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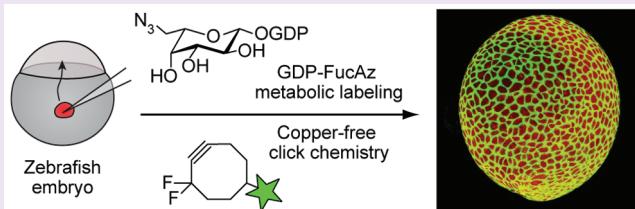
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Supporting Information

ABSTRACT: Many developmental processes depend on proper fucosylation, but this post-translational modification is difficult to monitor *in vivo*. Here we applied a chemical reporter strategy to visualize fucosylated glycans in developing zebrafish. Using azide-derivatized analogues of fucose, we metabolically labeled cell-surface glycans and then detected the incorporated azides *via* copper-free click chemistry with a difluorinated cyclooctyne probe. We found that the fucose salvage pathway enzymes are expressed during zebrafish embryogenesis but that they process the azide-modified substrates inefficiently. We were able to bypass the salvage pathway by using an azide-functionalized analogue of GDP-fucose. This nucleotide sugar was readily accepted by fucosyltransferases and provided robust cell-surface labeling of fucosylated glycans, as determined by flow cytometry and confocal microscopy analysis. We used this technique to image fucosylated glycans in the enveloping layer of zebrafish embryos during the first 5 days of development. This work provides a method to study the biosynthesis of fucosylated glycans *in vivo*.



Fucosylation is essential for proper cell signaling and embryonic development. For example, the Notch receptor must be fucosylated for Notch signaling, which regulates stem cell fate decisions and organogenesis.^{1–3} Fucose also resides at the periphery of N- and O-glycans in structures such as Lewis x, an epitope also known as stage-specific embryonic antigen 1 (SSEA-1) that is found in mouse early embryogenesis.^{4,5} Lewis x is also present in the developing brain, where it is thought to be important for cell–cell interactions.^{4,6,7} In zebrafish, a mutant that is deficient in the *de novo* biosynthetic pathway for fucose exhibits neural migration defects.⁸ Mouse knockouts of fucose biosynthetic genes and fucosyltransferases have demonstrated that the sugar is necessary for diverse aspects of embryogenesis, organogenesis, and neurogenesis.^{2,9–12} However, determining the actual functions of fucosylation during these processes would benefit from an ability to analyze the fucose modification in a living organism.

Here we report a method for imaging fucosylated glycans *in vivo*. We chose the zebrafish as our model organism due to its well-defined developmental program, optically transparent embryos, and ease of microinjection with exogenous reagents.¹³ We applied the bioorthogonal chemical reporter strategy¹⁴ for fucose labeling, which we have previously used to image sialic acids and mucin-type O-glycans in developing zebrafish.^{15,16} In this strategy, azide-functionalized monosaccharides are incorporated into cell-surface glycans *via* the cells' own metabolic machinery. The azide is subsequently reacted with an imaging probe or pull-down reagent *via* copper-free click chemistry.¹⁷

Specifically, we utilized several analogues of fucose modified at the C6-position with an azide (FucAz, Figure 1). In mammalian cell culture, FucAz traverses endogenous metabolic pathways and is incorporated into cell-surface glycans.^{18,19} FucAz is utilized by the fucose salvage pathway, where it is first converted to FucAz-1-phosphate (FucAz-1-P) by fucose kinase (FUK) and then to GDP-FucAz by fucose-1-phosphate guanyltransferase (FPGT) (Figure 1). GDP-FucAz is transported into the Golgi apparatus, where it serves as a substrate for fucosyltransferases (FucTs) that install FucAz onto glycoproteins. At the cell surface, the azide-labeled glycans can be imaged *via* reaction with a difluorinated cyclooctyne reagent conjugated to Alexa Fluor 488 (DIFO-488).²⁰

For this strategy to be feasible in zebrafish, the salvage pathway and fucosyltransferase enzymes must be expressed in the embryo. The fucose salvage pathway, which is present in mammals but absent from *Drosophila*,²¹ has not been characterized in zebrafish, although salvage pathway candidate genes are registered in GenBank (accession numbers XP_001344272 and NP_0010-18590). The zebrafish mutant that is deficient in the *de novo* biosynthetic pathway for fucose retains the ability to fucosylate Notch, but its neural migration defect at 48 h postfertilization (hpf) is not rescued by injection of fucose.⁸ These observations suggest that fucose salvage occurs during early embryogenesis

