Literature seminar
(M2 part)

2009/10/7(Wed)
A.Nojiri(M2)

Labeling in living systems - New Tools for Glycobiology
~Focused on Carolyn R. Bertozzi's works~

Tools to visualize biomolecular interactions
and the resulting functional phenomena within the living cell, tissue or organism

Powerful tools

Genetically encoded fluorescent proteins are powerful tools.
(for example, the green fluorescent protein, GFP)
the 2008 Nobel Prize in Chemistry

Limitation

Many biomolecules(nucleic acids, lipids, and glycans)
cannot be monitored with genetically encoded reporters.

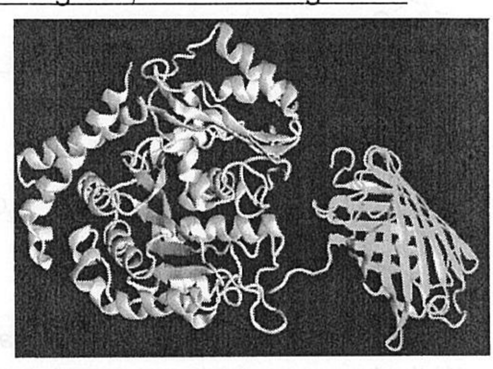


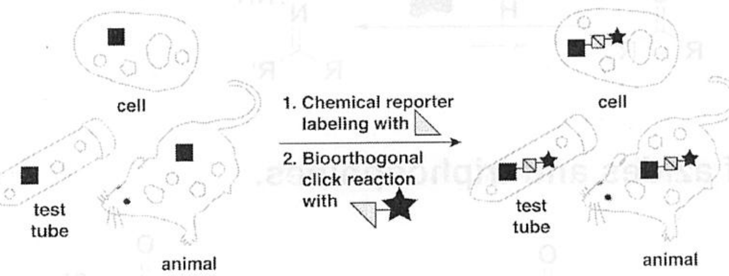
Figure GFP domain (right side)
connected to another protein(left)

Review
Angew. Chem. Int. Ed. 2009, 48, 6974

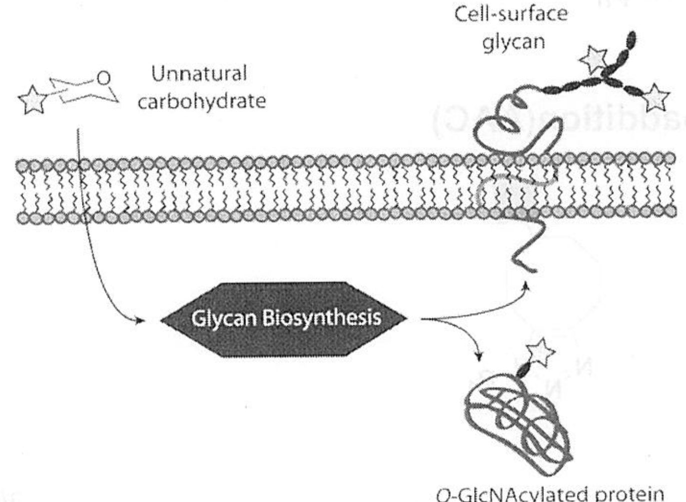
Today's theme
Tagging probe by using of *bioorthogonal chemical reactions* (that is, reactions that do not interfere with biological processes)

Chemical reporter Strategy by Carolyn R. Bertozzi

(1) Functional group (the **chemical reporter**) that does not interact with any biological functionality is incorporated into the target biomolecule using the cellfs metabolic pathways.
(2) The reporter is covalently tagged with an exogenous probe using a highly selective chemical reaction(**bioorthogonal reaction**)



- Thier focus is molecular imaging of glycans because of their incompatibility with genetically encoded reporters.
- They paid attention that unusual oligosaccharides can be metabolically installed.



Keyword:Bioorthogonal chemical reactions = 生体直交型化学反応

Non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes

A + B ⟶ A–B

Requirement for *bioorthogonal chemical reactions*

(1) the two components are non-interacting (orthogonal) to the functionality presented in biological systems.

(2) Proceed in water at neutral or near-neutral pH at temperatures ranging from 25 to 37 °C without any cytotoxic reagents or byproducts.

Contents Tag

1 The condensation of ketones and aldehydes with amine nucleophiles

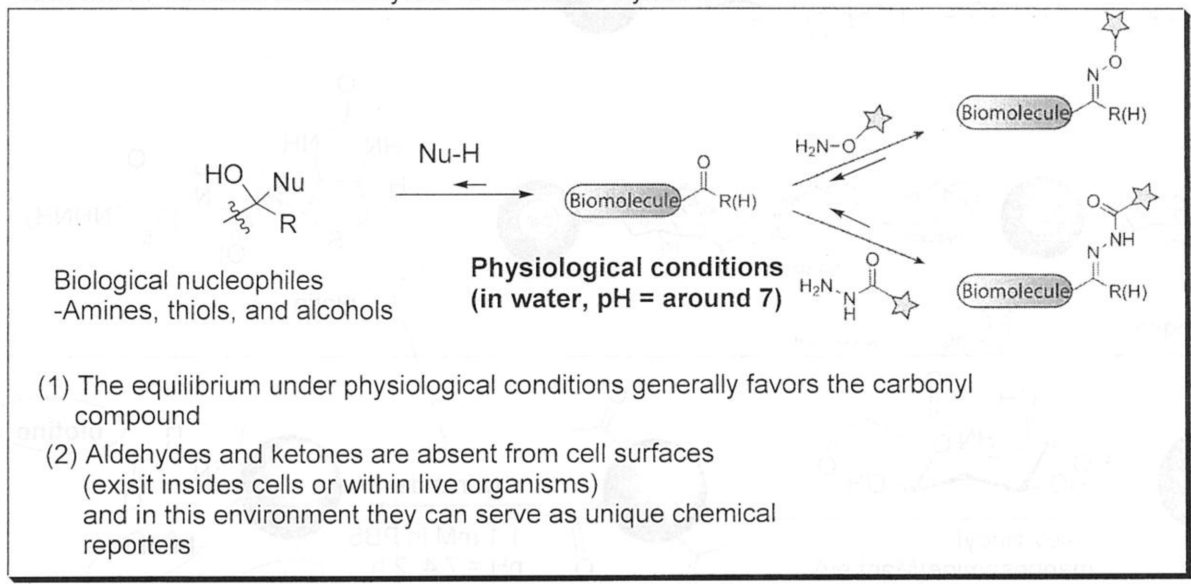
2 The Staudinger Ligation of azides and triphosphines.

