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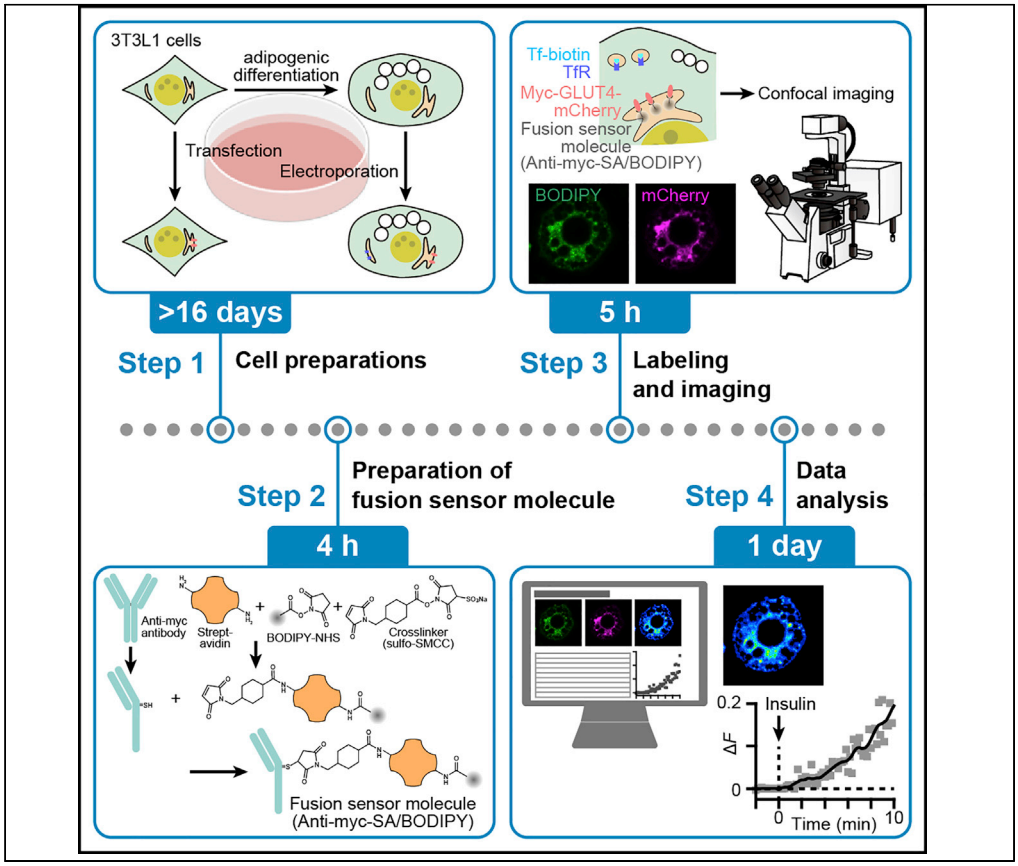
Protocol for preparing sensor molecules and analyzing heterotypic endomembrane fusion in insulin-responsive cells using live-cell imaging



Heterotypic endomembrane fusion between static GLUT4-containing vesicles and traveling transferrin receptor-containing endosomes triggers insulin-responsive translocation of the GLUT4 glucose transporter. Here, we provide a protocol for preparing BODIPY-based fluorescent sensor molecules allowing detection of heterotypic endomembrane fusion through dequenching via streptavidin-biotin binding and ratiometrically analyzing insulin-responsive events with live-cell imaging. Although this protocol is for evaluating specific fusion processes relating GLUT4 translocation, it is also applicable to assessing other processes so long as sensor molecules can properly label target molecules.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Heterotypic endomembrane fusion triggers insulin-responsive GLUT4 translocation

Preparing BODIPY-based sensor showing dequenching via streptavidin-biotin binding

Ratiometric analysis of insulin-responsive heterotypic endomembrane fusion events