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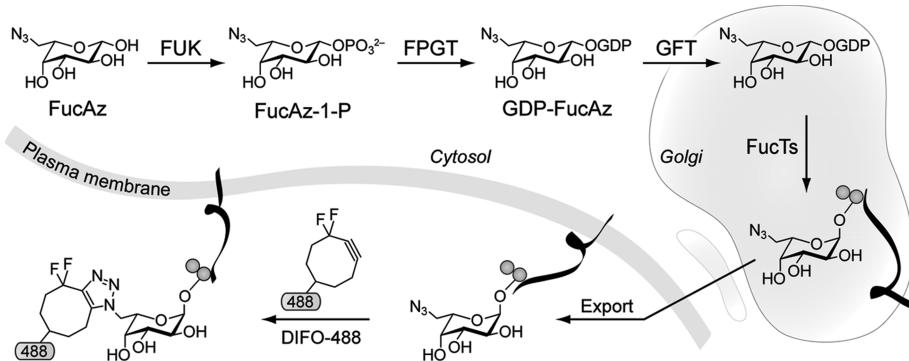


Figure 1. Pathway for metabolic labeling of fucosylated glycans using 6-azido fucose (FucAz). In the fucose salvage pathway, FucAz is converted to FucAz-1-phosphate (FucAz-1-P) and then to GDP-FucAz by the sequential actions of fucose kinase (FUK) and fucose-1-phosphate guanyltransferase (FPGT). GDP-FucAz is then transported into the Golgi lumen by the GDP-fucose transporter (GFT). Within the Golgi compartment, fucosyltransferases (FucTs) add FucAz to glycoproteins, which are then exported to the cell surface or secreted. To image FucAz incorporated into cell-surface glycans, the azide is reacted with an Alexa Fluor 488 conjugate of DIFO (DIFO-488).

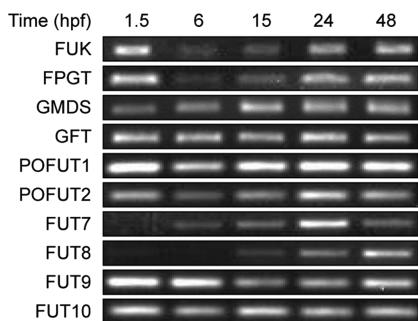


Figure 2. Expression of fucosylation pathway proteins during zebrafish embryogenesis. Transcripts were detected by RT-PCR analysis at various developmental stages (hpf, hours postfertilization). FUK, fucose kinase; FPGT, fucose-1-phosphate guanyltransferase; GMDS, GDP-mannose 4,6-dehydratase; GFT, Golgi GDP-fucose transporter; POFUT1 and 2, protein O-fucosyltransferases; FUT7–10, various fucosyltransferases.

but perhaps at very low levels. Indeed, in mammalian cell lines, fucose salvage has been shown to account for only 10% of total fucosylation.²²

We began our analysis by directly analyzing the expression of fucose salvage pathway enzymes. We used RT-PCR to detect mRNA transcripts at several stages of development (Figure 2). We found that both salvage pathway enzymes, FUK and FPGT, had detectable transcripts at the 16–32-cell stage, before zygotic transcription begins,²³ indicating that these transcripts are provided maternally. Zygotic expression of FUK and FPGT was detectable after midsegmentation stages. In contrast, transcripts for GDP-mannose 4,6-dehydratase (GMDS), a key enzyme in the *de novo* biosynthesis of GDP-fucose,²⁴ were detected at all of the stages tested. Transcripts of the Golgi GDP-fucose transporter GFT were found at all stages as well.

We also examined the expression of putative fucosyltransferase genes that are predicted from genome analysis.⁸ The corresponding enzymes are classified on the basis of the type of fucosyl linkage that they are predicted to generate. The protein O-fucosyltransferases POFUT1 and POFUT2, which catalyze the addition of fucose to the hydroxyl groups of serine and threonine residues, were expressed at all of the stages tested. The former is required for Notch signaling,² and the latter fucosylates