3. Azides-Alkynes 1,3-dipolar cycloaddition(AAC)

4. Summary

1 The condensation of ketones and aldehydes with amine nucleophiles

Conversion "Ketones and aldehydes" to oximes or hydrazones



(1) The equilibrium under physiological conditions generally favors the carbonyl compound

(2) Aldehydes and ketones are absent from cell surfaces (exisit insides cells or within live organisms) and in this environment they can serve as unique chemical reporters

<Representative example>

Engineering Chemical Reactivity on Cell Surfaces Through Oligosaccharide Biosynthesis

Lara K. Mahal, Kevin J. Yarema, Carolyn R. Bertozzi*

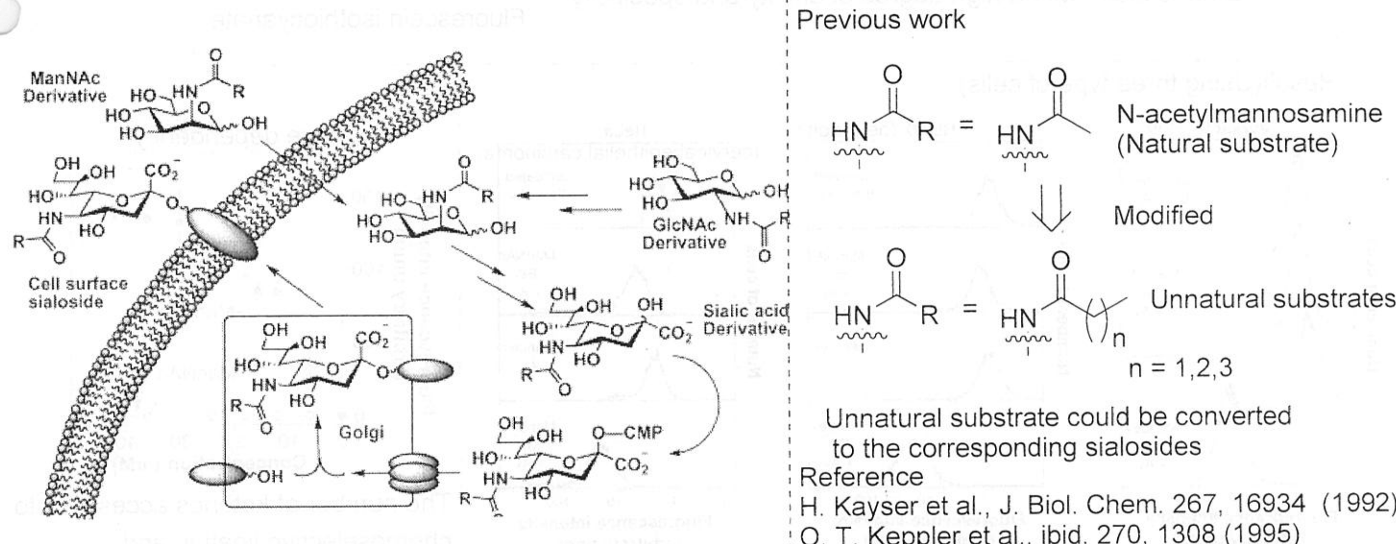
Science 276, 1125 (1997)

Main theme

Cell surface oligosaccharides can be engineered to display unusual functional groups for the selective chemical remodeling of cell surfaces

Sialic acid biosynthesis

They focused on **sialic acid** because
(1) They are the most abundant terminal components of oligosaccharides on mammalian glycoproteins and glycolipids,
(2) The enzymes that participate in sialic acid metabolism are permissive for simple unnatural substrates.



To use the condensation of ketones and aldehydes with amine nucleophiles, they decided to introduce the ketone functionality at the position normally occupied by the N-acetyl group in the natural substrate

Plan

A

Ketone

ManLev

Cell

Ketone

Cell

B

Cell

Hydrazide

N-Acyl hydrazone

Cell

$-(CH_2)_4-CONH-(CH_2)_5-$ = R

biotin

<Methodology>

Cells

N-levulinoyl mannosamine(ManLev)
5 mM, 48 h

hydrazide
1.1 mM in PBS
pH = 7.4, 2 h

biotine

hydrazone

FITC-avidin

Analyzed by flow cytometry

FITC-avidin = fluorescence protein to bind the biotin specifically.

FITC = Fluorescein isothiocyanate

Avidin =The tetrameric protein
Bind to biotin with a high degree of affinity and specificity.

Fluorescein isothiocyanate

Result(Using three type of cells)

Jurkat (T cell)

HL-60 (neurtophil)

HeLa (cervical epithelial carcinoma)

Untreated Bio + Av

ManNAc Bio + Av

ManLev Av

ManLev Bio + Av

Number of cells

Fluorescence intensity (arbitrary units)

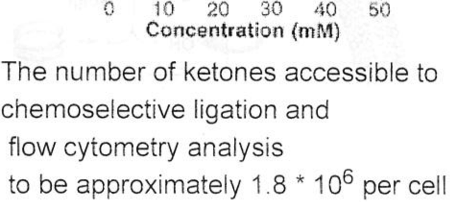
Dose dependency

Fluorescence intensity (arbitrary units)

ManLev

ManNAc

Concentration (mM)



The number of ketones accessible to chemoselective ligation and flow cytometry analysis to be approximately $1.8 * 10^6$ per cell

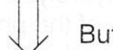
All results showed a large increase in fluorescence intensity compared to cells treated with buffer or the natural derivative ManNAc.

