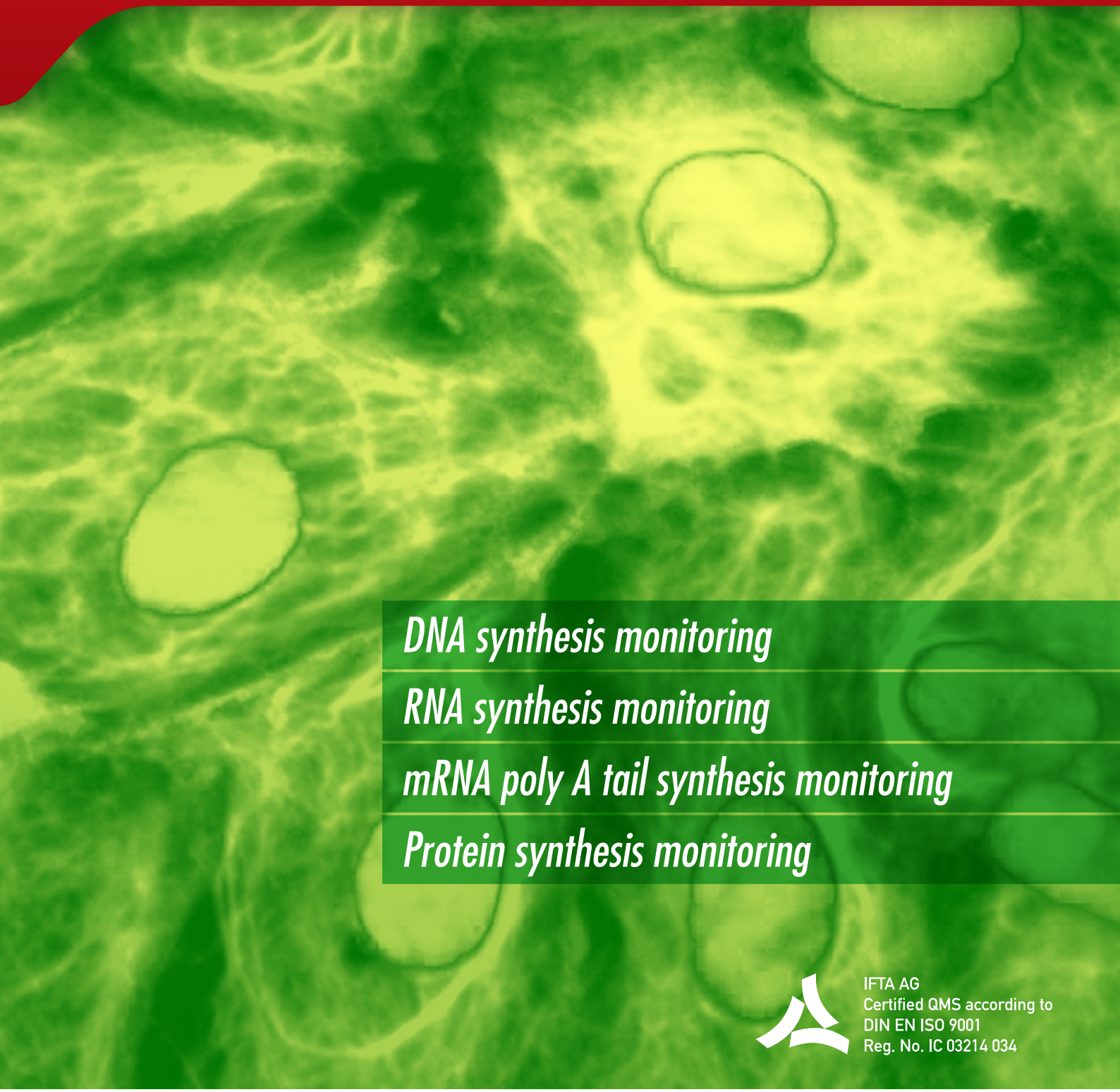
CLICK-labeling of cellular metabolites



DNA synthesis monitoring

RNA synthesis monitoring

mRNA poly A tail synthesis monitoring

Protein synthesis monitoring

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CLICK-labeling of cellular metabolites

The dynamics of global *de novo* DNA, RNA and protein synthesis are critical parameters to analyze the cellular response under various physiological and pathological conditions.