thrombospondin type I repeats found in many extracellular matrix proteins from *Caenorhabditis elegans* to humans.²⁵ Among the predicted α 1,3-fucosyltransferases, which catalyze the addition of fucose to terminal glycan structures such as Lewis x, FUT9 was expressed at all of the stages tested, and FUT7 was expressed from 6 hpf onward. These results are consistent with existing *in situ* expression data for POFUT1 and FUT9.²⁶ Two additional α 1,3-fucosyltransferases in zebrafish, FT1 and FT2, have been shown to synthesize Lewis x *in vitro* and are expressed only at 15–18 and 72 hpf, respectively.²⁷ Additionally, the predicted core N-glycan α 1,3-fucosyltransferase FUT10 was expressed at all of the stages tested, and transcripts of the core N-glycan α 1,6-fucosyltransferase FUT8 were detected from 15 hpf onward. The presence of these transcripts, along with those of the salvage pathway and *de novo* biosynthetic enzymes, suggests that fucosylated glycans are synthesized during early development and that the necessary machinery for metabolic labeling is present.

Encouraged by these expression data, we attempted to label fucosylated glycans by treatment with FucAz. We bathed embryos in medium containing cell-permeable, per-O-acetylated FucAz for 2 days and then reacted the embryos with DIFO-488 to visualize FucAz that had been incorporated into cell-surface glycans. However, these embryos exhibited no azide-dependent cell-surface labeling (Supplementary Figure 1).

This result suggested that one of the proteins in the biosynthetic pathway might not accept the unnatural azide-modified substrate. Because human FucTs can tolerate large modifications at the C6 position of fucose,^{28,29} we suspected that one of the salvage pathway enzymes or the Golgi GDP-fucose transporter formed a bottleneck. We therefore sought to systematically bypass enzymes in the pathway by metabolic labeling with the downstream intermediates FucAz-1-P and GDP-FucAz. Because these reagents are not cell-permeable, we microinjected them into the yolk of 1–8-cell zebrafish embryos. At this stage, small molecules injected into the yolk are taken up into the first cells of the organism and subsequently distributed to all of the daughter cells over the course of development.^{30,16}

We previously demonstrated that microinjection of zebrafish embryos with other azidosugars followed by reaction with DIFO-488 enables fluorescence imaging of cell-surface glycans in the enveloping layer.¹⁶ Using a similar method, embryos were microinjected with FucAz-1-P, GDP-FucAz, or vehicle alone and