Limitations
(1) pH optimum is 5 - 6 (physiological pH is typically 7 - 8)
(2) The linkages are reversible
(3) Certain biological milieus - notably the inside of most cells - contain endogenous ketones or aldehydes

2.2 The Staudinger Ligation of azides and triphosphines.

Azide

Azides have been neglected for a long time because of safe concern. (Explosive character)



But

Suitable functional group for bioorthogonal reaction

- Azide is totally absent from biological systems
- Azide also possesses orthogonal reactivity to the majority of biological functional groups
- The azide group is small
- Stable toward water and oxygen.

The Staudinger Ligation

Classical staudinger reaction (Staudinger, 1919)

azide + phosphine (X_3P:) → phosphazide → $R-N=PX_3$ / $N=NH$ → ($-N_2$) → aza-ylide → (H_2O) → RNH_2 + $O=PX_3$

Modified Staudinger reaction (Bertozzi, 2000)

Electrophlic trap

$-N_2$; H_2O, $-MeOH$; $-MeOH$; H_2O

OMe, Ph, Ph, R, R', $^{-}N=N^{+}=N$, P=O, N-R', OH

Appropriately situated electrophilic methyl ester, within the phosphine structure, capture the nucleophilic aza-ylide by intramolecular cyclization.

<Representative report>

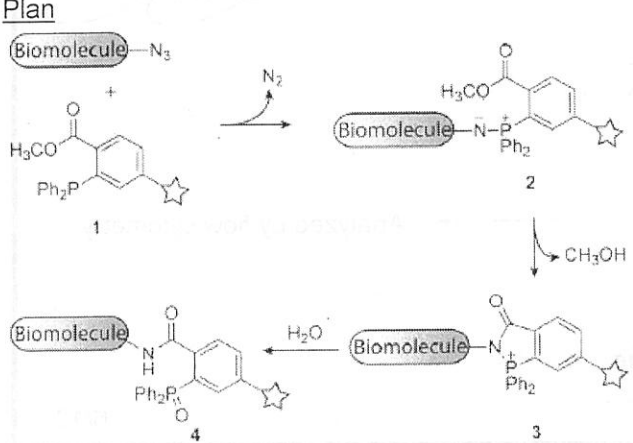
Cell Surface Engineering by a Modified Staudinger Reaction

Eliana Saxon and Carolyn R. Bertozzi*

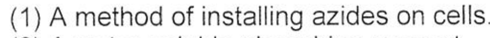
Bertozzi et al, Science **2000**, *287*, 2007

- First report to use Staudinger reaction for formation of amide bond.

Plan



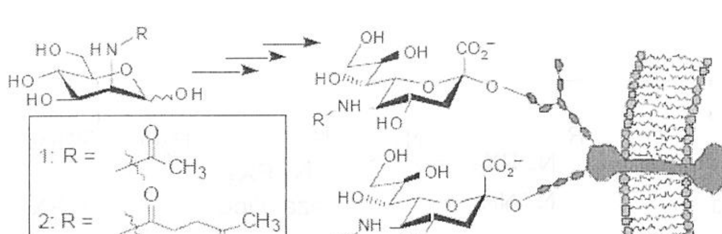
<Model study>



- Only amide-linked product was obtained.
- No evidence of aza-ylide hydrolysis.
- The limited water solubility of the phosphine necessitated an organic cosolvent(THF).

<Requirements to test the modified Staudinger reaction in ***the cell surface*** (far more demanding environment)>

(1) A method of installing azides on cells.
(2) A water-soluble phosphine reagent.



About (1)

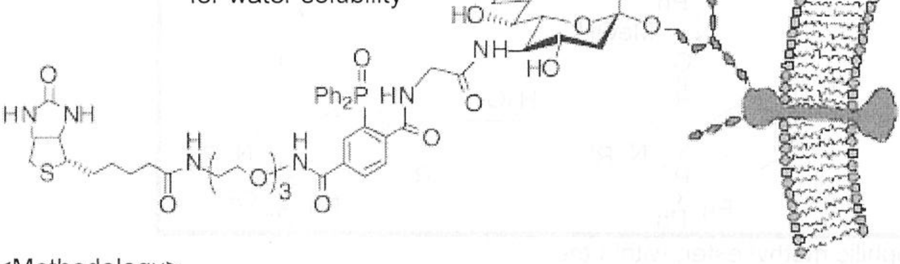
By using azidosugars as metabolic precursors, N-azidoacetylsilic acid should be installed.