Traditional analyses rely on radioactivity or tedious Br(d)U-/antibody-based experiments that raise safety issues, are limited with regard to their read out options or require a detection procedure that is destructive to cell morphology thus preventing subsequent analysis. These drawbacks can be circumvented by Click Chemistry-based labeling methods that provide a superior alternative to traditional global DNA, RNA and protein synthesis monitoring approaches.

Principle of Click Chemistry-based labeling of cellular metabolites

Click Chemistry[1] describes pairs of functional groups that rapidly and selectively react ("click") with each other under mild, aqueous conditions. The principle of Click Chemistry-based labeling of cellular metabolites relies on the Cu(I)-catalyzed terminal Alkyne-Azide Click Chemistry (CuAAC) reaction: A terminal Alkyne-functionalized molecule A is intracellularly incorporated into a metabolite (DNA, RNA or protein) and the terminal Alkyne-functionalized metabolite is subsequently visualized by an Azide functionalized detection molecule B (Fig. 1).

Terminal Alkyne-functionalized molecules are ideally suited for the intracellular functionalization of metabolites (DNA, RNA, protein) through various enzymatic pathways due to their cell-permeability and excellent substrate properties. The detection via small-sized Azide-functionalized labels allows a flexible readout (Biotin or FLAG tag labeling for subsequent purification tasks or fluorescent labeling for subsequent microscopic imaging) with minimal background staining.

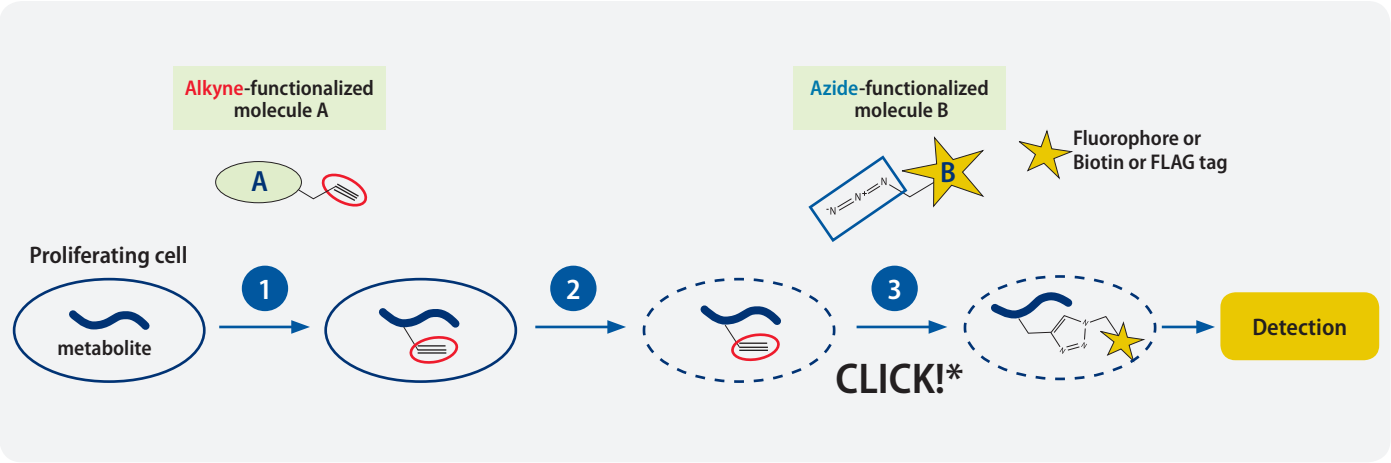


Figure 1 Cellular metabolites can be detected by a three-step procedure: **1)** Intracellular Alkyne-functionalization of metabolites, **2)** Cell fixation & permeablilization, **3)** CuAAC*-mediated CLICK-labeling via an Azide-functionalized detection molecule.

**CuAAC: (Cu(I))-catalyzed terminal Alkyne-Azide Click Chemistry reaction*

Since terminal Alkynes are fairly unreactive towards Azides, the efficiency of a CuAAC reaction strongly depends on the presence of a metal catalyst such as copper (Cu) in the +1 oxidation state (Cu(I)).

Different copper sources and reduction reagents are available however, the Cu(II) salt $CuSO_4$ as copper source in combination with sodium ascorbate as reduction reagent has been recommended for most biomolecule labeling applications. Potential Cu(I) cytotoxicity is partially overcome by the use of Cu(I) chelating ligands such as THPTA that serve a dual purpose: 1) Acceleration of the CuAAC reaction by maintaining the Cu(I) oxidation state and 2) Protection of the biomolecule from oxidative damage.