Applicable to assessing other endomembrane fusion processes based on our scheme

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Protocol for preparing sensor molecules and analyzing heterotypic endomembrane fusion in insulin-responsive cells using live-cell imaging

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SUMMARY

Heterotypic endomembrane fusion between static GLUT4-containing vesicles and traveling transferrin receptor-containing endosomes triggers insulin-responsive translocation of the GLUT4 glucose transporter. Here, we provide a protocol for preparing BODIPY-based fluorescent sensor molecules allowing detection of heterotypic endomembrane fusion through dequenching via streptavidin-biotin binding and ratiometrically analyzing insulin-responsive events with live-cell imaging. Although this protocol is for evaluating specific fusion processes relating GLUT4 translocation, it is also applicable to assessing other processes so long as sensor molecules can properly label target molecules.

For complete details on the use and execution of this protocol, please refer to Hatakeyama et al. (2022).

BEFORE YOU BEGIN

GLUT4 is the glucose transporter that mediates insulin-dependent facilitation of glucose uptake in cells displaying the highest levels of insulin-responsive glucose uptake including adipocytes and skeletal muscle cells in which this action is demonstrated by insulin-responsive translocation from a specialized intracellular pool called the GLUT4 storage compartment to the plasma membrane. Nanometric assessment of intracellular GLUT4 behavior in comparison to that of the transferrin receptor (TfR), which is a general recycling protein that does not show as much insulin-responsive translocation as GLUT4, demonstrated that, in the absence of insulin stimulation, most GLUT4 molecules are statically sequestered and show spatially restricted movement especially around the perinuclear trans-Golgi network region (Fujita et al., 2010; Hatakeyama and Kanzaki, 2011, 2017). Upon insulin stimulation, such statically sequestered GLUT4-containing vesicles undergo "heterotypic endomembrane fusion" with traveling endosomes such as TfR-containing endosomes that are briskly recycled among the plasma membrane, early/sorting endosomes, and endocytic recycling compartment, leading to liberation of the static GLUT4 and incorporation into a trafficking itinerary involving the fused endosomes (Hatakeyama and Kanzaki, 2017; Hatakeyama et al., 2022). Heterotypic endomembrane fusion is thus the essential first trigger for insulin-responsive GLUT4 redistribution, indicating the importance of accurate measurement of this process. The protocol below describes specific steps for preparing our fusion sensor molecule, streptavidin/BODIPY-conjugated anti-myc antibody (hereafter anti-myc-SA/BODIPY) and analyzing heterotypic endomembrane fusion between GLUT4-containing vesicles and TfR-containing endosomes in cells displaying insulin-responsive liberation of static GLUT4, i.e., 3T3L1 adipocytes and the reconstitution model of

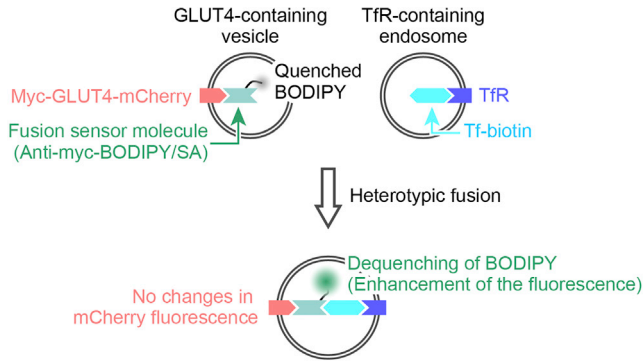

Figure 1. Schema for the fusion experiments

Prior to stimulation, myc-GLUT4-mCherry and transferrin receptor (TfR) are labeled with the fusion sensor molecule anti-myc-streptavidin/BODIPY and transferrin-biotin (Tf-biotin), respectively. After heterotypic fusion between myc-GLUT4-mCherry-containing vesicles and transferrin receptor-containing endosomes, biotin-streptavidin binding dequenches BODIPY fluorescence, such that increases in BODIPY fluorescence can be observed without changes in mCherry fluorescence.

