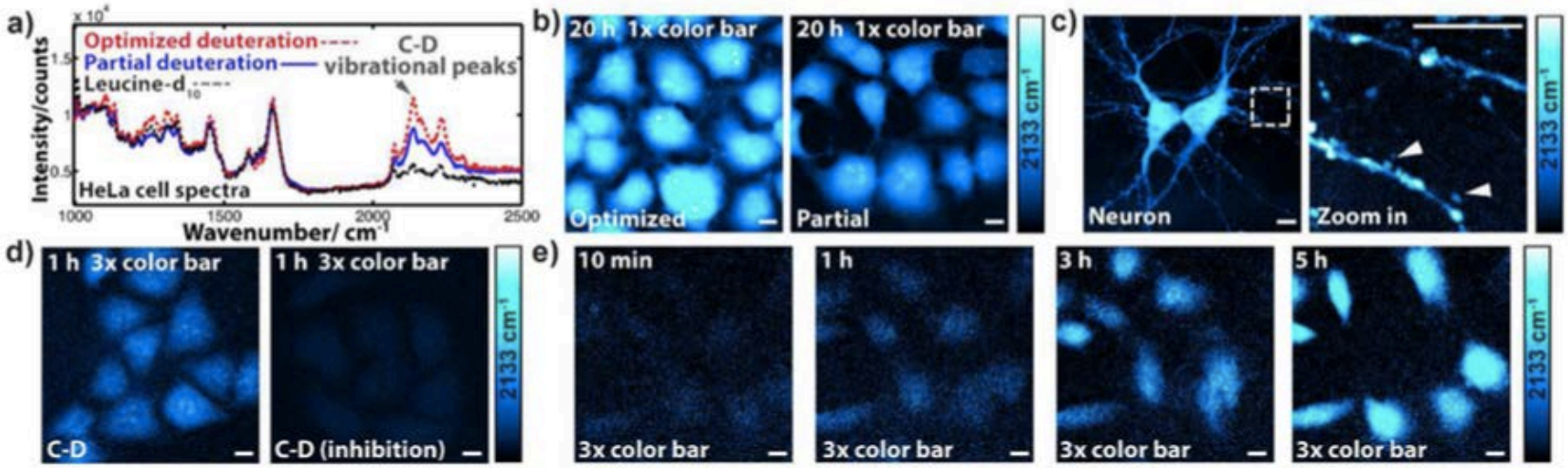
[‡]Department of Pediatrics, Columbia University, New York, New York 10032, United States