Presolski *et al.*[2] and Hong *et al.*[3] provide a general protocol for Cu(I)-catalyzed click chemistry reactions that may be used as a starting point for the set up and optimization of individual assays.

Selected References:

[1] Kolb *et al.* (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed.* **40(11)**:2004.

[2] Presolski *et al.* (2011) Copper-Catalyzed Azide-Alkyne Click Chemistry for Bioconjugation. *Current Protocols in Chemical Biology* **3**:153.

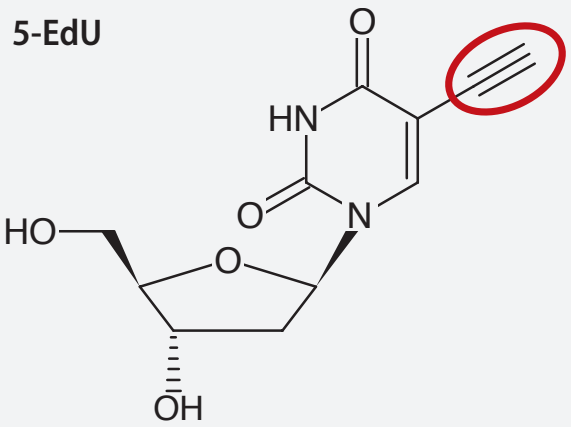
[3] Hong *et al.* (2009) Analysis and Optimization of Copper-Catalyzed Azide-Alkyne Cycloaddition for Bioconjugation. *Angew. Chem. Int. Ed.* **48**:9879.

DNA synthesis monitoring with 5-EdU

5-Ethynyl-2'-deoxyuridine (5-EdU) (Fig. 2) can be used as a replacement for ^{3}H-thymidine and 5-Bromo-2'-deoxyuridine (5-BrdU) to measure the global *de novo* DNA synthesis during the S-phase of the cell cycle[1,2] The cell-permeable 5-EdU is intracellularly metabolized into its triphosphate form via the nucleotide salvage pathway followed by incorporation into the replicating DNA by cellular polymerases instead of its natural analog thymidine.

The resulting ethynyl-functionalized DNA can be detected via Cu(I)-catalyzed click chemistry by introduction of

- a Biotin group (via Azides of Biotin)
- a FLAG-tag (via FLAG-Azides) for purification or
- a fluorescent group (via Azides of fluorescent dyes) for microscopic imaging.



Features:

- non-destructive & therefore suitable for multiparametric analysis (detection with small-sized labeled Azides
- no harsh permeabilization & nucleic acid denaturation required as for the antibody-based 5-BrdU detection
- significantly faster detection procedure (~ 2 h instead of 4 h+)
- compatible with flow cytometry & microscopy[1,2]

Figure 2 5-EdU (5-Ethynyl-2'-deoxyuridine) bypasses limitations of 5-BrdU-based monitoring. Red: Terminal Alkyne group.

Table 1: Selected cell lines & organisms analyzed with 5-EdU

Cell line / Organism	Final 5-EdU concentration
HeLa cells	10 µM[1]
CEM cells	20 µM[2]
BT474 cells	0.1-20 µM[3]
Jurkat cells	10 µM[4]
NIH3T3 cells	10 µM[1]
SK-BR-3 cells	0.1-10 µM[3]
Primary human fibroblast 48BR (normal), 1BR (normal), XP15BR (XP-A), XP20BE (XP-G), XP13BR (XP-C), XP12BR (XP-D)	10 µM[5]
Mouse embryo	10-200 mg/kg[6]
Chicken embryo	500 µM[7]
Primary valvular interstitial cells	10 µM[8]
Drosophila (neuroblasts, salivary glands, and wing discs)	15 µM[9]
Plants (Alfalfa, *Arabidopsis*, grape, maize, rice and tobacco)	10 µM[10]

Selected References:

[1] Salic *et al.* (2008) A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad.Sci.* USA **105**: 2415.
[2] Diermeier-Daucher *et al.* (2009) Cell Type Specific Applicability of 5-Ethynyl-2'-deoxyuridine (EdU) for Dynamic Proliferation Assessment in Flow Cytometry. Cytometry A **75A**:535
[3] Hamelik *et al.* (2009) Click-iTTM Assay with Improved DNA Distribution Histograms. *Cytometry* A **75A**:862.
[4] Buck *et al.* (2008) Detection of S-Phase cell cycle progression using 5'-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5'-bromo-2'-deoxyuridine. *Biotechniques* **44(7)**:927.
[5] Limsirichaikul *et al.* (2009) A rapid non-radioactive technique for measurement of repair synthesis in primary human fibroblasts by incorporation of ethynyl deoxyuridine (EdU). *Nucleic Acids Res.* **37(4)**:e31.
[6] Zeng *et al.* (2010) Evaluation of 5-ethynyl-2'-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. *Brain Research.* **1319**:21.
[7] Warren *et al.* (2009) Chick embryo proliferation studies using EdU labeling. *Dev Dyn.* **238(4)**:944.
[8] Monzack *et al.* (2012) A time course investigation of the statin paradox among valvular interstitial cell phenotypes. *American Journal of Physiology.* **303(7)**:H903.
[9] Gouge *et al.* (2010) Detection of S Phase in multiple Drosophila tissues utilizing the EdU labeling technique. *Dros. Inf. Serv.* **93**:213.
[10] Kotogány *et al.* (2010) A rapid and robust assay for detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl deoxyuridine. *Plant Methods* **6**:5.

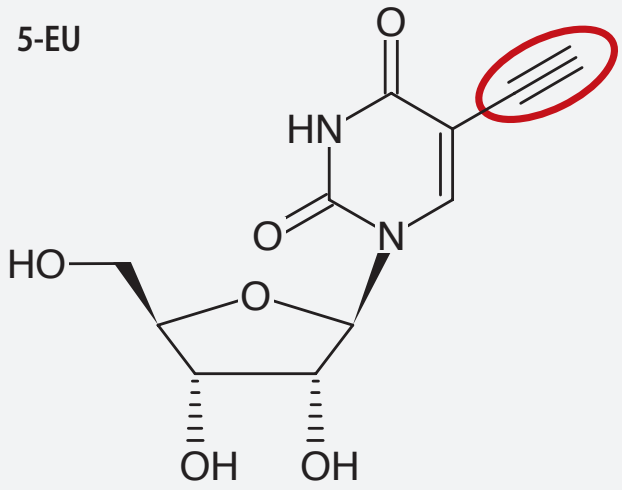
RNA synthesis monitoring with 5-EU

5-Ethynyl-uridine (5-EU) (Fig. 3) can be used as a replacement for ^{3}H-Uracil and 5-Bromo-2'-uridine (5-BrU) to measure the global *de novo* RNA synthesis[1].

The cell-permeable 5-EU is intracellularly metabolized into its triphosphate form via the nucleotide salvage pathway followed by incorporation into nascent RNA by cellular polymerases instead of its natural analog uracil.

The resulting ethynyl-functionalized RNA can be detected via Cu(I)-catalyzed click chemistry by introduction of

- a Biotin group (via Azides of Biotin)
- a FLAG-tag (via FLAG-Azides) for purification or
- a fluorescent group (via Azides of fluorescent dyes) for microscopic imaging.



Features:

- non-destructive & therefore suitable for multiparametric analysis (detection with small-sized labeled Azides)
- no harsh permeabilization & nucleic acid denaturation required as for the antibody-based 5-BrU detection
- significantly faster detection procedure (~2 h instead of 4 h +)
- compatible with flow cytometry & microscopy[1,2]

Figure 3 5-EU (5-Ethynyl-uridine) bypasses limitations of 5-BrU-based monitoring. Red: Terminal Alkyne group.

Table 2: Selected cell lines & organisms analyzed with 5-EdU

Cell line / Organism	Final 5-EU concentration
A549 cells	0. 2 mM[3]
BEAS-2B cells	1 mM[2]
HEK 293T cells	1 mM[1]
LR7 cells	0.25-4 mM[4]
NIH3T3 cells	1 mM[1]
murine ES cell line R1	1 mM[5]
Granylocytes (isolated from PBMCs)	1 mM[6]
Mouse (intestine, kidney, liver, spleen)	2 mg/mouse[1]
Plants *Vicia faba*	1 mM[7]

Selected References:

[1] Jao *et al.* (2008) Exploring RNA transcription and turnover in vivo by using click chemistry. *Proc. Natl. Acad.Sci. USA* **105 (41)**:15779.
[2] Abe *et al.* (2012) Analysis of interferon-beta mRNA stability control after poly(I:C) stimulation using RNA metabolic labeling by ethynyluridine. *Biochem Biophy Res Com* **428(1)**:42.
[3] Xing *et al.* (2012) Foreign RNA Induces the Degradation of Mitochondrial Antiviral Signaling Protein (MAVS): The Role of Intracellular Antiviral Factors. *PLoS ONE* **7(9)**:e45136.
[4] Hagemeijer *et al.* (2012) Visualizing Coronavirus RNA Synthesis in Time by Using Click Chemistry. *Journal of Virology* **86(10)**:5808.
[5] Vizlin-Hodzic *et al.* (2011) SAF-A Forms a Complex with BRG1 and Both Components Are Required for RNA Polymerase II Mediated Transcription. *PLoS ONE* **6(12)**:e28049.
[6] Feng *et al.* (2013) Impairment of FOS mRNA Stabilization Following Translation Arrest in Granulocytes from Myelodysplastic Syndrome Patients. *PLoS ONE* **8(4)**:e61107.
[7] Winnicki *et al.* (2012) Behavior of RNAs transcripts during nucleolus assembly and disassembly in Vicia faba root meristematic cells under normal conditions and after colchicine treatment. *Acta Physiol Plant* **34**:1401.

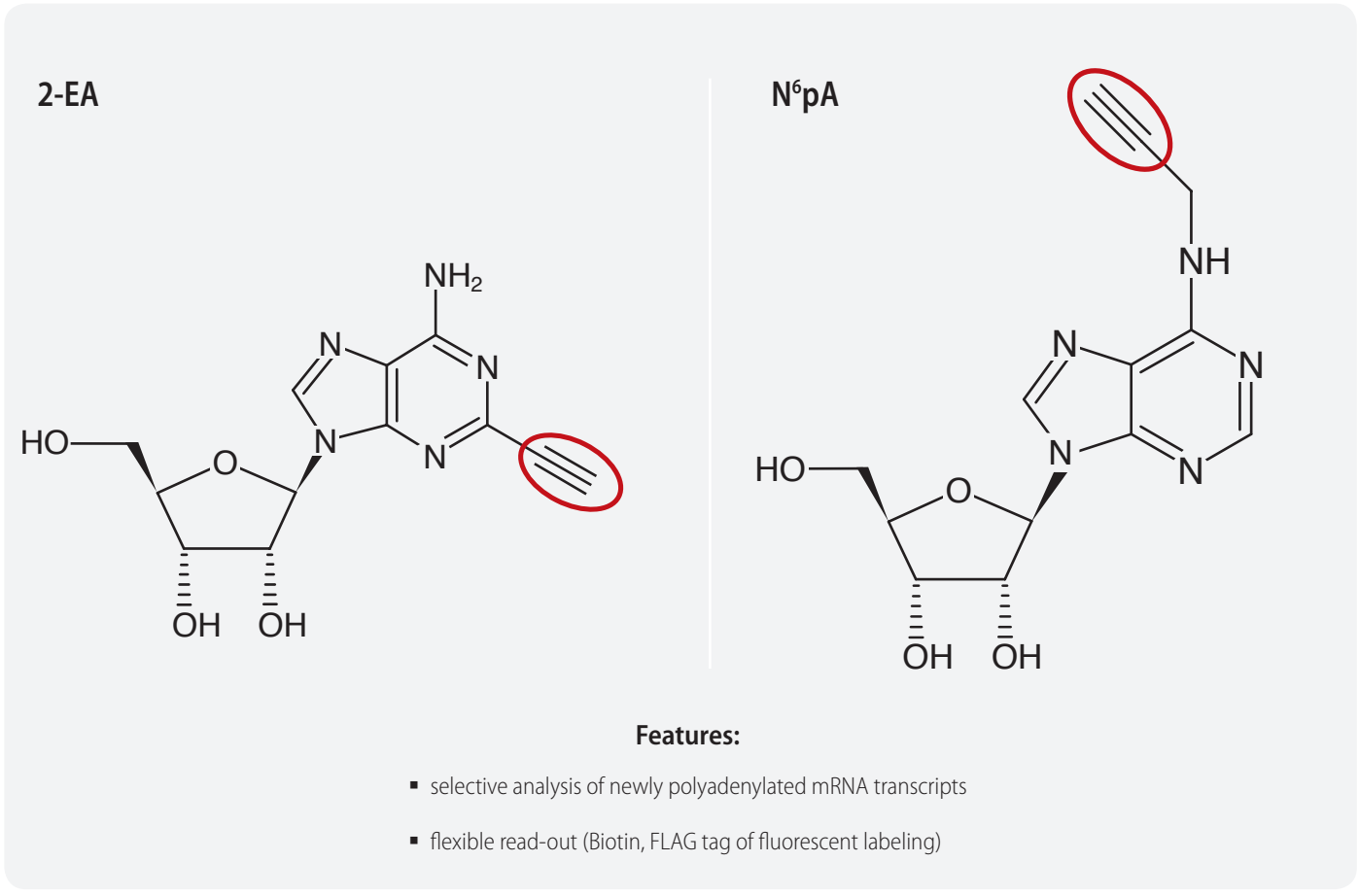
mRNA poly A tail synthesis monitoring with 2-EA or N⁶pA

