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# Click labeling of unnatural sugars metabolically incorporated into viral envelope glycoproteins enables visualization of single particle fusion

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A B S T R A C T

Enveloped viruses infect target cells by fusing their membrane with cellular membrane through a process that is mediated by specialized viral glycoproteins. The inefficient and highly asynchronous nature of viral fusion complicates studies of virus entry on a population level. Single virus imaging in living cells has become an important tool for delineating the entry pathways and for mechanistic studies of viral fusion. We have previously demonstrated that incorporation of fluorescent labels into the viral membrane and trapping fluorescent proteins in the virus interior enables the visualization of single virus fusion in living cells. Here, we implement a new approach to non-invasively label the viral membrane glycoproteins through metabolic incorporation of unnatural sugars followed by click-reaction with organic fluorescent dyes. This approach allows for efficient labeling of diverse viral fusion glycoproteins on the surface of HIV pseudoviruses. Incorporation of a content marker into surface-labeled viral particles enables sensitive detection of single virus fusion with live cells.

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## 1. Introduction

Enveloped viruses infect cells by fusing their envelope membrane with a host cell membrane, a process that culminates in the release of the nucleocapsid into the cytoplasm. Viral envelope glycoprotein-mediated merger of viral and cellular membranes is generally asynchronous and relatively inefficient. As a result of this, most viruses fail to productively enter the cells and are eventually degraded. Single virus imaging has emerged as a powerful tool to delineate the virus entry pathways into host cells in spite of virus heterogeneity and the stochastic nature of viral fusion. A number of virus labeling strategies suitable for imaging single particle entry have been introduced and validated in the recent years (reviewed in (Brandenburg and Zhuang, 2007; Huang and Xie, 2014)). Among these, tagging viral proteins with GFP or other fluorescent proteins and incorporation of lipophilic dyes into the viral membrane have been most widely implemented (see for example (Burdick et al., 2013; Ewers et al., 2005; Floyd et al., 2008; Lakadamyali et al., 2003; Lampe et al., 2007; McDonald et al., 2002; Miyauchi et al., 2009)).

In order to identify particles that undergo fusion, which is a prerequisite for the release of the nucleocapsid into the cytosol, viruses must be co-labeled with two distinct viral determinants—one that is released upon fusion (referred to as content marker) and a reference marker that is, at least transiently, retained by a virus or remains at the site of viral fusion (Campbell et al., 2007; Dale et al., 2011; Miyauchi et al., 2009; Padilla-Parra et al., 2013). Retroviruses are ideally suited for introducing a releasable viral content marker into virions, since their Gag polyprotein is cleaved at several locations by the viral protease upon virus maturation (Freed, 2001; Sundquist and Krausslich, 2012). Insertion of a fluorescent protein flanked by a protease cleavage site produces free fluorescent proteins that are released into the cytoplasm upon virus-cell fusion (Dale et al., 2011; Miyauchi et al., 2009; Padilla-Parra et al., 2013). Two strategies have been employed to provide a reference marker for tracking loss of viral content—labeling the retroviral membrane or the viral core (Albanese et al., 2008; Burdick et al., 2013; Campbell et al., 2007; Lampe et al., 2007; McDonald et al., 2002; Melikyan et al., 2005; Miyauchi et al., 2009; Padilla-Parra et al., 2013; Zhou et al., 2012).

Labeling of proteins in living cells is an active area of research. The goal is to achieve non-invasive, site-specific labeling with different photostable fluorescent dyes. The labeling strategies include direct labeling with amine-reactive dyes (for indiscriminate

*Abbreviations:* $Ac_4$ManNAz, peracylated azidomannosamine; ASLV, Avian Sarcoma and Leukosis Virus; Env, viral envelope glycoprotein; SiaNAz, *N*-azidosialic acid; VSV, Vesicular Stomatitis Virus; YFP, yellow fluorescent protein.