⇓

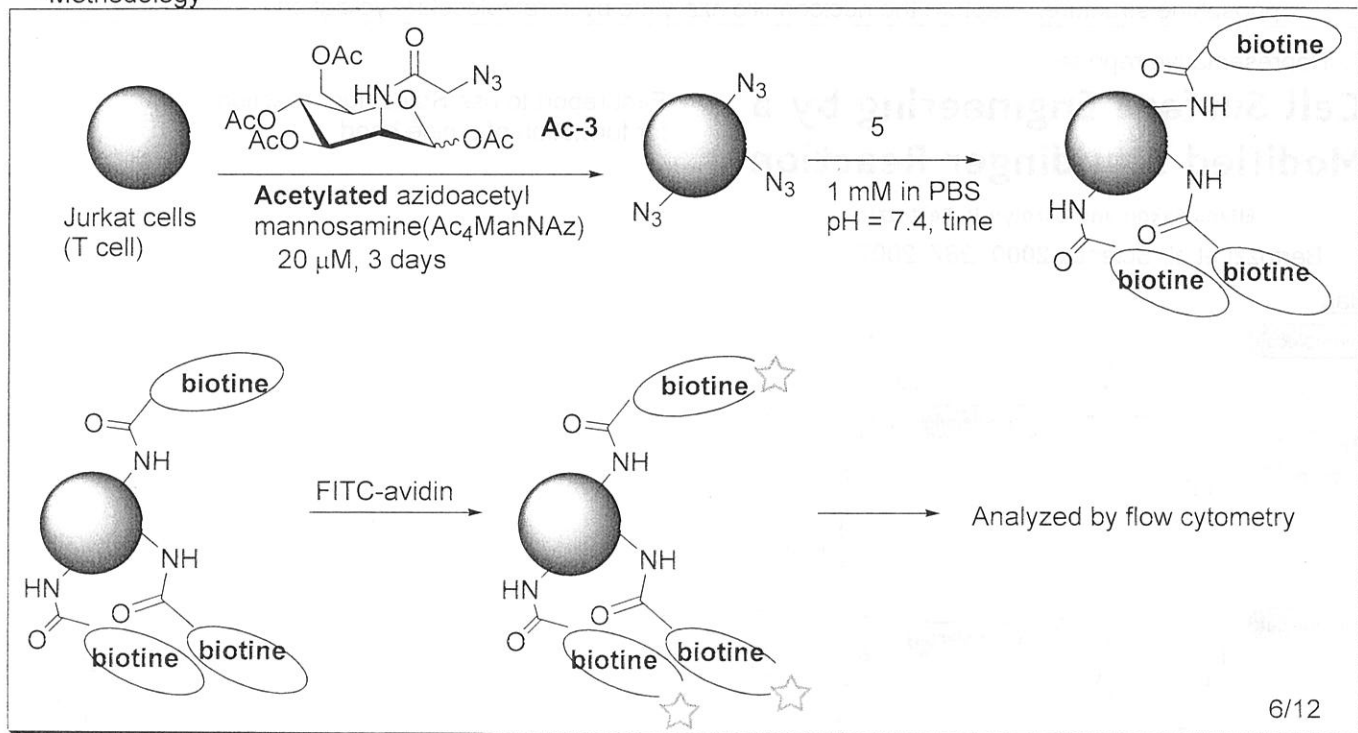
N-azidoacetylmannosamine could be well tolerated by the sialic acid biosynthetic machinery

About (2)

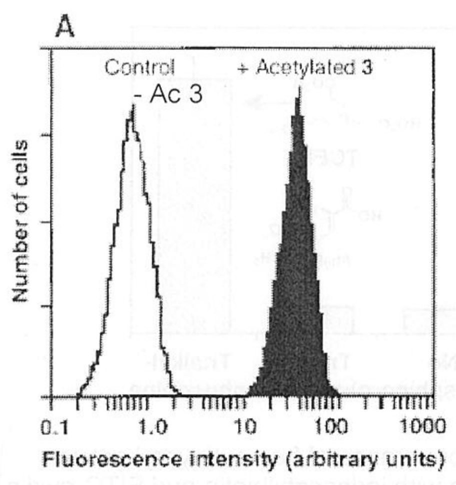
Biotinylated phosphine with tetraethyleneglycol linker was used for water solubility and detection of the ligated cell-surface product.

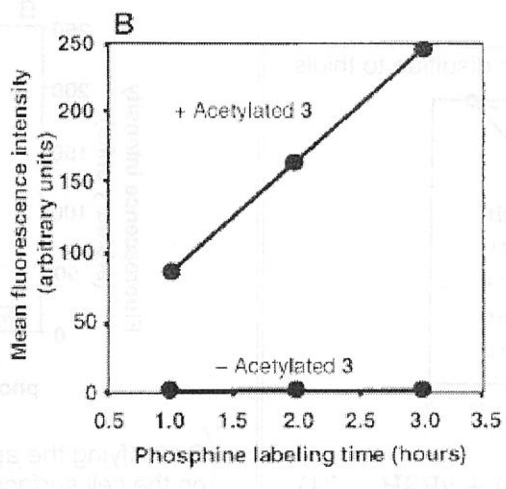


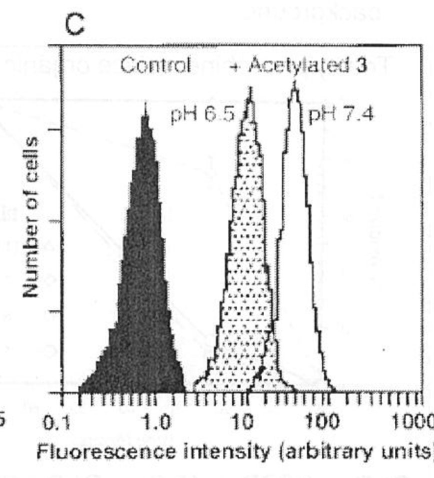
<Methodology>



<Result in vitro analysis>







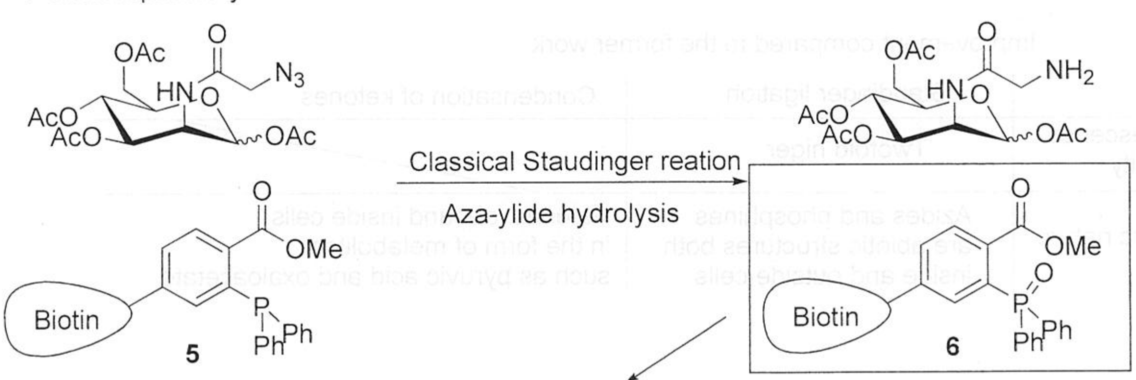
A Marked increase in fluorescence that indicated the accumulation of biotin moieties on the cell surface.

B ~850,000 biotin moieties on the cell surface(in 1h)
This value places a lower limit on the number of azides present on the cell surface,

C This is consistent with previous observations that protonation of azaylides facilitates their hydrolysis, a competing side reaction of the modified Staudinger process.