Poly(A) tails, a long chain of adenosine nucleotides of variable length, are post-transcriptionally added to the 3'-end of eukaryotic mRNAs by poly(A) polymerase (polyadenylation). This modification plays a crucial role in mRNA metabolism and thus gene regulation by influencing mRNA stability, nuclear export or translation efficiency[1].

Traditional methods for the analysis of poly(A) tails do not allow the discrimination between pre-existing and newly polyadenylated mRNA transcripts. The Alkyne-functionalized adenosine analogs 2-Ethynyl-adenosine (2-EA)[2] and N⁶-Propargyl-adenosine (N⁶pA)[3] are suitable substrates for global *de novo* poly A tail synthesis monitoring in mammalian cells (Fig. 4). 2-EA and N⁶pA are cell permeable and incorporate into nascent mRNA transcripts instead of their natural analog adenosine both transcriptionally by RNA polymerase I, II and III and post-transcriptionally by poly(A) polymerase.

The resulting C-terminal alkyne labeled proteins can be detected via Cu(I)-catalyzed click chemistry by introduction of

- a Biotin group (via Azides of Biotin)
- a FLAG-tag (via FLAG-Azides) for purification or
- a fluorescent group (via Azides of fluorescent dyes) for microscopic imaging.



Features:

- selective analysis of newly polyadenylated mRNA transcripts
- flexible read-out (Biotin, FLAG tag of fluorescent labeling)

Figure 4 2-EA (2-Ethynyl-adenosine) and N⁶pA (N⁶-Propargyl-adenosine) allow monitoring of newly synthesized poly A tails. Red: Terminal Alkyne group.

Table 3: Selected cell lines & organisms analyzed with 2-EA

Cell line / Organism	Final 2-EA concentration
HEK 293	10-300 µM[2]
Rat E18 cortical neuron cultures	100 µM[2]
***Xenopus* oocytes**	300 µM[2]

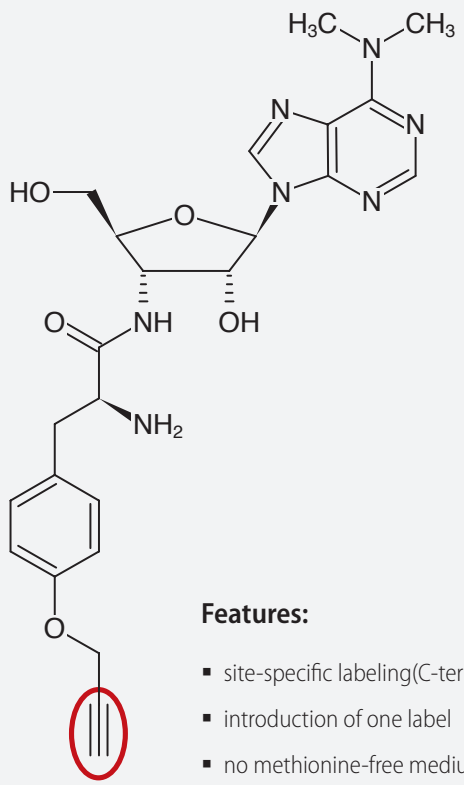
Table 4: Selected cell lines & organisms analyzed with N⁶pA.

Cell line / Organism	Final N⁶pA concentration
HEK 293	200 µM[3]
HeLa	10-100 µM[3]

Selected References:

[1] Colgan *et al.* (1997) Mechanism and regulation of mRNA polyadenylation. *Genes Dev.* **11**:2755.
[2] Curanovic *et al.* (2013) Global profiling of stimulus-induced polyadenyation in cells using a poly(A) trap. *Nature Chemical Biology* **9**:671.
[3] Grammel *et al.* (2012) Chemical reporters for monitoring RNA synthesis and poly(A) tail dynamics. *ChemBioChem* **13**:1112.