insulin-responsive GLUT4 behavior in 3T3L1 fibroblasts. We basically utilized cells exogenously expressing myc-GLUT4-mCherry, with additional expression of either 1) TfR to accurately evaluate the overall endosomal trafficking properties that TfR normally exhibits in adipocytes (Shigematsu et al., 2003) in which endogenous TfR expression is markedly reduced upon adipogenic differentiation (El-Jack et al., 1999) or 2) HA-sortilin and HaloTag-AS160 in fibroblasts to prepare the reconstitution model that shows insulin-responsive liberation of static GLUT4 (Hatakeyama and Kanzaki, 2011). The cells were first labeled with the fusion sensor molecule, and after allowing the labeled myc-GLUT4-mCherry to recycle back to their stationary compartments, TfR was labeled with biotinylated transferrin (hereafter Tf-biotin). Our fusion sensor molecule is based on enhancement/dequenching of the fluorescence of BODIPY; the fluorescence of BODIPY in fusion sensor molecules is quenched by energy transfer from streptavidin, and the binding of biotin in Tf-biotin to streptavidin in fusion sensor molecules leads to dequenching (i.e., enhancement) of BODIPY fluorescence (Emans et al., 1995). When heterotypic endomembrane fusion occurs between GLUT4-containing vesicles and TfR-containing endosomes, BODIPY fluorescence increases, whereas mCherry fluorescence remains unchanged (Figure 1). The fluorescence of BODIPY and mCherry as well as biotin–streptavidin binding is known to be highly resistant to a wide range of pH change (Chaiet and Wolf, 1964; Shaner et al., 2004; Urano et al., 2009). Moreover, the dequenching property of streptavidin-conjugated BODIPY through biotin binding was reported to be insensitive to changes in pH (Emans et al., 1995). Therefore, we calculated the fusion index, based on the fluorescence ratio of BODIPY and mCherry which reflects the occurrence of heterotypic endomembrane fusion but not pH changes along the endosomal pathway. Although the present study shows an application that allows the user to assess specific heterotypic endomembrane fusion occurring between GLUT4-containing vesicles and TfR-containing endosomes, it merits emphasis that the present technique is also applicable to assessing other fusion processes involving endomembrane systems so long as the vesicles of interest can be properly labeled with SA/BODIPY and biotin.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Streptavidin	ProSpec	Cat#PRO-791
BODIPY FL-NHS	Thermo Fisher Scientific	Cat#D2184
DMSO, anhydrous	Thermo Fisher Scientific	Cat#D12345

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
2-Mercaptoehylamine-HCl	Thermo Fisher Scientific	Cat#20408
Sulfo-SMCC	Thermo Fisher Scientific	Cat#A39268
Transferrin-biotin	Thermo Fisher Scientific	Cat#T23363
Cellmatrix Type IV collagen	Nitta gelatin	Cat#638-05921
Insulin	Sigma	Cat#I5500
Dexamethasone	Sigma	Cat#D1756
IBMX	Sigma	Cat#I7018
DMEM, high glucose	Wako	Cat#044-29765
RPMI1640 medium	Wako	Cat#189-02025
Ultra low-IgG fetal bovine serum	Thermo Fisher Scientific	Cat#16250078
Lipofectamine 3000 transfection reagent	Thermo Fisher Scientific	Cat#L3000008
Experimental models: Cell lines		
3T3L1 cells	ATCC	Cat#CCL-92.1, RRID: CVCL_0123
Myc 1-9E10.2 hybridoma cells	ATCC	Cat#CRL-1729, RRID: CVCL_G671
Recombinant DNA		
Myc-GLUT4-mCherry	Hatakeyama et al., 2022	N/A
Transferrin receptor	Fujita et al., 2010	N/A
HA-sortilin	Hatakeyama and Kanzaki, 2011	N/A
HaloTag-AS160	Kazusa DNA Research Institute	Clone Name: pFN21AA0603, GenBank Accession# AB463305
Software and algorithms		
Fiji ImageJ	NIH	RRID: SCR_002285
ORIGIN	OriginLab	RRID: SCR_014212
Other		
Electroporator	BEX	CUY21EDITII
Plate electrode for adherent cells	BEX	LF513-5
Glass bottom dishes	Matsunami	Cat#D11130H
Amicon Ultra-4 10k MWCO	Merck	Cat#UFC801008
Amicon Ultra-4 100k MWCO	Merck	Cat#UFC810008