<Control experiments>

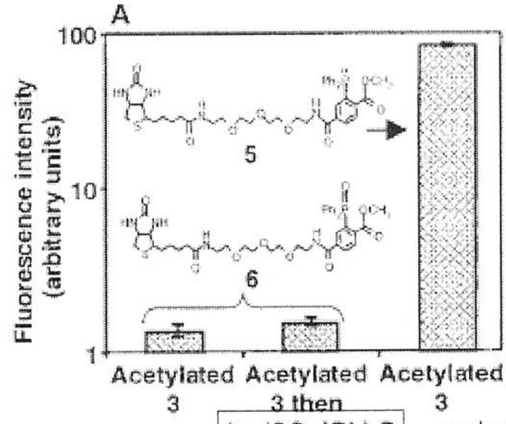
1 Check specificity



this compound might be nonspecifically acylate cell surface amines

⟹ In this case, selective labeling cannot be realized.

Experiment



→ water-soluble phosphine to intentionally reduce the azides to amine

(Left) Compound **6** didn't acylate cell surface amines.

(Center) Amines derived from classical Staudinger reaction were not acylated by compound **6**

(Right) Control

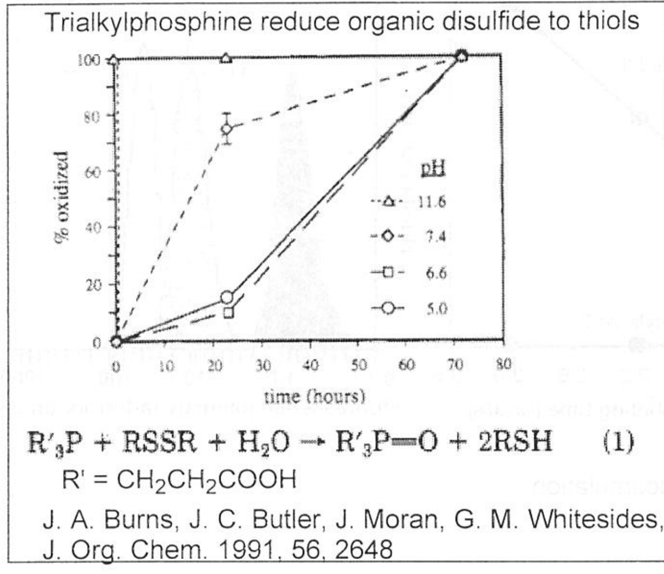
⇓

The chemoselective ligation reaction proceeds as designed without complications arising from nonspecific amine acylation

2 Check bioorthogonality

background

Trialkylphosphine reduce organic disulfide to thiols

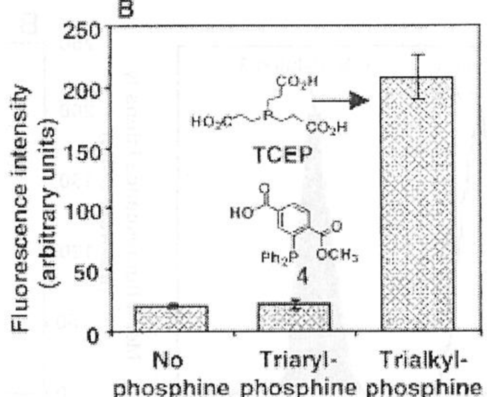


$$R'_3P + RSSR + H_2O \rightarrow R'_3P{=}O + 2RSH \quad (1)$$

$R' = CH_2CH_2COOH$

J. A. Burns, J. C. Butler, J. Moran, G. M. Whitesides, J. Org. Chem. 1991, 56, 2648

Check the reactivity of arylphosphine



(Quantifying the appearance of free sulfhydryl groups on the cell surface with iodoacetylbiotin and FITC-avidin)

Result

In the presence of the arylphosphine or abesent, RSH was not detected.

In the presence of the alkylphosphine, RSH was detected.

→ Triarylphosphines are essentially unreactive toward disulfide bonds under these conditions, rendering ligation with azides the predominant pathway for reactivity

Improvement compared to the former work.

	Staudinger ligation	Condensation of ketones
Fluorescence intensity	Twofold higer	
Abiotic nature	Azides and phosphines are abiotic structures both inside and outside cells	Ketones abound inside cells in the form of metabolites such as pyruvic acid and oxaloacetate

Limitations

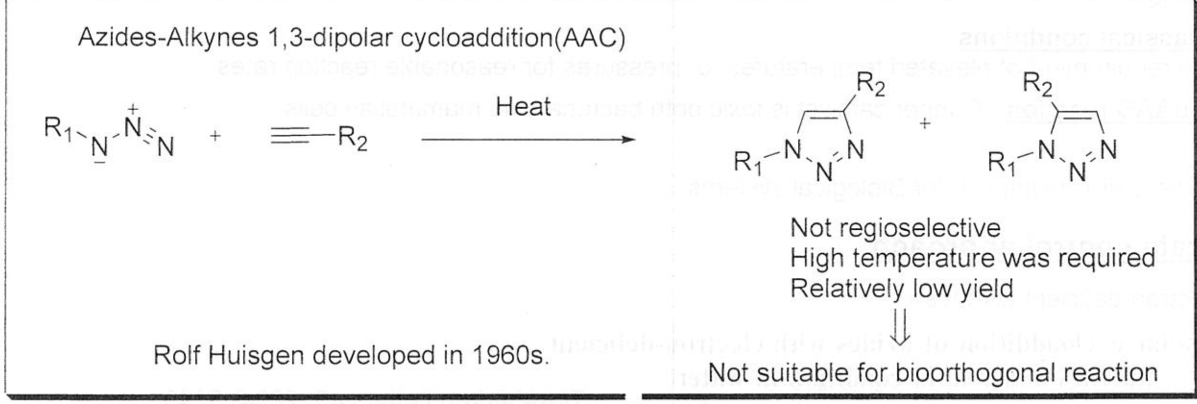
(1) Suffers from the inconvenience of air oxidation of the phosphine reagents.
 ~ Limit their shelf life