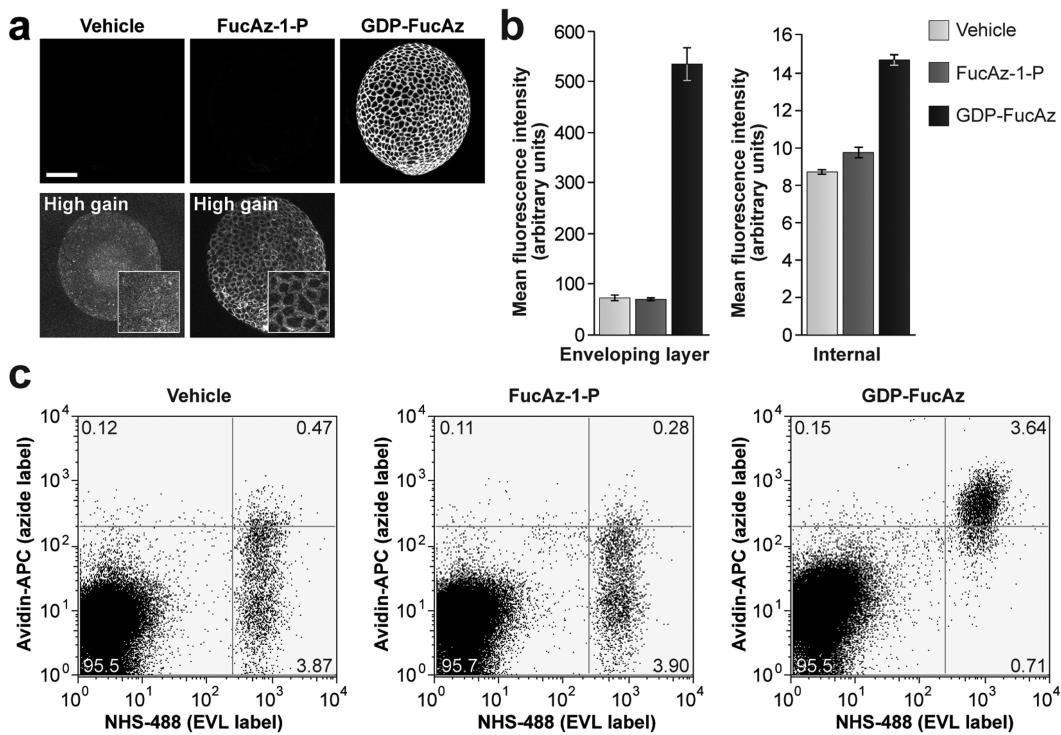


Figure 3. GDP-FucAz metabolically labels cell-surface glycans of the enveloping layer more efficiently than FucAz-1-P. (a) Fluorescence images of embryos microinjected with GDP-FucAz or FucAz-1-P. Zebrafish embryos were microinjected with 75 pmol of GDP-FucAz, FucAz-1-P, or vehicle alone, allowed to develop to 10 hpf, and reacted with DIFO-488. Shown are z-projection images of DIFO-488 fluorescence in the enveloping layer (EVL). Top: laser and detector gain settings optimized for fluorescence of GDP-FucAz-treated embryos. Bottom: laser and detector gain settings optimized for fluorescence of FucAz-1-P-treated embryos. Scale bar: 200 μ m. (b) Flow cytometry analysis of EVL and internal cells after metabolic and chemical labeling. Zebrafish embryos were microinjected with 75 pmol of the indicated sugar and allowed to develop to 15 hpf. Cells of the EVL were labeled with NHS-488. The embryos were dissociated, and the resulting cell suspension was reacted with DIFO-biotin, incubated with avidin-APC, and analyzed by flow cytometry. Error bars represent standard error for three replicates. (c) FucAz labeling is most pronounced on EVL cells (detected with NHS-488). Shown are representative flow cytometry plots corresponding to data shown in panel b. Each plot includes at least 50,000 cells. Numbers in the corners of quadrants represent the percent of total.

were allowed to develop for 10 h. The embryos were then bathed in a solution of DIFO-488 to react with azide-labeled glycans in the enveloping layer and imaged by confocal microscopy.

We observed robust cell-surface DIFO-488 fluorescence in the enveloping layer of embryos injected with GDP-FucAz and no detectable signal from embryos injected with vehicle alone (Figure 3, panel a, top). This result is consistent with a recent report of metabolic labeling using an alkynyl derivative of fucose.³¹ Embryos injected with FucAz-1-P displayed weak cell-surface fluorescence that was detectable only using higher laser power and detector gain (Figure 3, panel a, bottom). This result suggests that conversion of FucAz-1-P to GDP-FucAz is inefficient in zebrafish. Even when the FucAz-1-P-injected embryos were allowed to develop for 24–120 hpf before reaction with DIFO-488, negligible cell-surface fluorescence was observed (Supplementary Figure 2). This observation contrasts with the relatively efficient enzymatic conversion of azide-modified sialic acid precursors to sialosides in mammalian systems.³²

For a more quantitative comparison of the efficiencies of FucAz-1-P and GDP-FucAz metabolism, we analyzed cells from metabolically labeled and then dissociated zebrafish embryos using flow cytometry. Embryos were microinjected with FucAz-1-P, GDP-FucAz, or vehicle alone and were then allowed to develop to 15 hpf. The embryos were incubated with the *N*-hydroxysuccinimidyl ester of Alexa Fluor 488 (NHS-488), a charged dye that reacts with

amines on the organism's surface, to specifically label cells of the enveloping layer. The embryos were then dissociated by treatment with ethylenediaminetetraacetic acid (EDTA), the cell suspension was labeled with DIFO-biotin followed by avidin-allophycocyanin (avidin-APC), and cellular fluorescence was quantified by flow cytometry.

Enveloping layer cells (identified by their high NHS-488 fluorescence) from GDP-FucAz-injected embryos displayed over 7-fold higher avidin-APC signal than cells from embryos injected with FucAz-1-P or vehicle alone (Figure 3, panel b, left). This result is consistent with the imaging analysis of the enveloping layer. Cells from the interior of the organism (identified by their low NHS-488 fluorescence) also exhibited avidin-APC signal that was higher in GDP-FucAz-injected embryos than in FucAz-1-P- or vehicle-injected embryos (Figure 3, panel b, right). This observation indicates that internal cells also incorporate FucAz into their glycans by 15 hpf, although apparently to a lesser extent than enveloping layer cells (Figure 3, panel c). However, a direct comparison of enveloping layer and internal cells by flow cytometry is difficult because the analysis involves a heterogeneous population with different cell types, sizes, and granularities.