MATERIALS AND EQUIPMENT

Alternatives: We purified anti-myc antibodies from the conditioned media of the hybridoma cell culture (step 4), but commercial antibodies can be used as well. However, the antibodies must be dissolved in PBS containing only EDTA to stabilize the half-antibodies to be prepared, with additives such as BSA removed in advance for the conjugation reaction.

Alternatives: Electroporation into 3T3L1 adipocytes was performed with a CUY21EDITII electroporator (BEX) and a plate electrode for adherent cells (LF513-5, BEX). We also performed electroporation with a third-party electroporator and standard cuvettes, but the high cytotoxicity under our protocol conditions made it difficult to conduct efficient experiments. Other electroporators, electrodes and cuvettes can be used as well with optimized conditions. Furthermore, methods for gene delivery, such as viral transfection, can be used. However, efficiently transfecting 3T3L1 adipocytes by lipofection is difficult.

Alternatives: Imaging experiments were performed with a Leica SP8 confocal microscope system equipped with an inverted microscope, a 63× oil-immersion objective, a white-light pulsed laser and hybrid detectors. BODIPY and mCherry fluorescence were excited at 488 nm and 561 nm and collected at 495–565 nm and 580–650 nm, respectively, with the hybrid detectors by line-sequential scanning with a 2× line average. The pixel size is typically set around 0.144 μm/pixel. Other confocal systems can be used so long as they are capable of similar excitation and fluorescence collection.

Alternatives: Stage and lens heaters (TOKAI HIT) were used for imaging experiments. Under our conditions, the temperature in the vicinity of the specimen was approximately 30°C when the heaters were set to 37°C. Any other heaters can be used as well.

Anti-myc antibody stock solution

Reagent	Final concentration	Amount
Anti-myc antibody	10 mg/mL	5 mg
PBS-EDTA	N/A	0.5 mL
Total	**N/A**	**0.5 mL**

Store at 4°C for up to 2 weeks. Purified antibodies can be stored for a long term at –20°C in PBS containing a cryoprotectant such as glycerol, but the cryoprotectant must be removed by ultrafiltration or other standard methods for buffer exchange prior to conjugation reaction. The presence of EDTA is critical for stabilizing half-antibodies to be prepared at step 5 since EDTA prevents oxidation of sulfhydryl groups.

Streptavidin stock solution

Reagent	Final concentration	Amount
Streptavidin	20 mg/mL	5 mg
Milli-Q water	N/A	0.25 mL
Total	**N/A**	**0.25 mL**

Keep in aliquots at –20°C up to 2 years. This reconstitution allows the preparation of a solution of 20 mg/mL streptavidin dissolved in 10 mM potassium phosphate buffer (pH6.5).

BODIPY FL-NHS stock solution

Reagent	Final concentration	Amount
BODIPY FL-NHS	10 mg/mL	5 mg
DMSO, anhydrous	N/A	0.5 mL
Total	**N/A**	**0.5 mL**

Keep in aliquots at –80°C for up to 1 month.

2-Mercaptoethylamine solution

Reagent	Final concentration	Amount
2-Mercaptoethylamine-HCl	60 mg/mL	6 mg
PBS-EDTA	N/A	0.1 mL
Total	**N/A**	**0.1 mL**

Prepare just before use.