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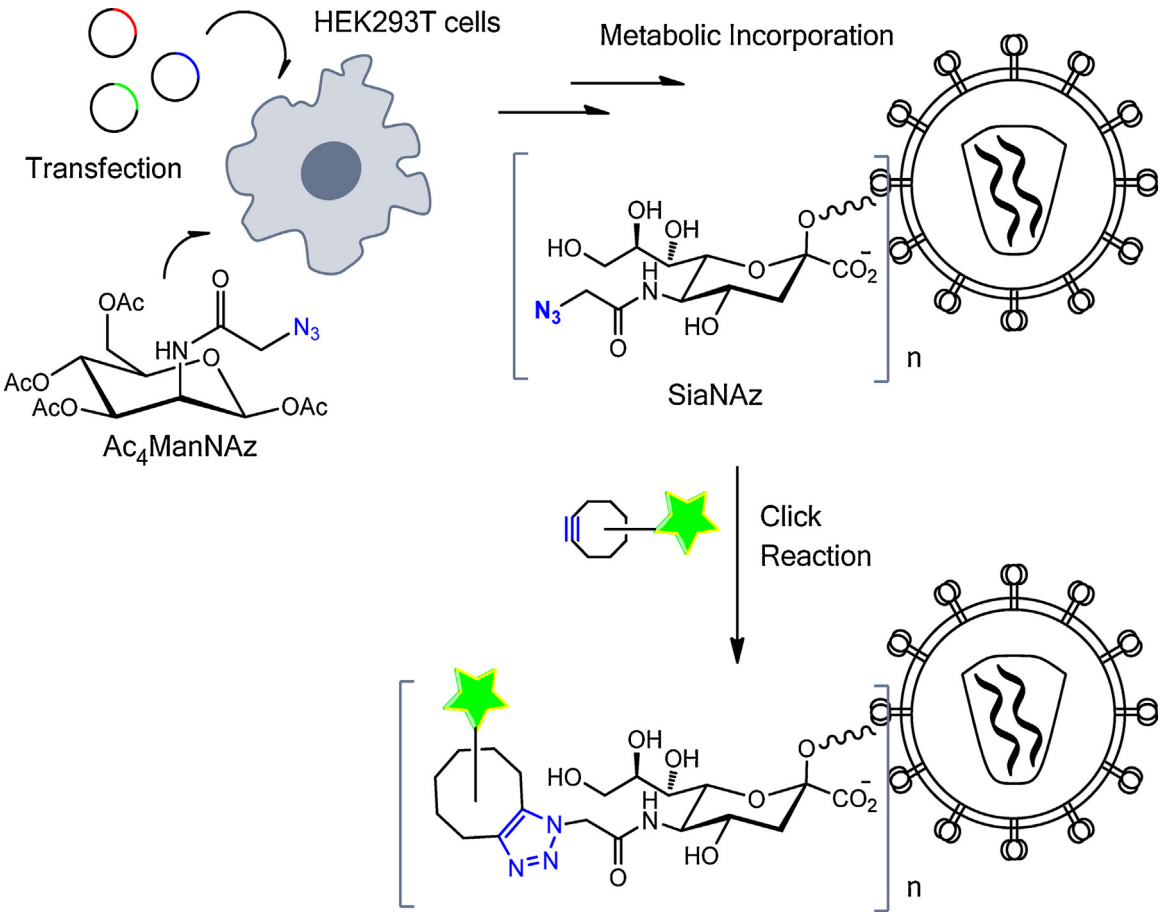


**Fig. 1.** Illustration of virus glycoprotein labeling by click reaction with a metabolically incorporated unnatural sugar.

labeling of proteins) and introduction of various tags (biotin, SNAP, Halo, tetracysteine and others) for subsequent site-directed labeling with fluorescent probes (reviewed in (Crivat and Taraska, 2012; Dean and Palmer, 2014)). These approaches have been adapted for labeling of enveloped viruses (Arhel et al., 2006; Joo et al., 2008, 2010; Mengistu et al., 2015; Munro et al., 2014). Successful insertion of fluorescent proteins into viral fusion proteins has also been reported (Lehmann et al., 2005; Nakane et al., 2015). These strategies involve genetic or chemical modifications of proteins that could compromise the function of metastable viral fusion proteins (Munro et al., 2014; Nakane et al., 2015). We therefore sought to label the variable carbohydrate chains of viral glycoproteins—a modification that is well tolerated (Chu et al., 2015).

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) ("click chemistry") has revolutionized the field of bioconjugation (Bertozzi, 2011; Best, 2009). Click chemistry is a highly selective chemical reaction that proceeds in the presence of a wide variety of other functionalities in the complex biological milieu (i.e., is bioorthogonal), and is well suited for the physiological reaction conditions such as aqueous solutions, pH ~7.0 and physiological temperatures. Azide and alkyne moieties are readily introducible into biomolecules due to their small size and biological inertness. CuAAC has been adopted to label living systems (Baskin et al., 2010; Breidenbach et al., 2010; Chang et al., 2009; Prescher and Bertozzi, 2005) with a combination of selective metabolic engineering techniques, which include cellular (Fernandez-Suarez et al., 2007; Luchansky et al., 2003) and viral surfaces (Banerjee et al., 2010, 2011a,b; Oum and Carrico, 2012). However, the requirement for a cytotoxic cuprous catalyst restricts the utility of CuAAC. Therefore, copper-free "click" reactions, such as the Staudinger ligation of azides with functionalized phosphines, strain-promoted alkyne-azide cycloaddition (SPAAC) and strain-promoted inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC), have been developed (Arumugam et al., 2011; Carpenter et al., 2011; Nikic et al., 2014; Sachin et al., 2012) as alternatives.

Two recent studies (Chu et al., 2015; Zhao et al., 2015) have demonstrated the utility of metabolic incorporation of peracylated azidomannosamine ($Ac_4$ManNAz) into viral glycoproteins. Metabolically labeled glycoproteins on the surface of lentiviral particles were selectively modified using click chemistry in order to retarget pseudoviruses to specific cell types. This approach has also enabled fluorescence labeling of the measles virus glycoproteins (Zhao et al., 2015). Here, we sought to explore the utility of click-labeling azido sugars on diverse viral proteins for single particle imaging in living cells. $Ac_4$ManNAz metabolically incorporated into viral glycoproteins was successfully click-labeled with fluorescent dyes without considerably compromising their function. This labeling strategy combined with incorporation of a viral content marker enables reliable visualization of single viral fusion events.

## 2. Materials and methods

### 2.1. Reagents and cells

All chemicals were obtained from commercial sources and used without further purification. Bafilomycin A1 and neuraminidase were obtained from Sigma-Aldrich (St. Louis, MO). Clickable dyes, Click-iT® Alexa Fluor® 488 DIBO and Click-iT® Alexa Fluor® 647 DIBO alkyne were purchased from Invitrogen (Carlsbad, CA). HEK 293T/17 cells were obtained from ATCC (Manassas, VA).