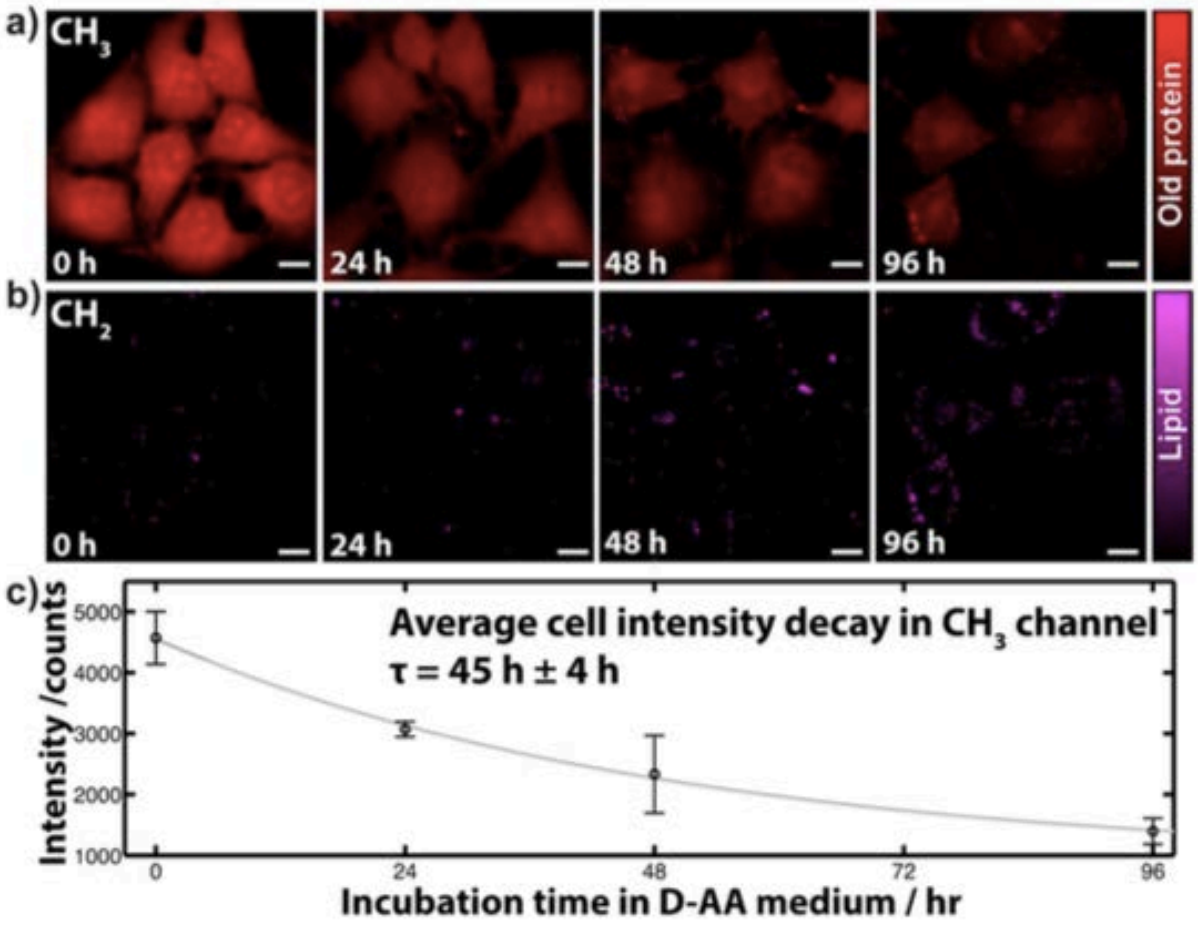
- ✓ Electro optic modulator
- ✓ Amplifier
- ✓ DAA-medium

Sensitivity Optimization and Time-Lapse Imaging of de Novo Proteome Synthesis Dynamics