The ability of GDP-FucAz to label fucosylated glycans implies that this unnatural substrate enters the Golgi lumen and is utilized by fucosyltransferase enzymes. Although the mechanism of entry into the secretory pathway is unknown, it is expected to

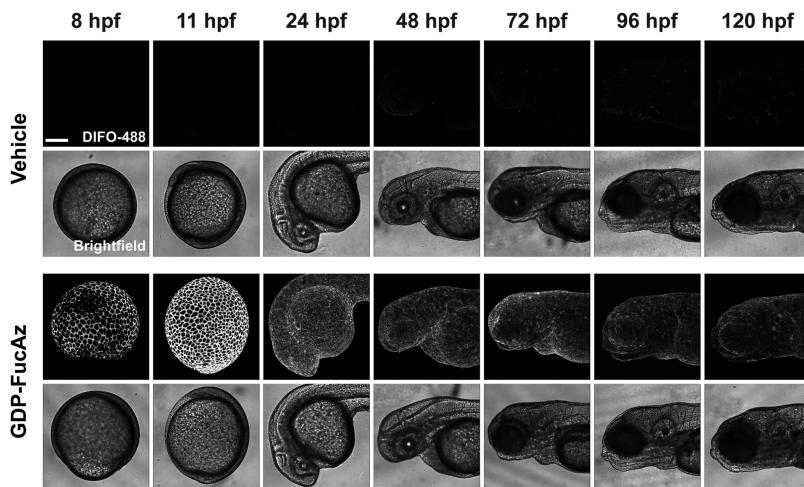


Figure 4. Microinjection of GDP-FucAz followed by copper-free click chemistry enables imaging of fucosylated glycans during the first 5 days of development. Zebrafish embryos were microinjected with vehicle alone (top) or 75 pmol of GDP-FucAz (bottom), allowed to develop, then treated with DIFO-488, and imaged at the time indicated. Shown are z-projection DIFO-488 fluorescence images of the EVL (rows 1 and 3) and corresponding brightfield images (rows 2 and 4). Scale bar: 200 μ m.

occur *via* active transport by the Golgi GDP-fucose transporter GFT.³³ We sought to determine whether GDP-FucAz labeling was dependent on GFT expression. Using a translation-blocking morpholino oligonucleotide, we suppressed GFT expression and observed no decrease in DIFO-derived signal in embryos injected with the GFT morpholino compared to control embryos (Supplementary Figure 3). This result suggests that there may be a second GDP-fucose transporter in zebrafish. Indeed, the zebrafish genome contains a homologue of Slc35c2, which has been proposed in recent studies to be a second GDP-fucose transporter in mammalian cells.³⁴

The lack of labeling by FucAz-1-P, in contrast to the robust labeling with GDP-FucAz, suggests that the salvage pathway enzyme FPGT does not tolerate the 6-azido modification. Indeed, human FPGT is 160-fold less efficient when using an analogue of fucose extended at the C6 position by a methyl group as a substrate.³⁵ This observation may also explain the weak labeling by FucAz in cultured mammalian cells.^{18,19} Alternatively, although RT-PCR analysis showed that transcripts for the salvage pathway enzymes were present, this method is not quantitative, and it is possible that salvage pathway enzyme levels were not high enough to yield efficient labeling. Notably, FucAz is toxic to mammalian cells in culture;¹⁸ however, we did not observe any adverse effects on zebrafish embryos during incubation with 5 mM per-O-acetylated FucAz in the medium or after microinjection of up to 125 pmol of FucAz-1-P or GDP-FucAz.

We used GDP-FucAz as a metabolic substrate to determine the onset and extent of fucose labeling during early development. Embryos were microinjected with GDP-FucAz or vehicle alone, then reacted with DIFO-488, and imaged at several stages of development. Cell-surface labeling of the enveloping layer was observed as early as 65% epiboly, or 7 hpf (Supplementary Figure 4). The labeling intensity increased from midgastrulation to early segmentation periods (Figure 4). These dynamics are similar to those observed for expression of mucin-type O-glycans¹⁶ and N-glycans,³⁶ both of which can be modified by fucosylation. Fluorescence derived from FucAz-labeled glycans in the enveloping layer decreased from 24 hpf onward (Figure 4). This decrease in signal could be due to depletion or degradation of the GDP-FucAz

substrate, or it could reflect decreasing levels of endogenous fucosylation. Indeed, labeling of fucosylated glycans in the enveloping layer by the fucose-binding *Aleuria aurantia* lectin (AAL) also decreased from 12 to 24 hpf, as determined by flow cytometry analysis (Supplementary Figure 5). Nevertheless, signal from FucAz-labeled glycans remained strong enough to observe FucAz-dependent signal over the first 5 days of development.