Sulfo-SMCC solution

Reagent	Final concentration	Amount
Sulfo-SMCC	5 mg/mL	2 mg
Milli-Q water	N/A	0.4 mL
Total	**N/A**	**0.4 mL**

Prepare just before use.

Insulin stock solution

Reagent	Final concentration	Amount
Insulin	1 mg/mL	1 mg
0.1 N HCl	N/A	1 mL
Total	**N/A**	**1 mL**

Keep in aliquots at –20°C for up to 2 years.

Dexamethasone stock solution		
Reagent	Final concentration	Amount
Dexamethasone	1 mg/mL	1 mg
95% Ethanol	N/A	1 mL
Total	**N/A**	**1 mL**
Keep in aliquots at –20°C for up to 6 months.		

Isobutylmethylxanthine (IBMX) solution		
Reagent	Final concentration	Amount
IBMX	250 mM	5.56 mg
0.35 M KOH	N/A	0.1 mL
Total	**N/A**	**0.1 mL**
Prepare just before use.		

Growth medium			
Reagent	Stock concentration	Final concentration	Amount
DMEM, high glucose	N/A	N/A	500 mL
Calf serum	N/A	10%	50 mL
L-glutamine	200 mM	2 mM	5 mL
Penicillin-Streptomycin	10,000 units/mL penicillin, 10,000 µg/mL streptomycin	100 units/mL penicillin 100 µg/mL streptomycin	5 mL
Store at 4°C for up to 1 month.			

Feed medium			
Reagent	Stock concentration	Final concentration	Amount
DMEM, high glucose	N/A	N/A	500 mL
Fetal bovine serum	N/A	10%	50 mL
L-glutamine	200 mM	2 mM	5 mL
Penicillin-Streptomycin	10,000 units/mL penicillin, 10,000 µg/mL streptomycin	100 units/mL penicillin 100 µg/mL streptomycin	5 mL
Store at 4°C for up to 1 month.			

Differentiation medium			
Reagent	Stock concentration	Final concentration	Amount
Feed medium	N/A	N/A	25 mL
Insulin	1 mg/mL	1 µg/mL	25 µL
Dexamethasone	1 mg/mL	0.1 µg/mL	2.5 µL
IBMX	250 mM	0.5 mM	50 µL
IBMX solution should be prepared just before use.			

Insulin medium			
Reagent	Stock concentration	Final concentration	Amount
Feed medium	N/A	N/A	25 mL
Insulin	1 mg/mL	1 µg/mL	25 µL
Prepare just before use.			

Serum-free medium			
Reagent	Stock concentration	Final concentration	Amount
DMEM, high glucose	N/A	N/A	500 mL
L-glutamine	200 mM	2 mM	5 mL
Penicillin-Streptomycin	10,000 units/mL penicillin, 10,000 μg/mL streptomycin	100 units/mL penicillin 100 μg/mL streptomycin	5 mL
Store at 4°C for up to 1 month.			

Antibody collection medium			
Reagent	Stock concentration	Final concentration	Amount
RPMI1640 medium	N/A	N/A	500 mL
Ultra-low IgG fetal bovine serum	N/A	2%	10 mL
Store at 4°C for up to 1 month.			

Other solutions	
Name	Reagents
Electroporation buffer	150 mM trehalose, 5 mM potassium phosphate buffer, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM ATP, 25 mM HEPES-KOH, 1% DMSO, pH 7.3
Imaging buffer	150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-NaOH, 5.5 mM D-glucose, pH7.4
Sodium bicarbonate buffer	100 mM $NaHCO_3$
PBS	100 mM sodium phosphate, 150 mM NaCl, pH7.2
PBS-EDTA	PBS, 5 mM EDTA
Tris-HCl	1 M Tris, Adjust pH to 8.5 with HCl
Glycine buffer	0.2 M Glycine, Adjust pH to 2.5 with HCl

Perifusion setup

In order to apply various types of solutions to the cells during imaging experiments, we set up a manual perifusion system on the microscope, which consists of a syringe for applying intended solutions, a circulating aspirator (Shibata Scientific; Cat#WJ-20) for aspirating excess solution, glass pipettes, tubings, and tube clampers (NARISHIGE; Cat#CAT-1) for holding glass pipettes and tubings (Figure 2). Other systems can be used.