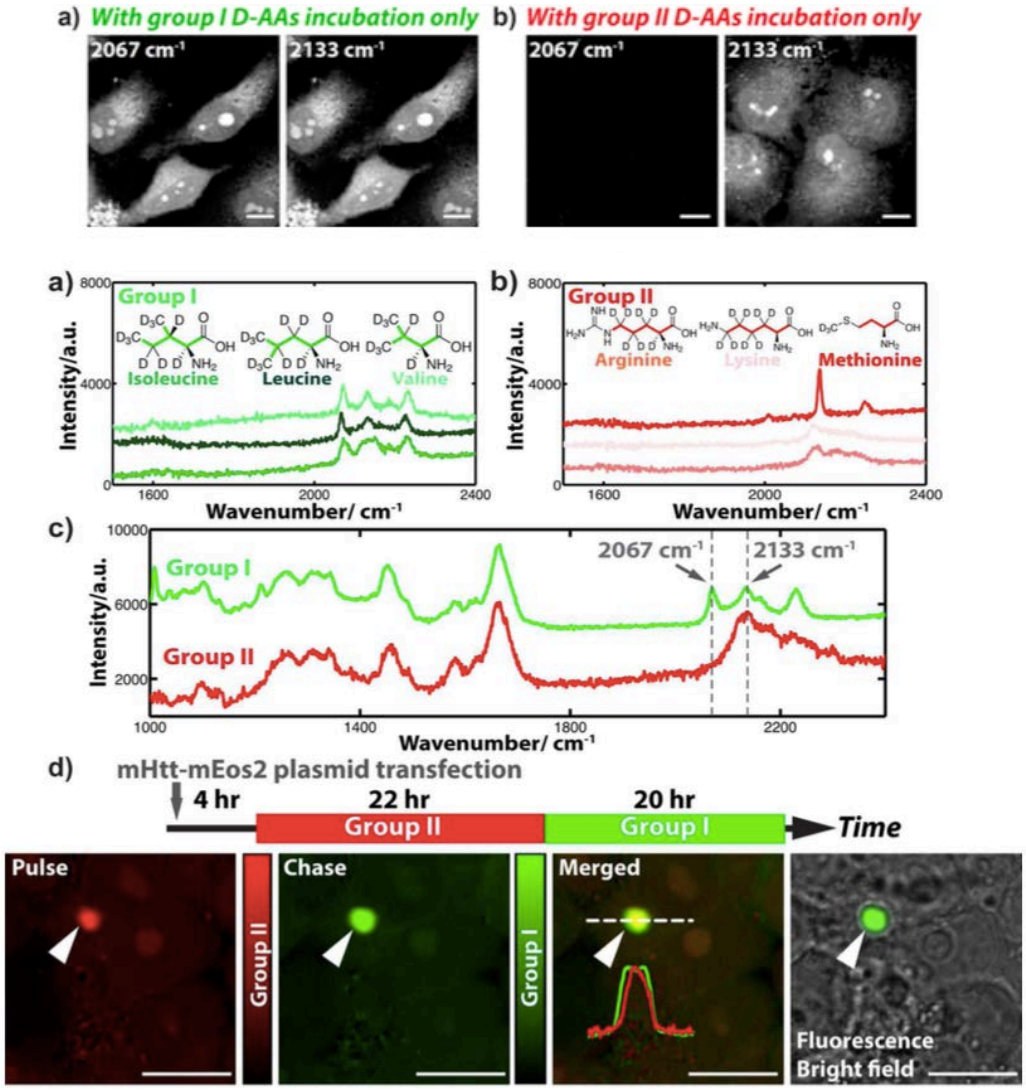


Long term time lapse imaging