During these studies we observed no toxicity or developmental defects due to FucAz incorporation into zebrafish glycans. This may suggest that the azide modification does not interfere with the normal functionality of fucosylated glycans or that FucAz replaces a small enough fraction of natural fucose that its biological effects are indiscernible. The efficiency of the subsequent reaction of cell-surface azides with DIFO-488 is enhanced by the intrinsically high bimolecular rate constant of copper-free click chemistry²⁰ and the molar excess of DIFO utilized in these experiments. Thus, metabolic labeling with FucAz followed by copper-free click chemistry is an effective and nontoxic method for imaging fucosylated glycans *in vivo*.

In conclusion, we have successfully applied the chemical reporter strategy to enable visualization of fucosylation during zebrafish development. During these studies, we discovered that the fucose salvage pathway is expressed in zebrafish embryos but does not process azide-modified substrates efficiently. In contrast, fucosyltransferase enzymes readily utilize GDP-FucAz, thereby incorporating the azide into newly synthesized glycoconjugates. In addition to its use in imaging applications, the azide modification can be exploited for isolation and identification of fucosylated glycoproteins during development.

METHODS

Metabolic Labeling of Zebrafish by Microinjection of GDP-FucAz and FucAz-1-P. Zebrafish embryos at the 1–8-cell stage were microinjected into the yolk with 1–5 nL of 25 or 50 mM solutions of GDP-FucAz, FucAz-1-P, or no sugar (for doses of 75–125 pmol) in 0.2 M KCl, with rhodamine-dextran (5% w/v) or phenol red (0.05% w/v) as tracer dyes. Once the embryos developed to 4 hpf, they were enzymatically dechorionated by incubating for 10 min in a 1 mg mL⁻¹ solution of

pronase in embryo medium (150 mM NaCl, 0.5 mM KCl, 1.0 mM CaCl₂, 0.37 mM KH₂PO₄, 0.05 mM Na₂HPO₄, 2.0 mM MgSO₄, 0.71 mM NaHCO₃ in deionized water, pH 7.4). All embryos younger than 12 hpf were maintained in 1% agarose-coated dishes. Embryos older than 24 hpf were incubated in embryo medium containing 131 μM N-phenylthiourea (PTU) to inhibit melanin production.

Detection of Cell-Surface Glycans by Copper-Free Click Chemistry and Confocal Microscopy. Embryos were reacted with 100 μM DIFO-488 in embryo medium for 1 h at 28.5 °C. The embryos were rinsed by transfer through six successive 15-cm tissue culture dishes containing embryo medium. For imaging by confocal microscopy, the embryos were mounted between two coverslips in a solution of 0.6% low melting point agarose in embryo medium. For embryos 24 hpf and older, 2.6 μM tricaine (ethyl 3-aminobenzoate methanesulfonate) was included in the medium and agarose for mounting.

Flow Cytometry of Zebrafish Embryos. Zebrafish embryos were microinjected with 75 pmol of the indicated sugar and phenol red as described. At 15 hpf, the embryos were reacted with NHS-488 (50 μM in embryo medium with 1% DMSO) for 30 min at 28.5 °C. After this reaction, the embryos were rinsed four times in embryo medium and were transferred to calcium-free Ringer's solution (116 mM NaCl, 2.6 mM KCl, 5 mM HEPES in deionized water, pH 7.0) and deyolked by passage through a 200-μL pipet tip. The embryos were then incubated for 15 min at 28.5 °C in a solution of 5 mM EDTA in phosphate-buffered saline (PBS), pH 7.6, to dissociate the cells. The reaction was stopped by the addition of 5% fetal bovine serum (FBS) and 1 mM CaCl₂ in PBS. The cells were rinsed once with labeling buffer (PBS, pH 7.6, 1% FBS). The cell suspension was reacted with DIFO-biotin (100 μM in labeling buffer) for 30 min at 28.5 °C. Following this reaction, the cells were rinsed once with labeling buffer and then incubated with avidin-APC (50 μg/mL in labeling buffer) for 15 min on ice. The cells were then rinsed and reacted a second time for 30 min with avidin-APC. The cells were washed three times and resuspended in 300 μL of labeling buffer and passed through a 35-μm filter before analysis by flow cytometry.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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