Protein synthesis monitoring with OPP



Features:

- site-specific labeling(C-terminal)
- introduction of one label
- no methionine-free medium required

Figure 5 OPP (O-Propargyl-puromycin) bypasses limitations of methionine analog-based labeling approaches. Red: Terminal Alkyne group

Traditional methods of global *de novo* protein synthesis monitoring rely on radioactive labeling with ^{35}S-methionine or indirect detection via DNA and mRNA microarrays. Liu *et al.* reported a non-radioactive alternative to analyze newly synthesized proteins in cell culture and whole organisms based on an alkyne analog of puromycin (Fig. 5)[1].

The cell-permeable O-Propargyl-puromycin (OPP) incorporates site-specifically into the C-terminus of translating polypeptide chains thereby stopping translation. The resulting C-terminal alkyne labeled proteins can be detected via Cu(I)-catalyzed click chemistry that offers the choice to introduce

- a Biotin group (via Azides of Biotin)
- a FLAG-tag (via FLAG-Azides) for purification or
- a fluorescent group (via Azides of fluorescent dyes) for microscopic imaging.

In contrast to previously reported non-radioactive methionine analog-approaches, methionine-free medium is not required for O-Propargyl-purmoycin-based monitoring of *de novo* protein synthesis.

Table 5: Selected cell lines & organisms analyzed with O-Propargyl-puromycin.

Cell line / Organism	Final O-Propargyl-puromycin concentration
NIH3T3	50 µM[1]
HeLa	25 µM[2]
Bone marrow or sorted cells	50 µM[3]
Mouse	50 mg/kg[3] 2 µmol[1]

Selected References:

[1] Liu *et al.* (2012) Imaging protein synthesis in cells and tissues with an alkyne analog of puromycin. *Proc. Natl. Acad. Sci. USA* **109(2)**:413.

[2] Seguin *et al.* (2014) Inhibition of autophagy, lysosome and VCP function impairs stress granule assembly. *Cell Death and Differentiation advance* online publication 18 July 2014; doi: 10.1038/cdd.2014.103

[3] Signer *et al.* (2014) Haematopoietic stem cells require a highly regulated protein synthesis rate. *Nature* **509**:49.

[4] Goodman *et al.* (2012) Imaging of protein synthesis with puromycin. *Proc. Natl. Acad. Sci. USA* **109(17)**:E989.

Products and Pricing

Product	Cat. No.	Amount	Price (EUR)
Alkyne-containing Nucleosides			
5-Ethynyl-uridine (5-EU)	CLK-N002-10	10 mg	101,38
	CLK-N001-25	25 mg	98,91
	CLK-N001-100	100 mg	165,00
	CLK-N001-500	500 mg	688,00
2-Ethynyl-adenosine (2-EA)	CLK-N005-1	1 mg	71,97
	CLK-N005-5	5 mg	287,87
N^6-Propargyl-adenosine (N^6pA)	CLK-N004-1	1 mg	71,97
	CLK-N004-5	5 mg	287,87
O-Propargyl-puromycin	NU-931-05	0,5 mg	140,00
	NU-931-5	10 x 0,5 mg	698,50
Selection of Fluorescent Azides			
3-Azido-hydroxycoumarin Abs/Em = 404/477 nm	CLK-FA047-1	1 mg	103,48
5/6-Fluorescein (5/6-Fam) Abs/Em = 492/517 nm	CLK-FA005-1	1 mg	160,75
5/6-Carboxyrhodamine 110-PEG_3-Azide Abs/Em = 501/525 nm	CLK-AZ105-5	5 mg	183,48
5/6-TAMRA-PEG_3-Azide Abs/Em = 546/565 nm	CLK-AZ109-5	5 mg	240,83
Sulfo-Cy3-Azide Abs/Em = 553/566 nm	CLK-AZ119-1	1 mg	124,03
5/6-Texas Red-PEG_3-Azide Abs/Em = 584/603 nm	CLK-AZ110-5	5 mg	240,83
Sulfo-Cy5-Azide Abs/Em = 647/663 nm	CLK-AZ118-1	1 mg	124,03
Selection of Biotin- and FLAG-tagged Azides			
Biotin-Azide	CLK-80111-25	25 mg	154,45
	CLK-80111-100	100 mg	340,40
Biotin-PEG_3-Azide	CLK-AZ104P4-25	25 mg	81,49
	CLK-AZ104P4-100	100 mg	289,32
FLAG-PEG_3-Azide	CLK-032-S	0,5 µmol	148,50
	CLK-032-L	5 x 0,5 µmol	596,60
Selection of Auxiliary Reagents			
$CuSO_4$	CLK-MI004-10	10 mg	13,02
THPTA-Ligand Tris(3-hydroxypropyltriazolylmethyl)amine	CLK-1010-100	100 mg	136,95
Sodium Ascorbate (Na-Ascorbate)	CLK-MI005-10	10 mg	13,67