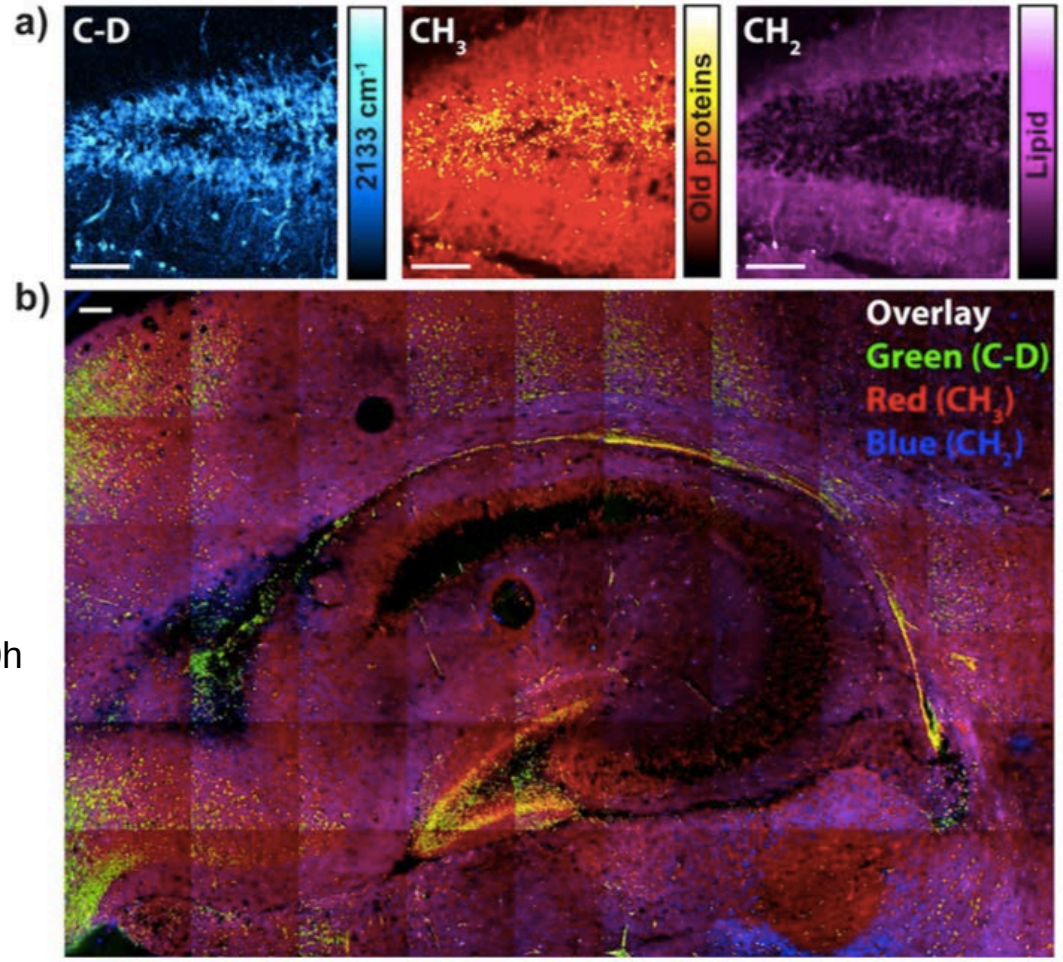
Time-dependent SRS imaging of protein degradation in live HeLa cells