(2) Sluggish reaction kinetics
 ~ Cannot use the reaction for dynamic monitoring of cellular processes that occur faster than the timescale of the chemical reaction

3 Azides-Alkynes 1,3-dipolar cycloaddition(AAC)

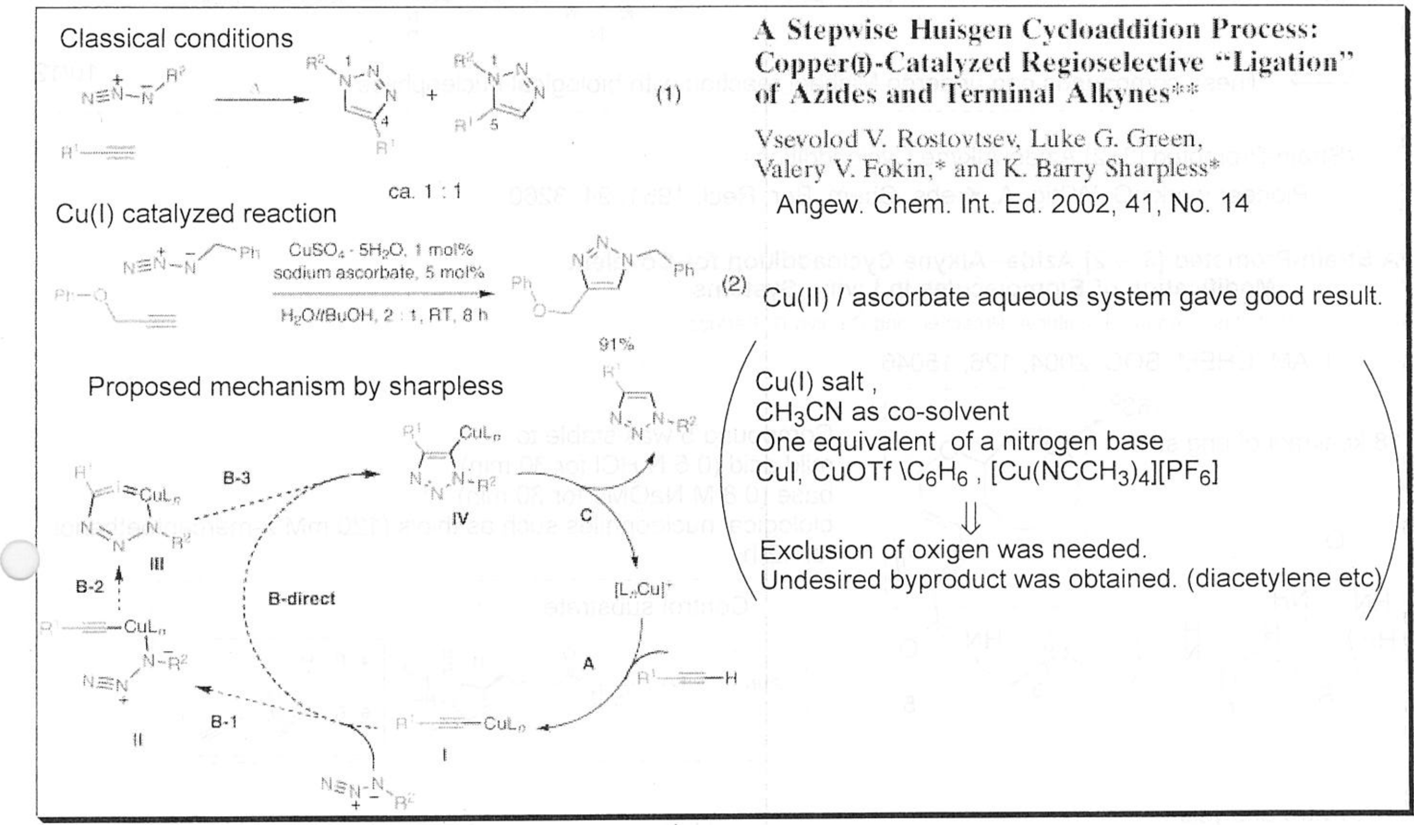
The synthesis of 1,2,3-triazoles by 1,3-dipolar cycloaddition of azides and alkynes was discovered by Arthur Michael

A. Michael, J. Prakt. Chem. 1893, 48, 94



Early works about CuAAC reactions(copper catalyzed azide-alkyne cycloadditions)

First report Morten Meldal et al J. Org. Chem. 2002, 67, 3057



Concept **Click Chemistry** (Sharpless et al.Angew. Chem. Int. Ed. 2001, 40, 2004)

Criteria of click reaction

(1) Simple reaction conditions (readily available starting materials and reagents, the use of no solvent or a solvent that is benign)
(2) Modular, wide in scope, very high yields, only inoffensive byproducts
(3) Easy purification(nonchromatographic methods, such as crystallization or distillation)
(4) Stable toward water and oxygen.

"The purpose is to accelerate the discovery of substances with useful properties."

CuAAC attracted attention as a true example of efficient and versatile "*Click chemistry*".
In some cases, CuAAC = click chemistry

Biological application

Azide and alkyne functions are, respectively, absent or relatively rare in the biological world

⇓

Azide-alkyne chemistry constitutes a very interesting chemoselective platform
for the functionalization or ligation of biological systems.

Problems

- **Classical conditions**:
  the requirement of elevated temperatures or pressures for reasonable reaction rates
- **CuAAC reaction**: Copper catalyst is toxic both bacterial and mammalian cells.