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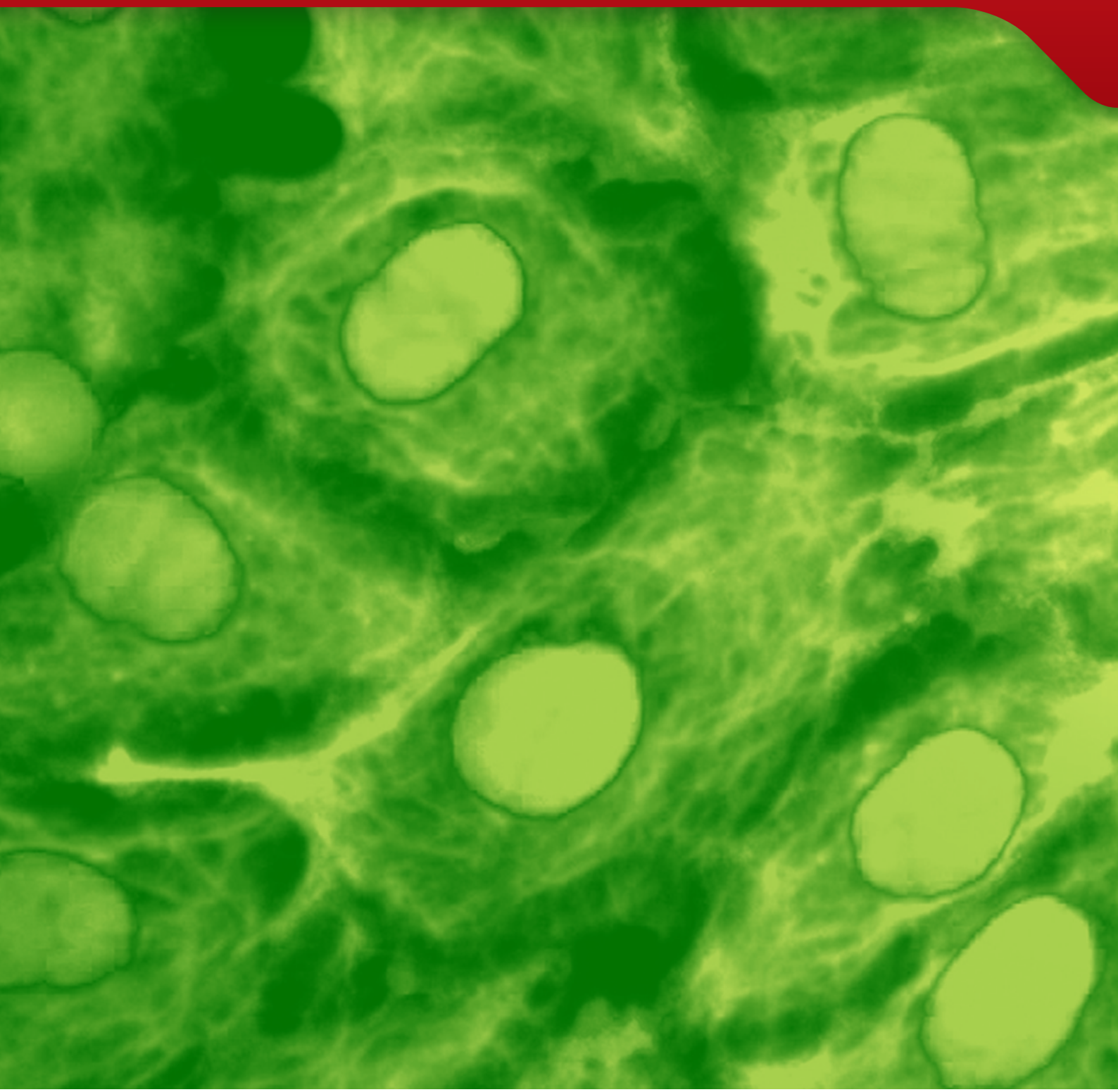

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