Two-Color Pulse−Chase SRS Imaging of Two Sets of Temporally Defined Proteins



SRS Imaging of Newly Synthesized Proteins in Live Mouse Brain Tissues

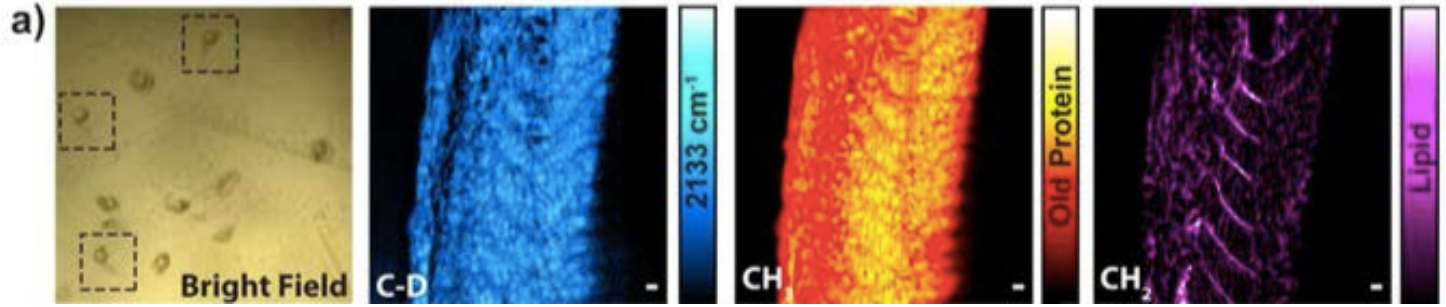


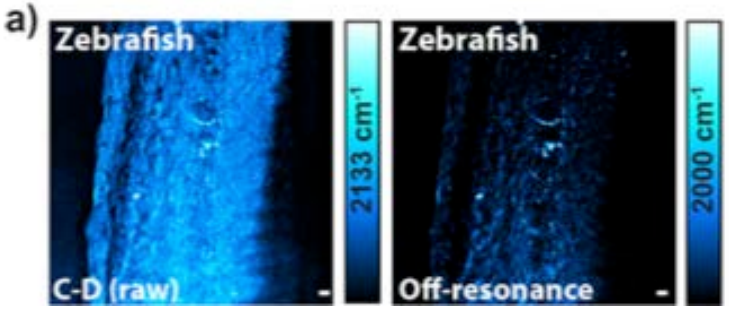
D-AA culture for 30h

SRS Imaging of Newly Synthesized Proteins *in Vivo*

150 ng/ul D-AA in
1 cell embryo

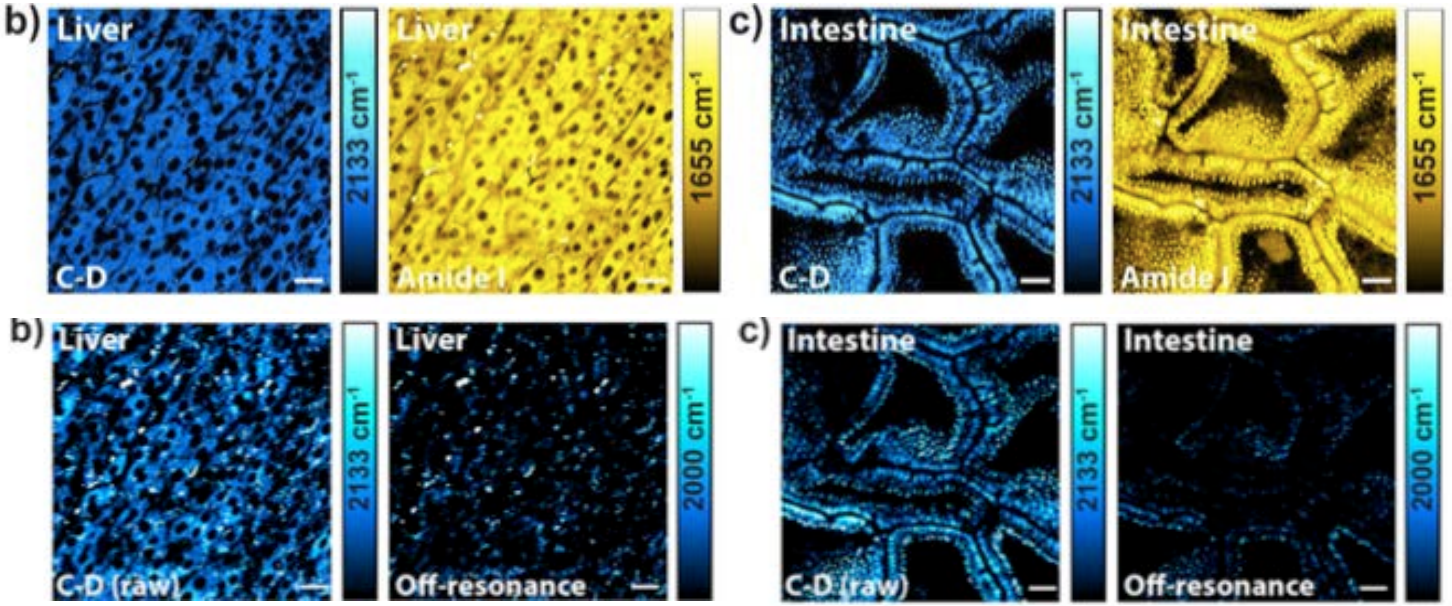
Imaging 24h later





SRS Imaging of Newly Synthesized Proteins *in Vivo*

D-AA in water or
IP injection



SRS microscopy

- ✓ Biocompatible
- ✓ Live imaging (video rate, 3s/frame)
- ✓ Low backgroung, high sensitivity

- ➢ Relatively long incubation time compared to FUNCAT-PLA
- ➢ Non selective for a specific target protein

Tag your newly generated ideas and track the good ones

Thank you for your attention!