There are limitations for Biological systems

Substrate control approach

<Electron-deficient alkenes>

1,3-Dipolar cycloaddition of azides with electron-deficient alkynes under mild condition in water

Zengmin Li,[a,b] Tae Seok Seo[a,b] and Jingyue Ju[a,b,*]

Tetrahedron Letters 45 (2004) 3143

N_3–(CH₂)₅–C(=O)–NH–(CH₂)₆–DNA + H–C≡C–C(=O)–OR →(Water, RT) triazole product (RO–C(=O), H, N–(CH₂)₅–C(=O)–NH–(CH₂)₆–DNA)

⟹ These compounds can undergo Michael reaction with biological nucleophiles

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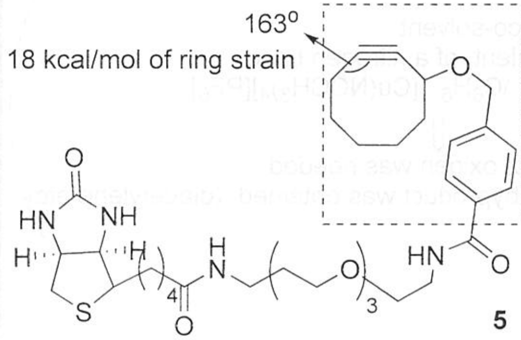
<Strain-Promoted [3+2] Azide-Alkyne Cycloaddition>

Pioneer works:G. Wittig, A. Krebs, Chem. Ber. Recl. 1961, 94, 3260

A Strain-Promoted [3 + 2] Azide–Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems

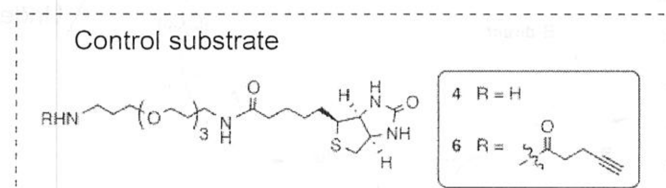
Nicholas J. Agard, Jennifer A. Prescher, and Carolyn R. Bertozzi*

J. AM. CHEM. SOC. 2004, 126, 15046



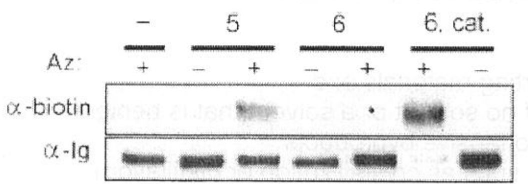
Compound 5 was stable to
mild acid (0.5 N HCl for 30 min),
base (0.8 M NaOMe for 30 min),
biological nucleophiles such as thiols (120 mM 2-mercaptoethanol for 12 h

Control substrate



<In vitro analysis>

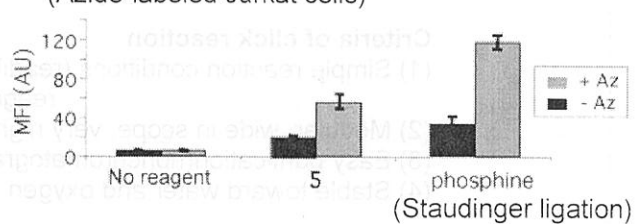
Reaction with azide-modified GlyCAM-Ig



5 Cyclooctyne
6 Acyclic octyne
6, cat conditions of CuAAC

++ Cyclooctyne(5) reacted with azide in vitro.
++ Cu effected the biological system.
(Copper damages the epitope recognized by HRP-α-IgG)

Comparison with Staudinger ligation
(Azide-labeled Jurkat cells)



(Staudinger ligation)

Staudinger reaction was twice faster than SPAAC.

⟹ Biocompatible but higher reactivity is needed.

<Improved reactivity (Strain-Promoted [3+2] Azide-Alkyne Cycloaddition)>

Agard NJ, Baskin JM, Prescher JA, Lo A, Bertozzi CR (2006) ACS Chem Biol,1,644

A — B

Electron-withdrawing groups

Slightly improved

A: $k = 0.0024\ M^{-1}\ s^{-1}$

B: $k = 0.0043\ M^{-1}\ s^{-1}$ (2-times greater)

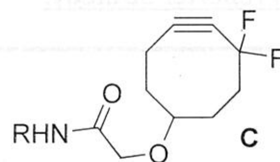
k: second order rate constant (reaction with benzyl azide)

<Improved reactivity and dynamic imaging in living cells>

Copper-free click chemistry for dynamic *in vivo* imaging

Jeremy M. Baskin*, Jennifer A. Prescher*, Scott T. Laughlin*, Nicholas J. Agard*, Pamela V. Chang*, Isaac A. Miller*, Anderson Lo*, Julian A. Codelli*, and Carolyn R. Bertozzi*†‡§¶

Proc. Natl. Acad. Sci. USA 2007, 104, 16793.

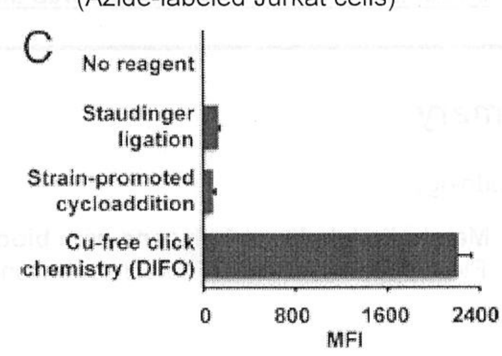


DIFO(Difluoronated Cyclooctyne)

$k = 0.076\ M^{-1}\ s^{-1}$ (30-times greater than **A**)

This value is similar to Cu-catalyzed reaction.

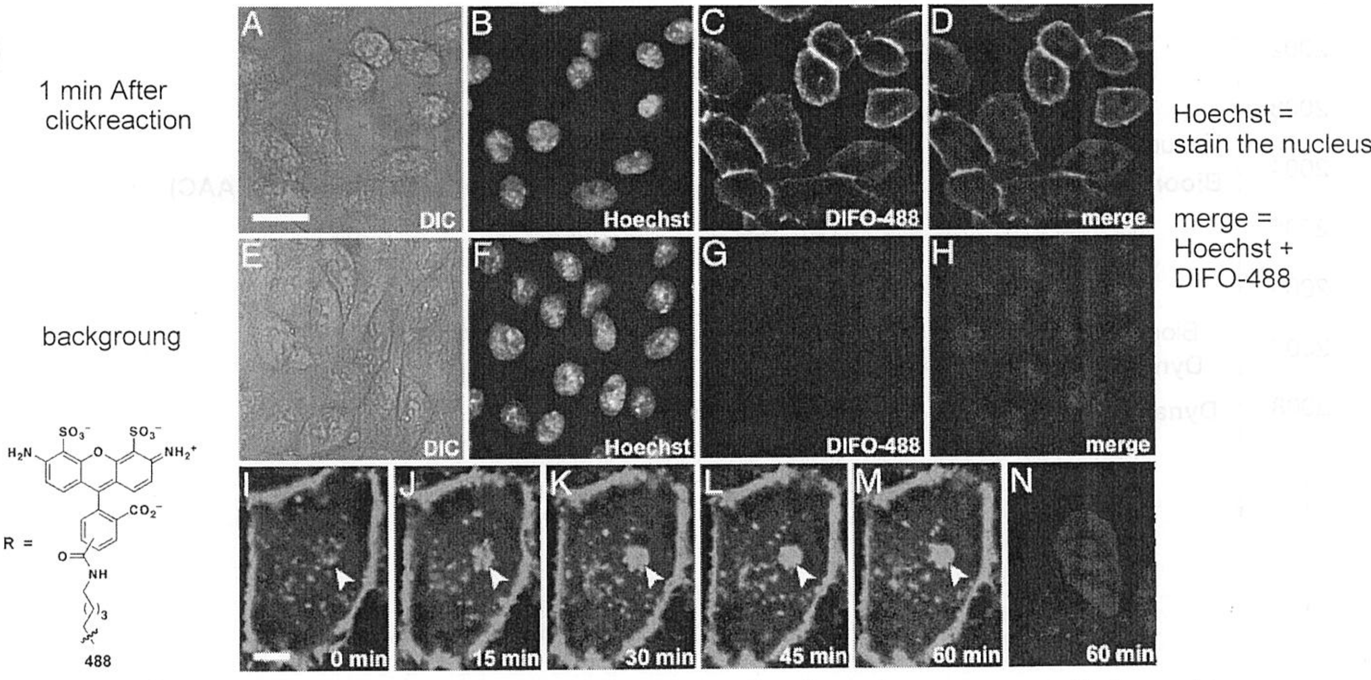
Living cell labeling
(Azide-labeled Jurkat cells)



R = biotine

Direct imaging

Chinese hamster ovary (CHO) cells bearing SiaNAz residues within their cell-surface glycans



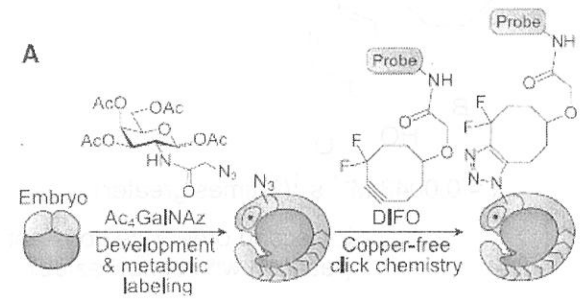
- After only 1 min of labeling, the azide-bearing cell-surface glycans were readily imaged by using DIFO-488, with negligible background fluorescence
- Recycle of cell-surface glycans to the Golgi apparatus was shown.

<In living animals>

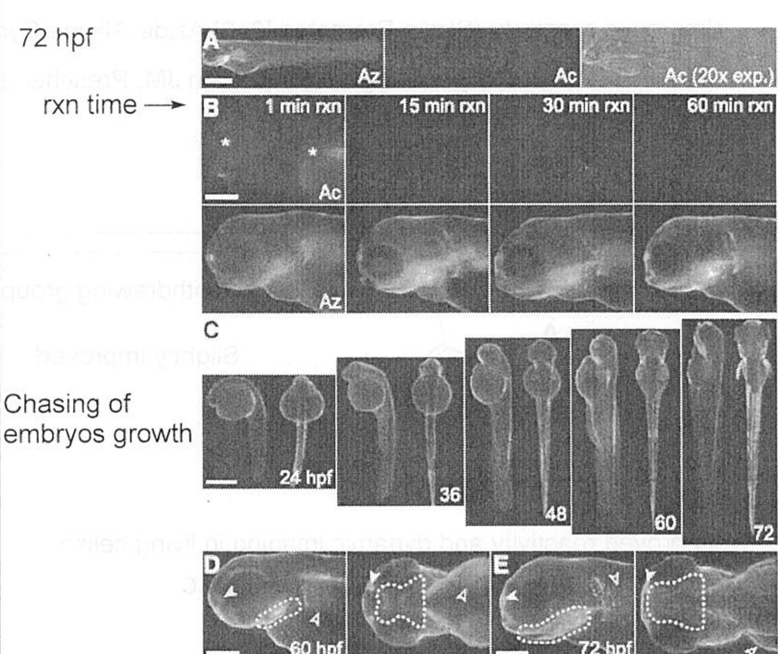
In Vivo Imaging of Membrane-Associated Glycans in Developing Zebrafish

Scott T. Laughlin,[1*] Jeremy M. Baskin,[1*] Sharon L. Amacher,[2] Carolyn R. Bertozzi[1,2,3,4]†

Science 320, 664 (2008)



$Ac_4GalNAz$ is metabolically incorporated into mucin⁻ type O-linked glycoproteins
(SiaNAz can be also imaged with Ac_4ManAz)



hpf = hours post-fertilization
label with Ac_4GalAz or Ac_4GalAc from 3 hpf

- No toxic effect was observed.
- ***Direct imaging in living animals was accomplished by her chemical reporter strategy.***

Summary

Methodology

Year	
1997	**Metabolic labeling of glycans with bioorthogonal chemical reporters** Bioorthogonal reaction:The condensation of ketones and aldehydes with amine nucleophiles
1998	
1999	
2000	**Bioorthogonal reaction:Staudinger Ligation**
2001	
2002	
2003	
2004	[Staudinger ligation in living animals(mice)] **Bioorthogonal reaction:Strain-Promoted [3+2] Azide-Alkyne Cycloaddition(SPAAC)**
2005	
2006	
2007	Bioorthogonal reaction:Dramatically improved reactivity of SPAAC **Dynamic imaging in living cells**
2008	**Dynamic imaging in living animals(Zebrafish)**