STEP-BY-STEP METHOD DETAILS

Cell preparations

Timing: 3T3L1 adipocytes, at least 14 days for differentiation and 1–2 days for electroporation; the reconstitution models in 3T3L1 fibroblasts, 2 days

This part of the protocol describes preparation of cell samples.

1. Preparation of collagen IV-coated glass bottom dishes.
   a. Dilute Cellmatrix Type IV collagen to 10× with 1 mM HCl.
   b. Cover the glass surface of the glass-bottom dishes with 150 μL of diluted Cellmatrix Type IV collagen solution.
   c. Keep the glass-bottom dishes for 1 h at 20°C–25°C.
   d. Remove excess fluid from the coated surface and allow them to dry for 16–20 h at 4°C.
   e. Rinse with PBS twice before introducing cells and medium.
2. Preparation of 3T3L1 adipocytes (Figure 3) Troubleshooting 1.
   a. Seed 2×10^5 undifferentiated 3T3L1 fibroblasts in growth medium into a 150-mm dish (day 0).
   b. Incubate the cells at 37°C at 8% CO_2 for 8 days with medium change at day 4.

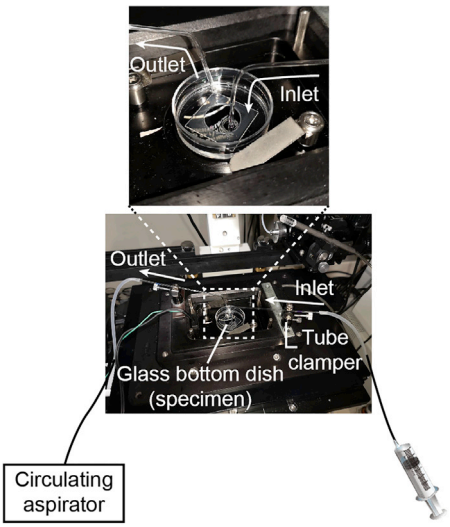

Figure 2. Perifusion setup

Note: Differentiation and maintenance of differentiation ability of 3T3L1 cells appears to be better under CO_2 concentrations of 8% than 5%.

c. At day 8, switch to differentiation medium, and incubate the cells at 37°C at 8% CO_2 for 4 days.
d. At day 12, switch to insulin medium, and incubate the cells at 37°C at 8% CO_2 for at least 2 days.
e. Confirm the accumulation of lipid droplets in the cells with brightfield observation.
f. Harvest the differentiated adipocytes.
g. Dilute the cell suspension to 4×.
h. Seed the cells (200 μL each) onto collagen IV-coated glass bottom dishes.
i. Incubate the cells at 37°C at 8% CO_2 for 1 day.
j. Dilute each 10–25 μg of myc-GLUT4-mCherry and TfR plasmid DNAs into 100 μL of electroporation buffer in total.
k. Remove the culture medium and add the plasmid solution.
l. Apply electroporation pulses with an electrode as follows: Poration pulse at 200 V for 10 ms, followed by five driving pulses at –30 V for 10 ms at 50-ms intervals.

Note: This solution can be reused for electroporation into at least three glass bottom dishes.

Note: Electroporation for other types of the cells can be performed according to previous reports (Matsubara et al., 2006; Tsai et al., 2009; Usui et al., 2000) and manufacturer's website (https://www.bexnet.co.jp/english/).

m. Add 2 mL of feed medium.
n. Incubate cells for 1–2 days.

3. Preparation of the reconstitution model in 3T3L1 fibroblasts.
   a. Seed 8×10^4 3T3L1 fibroblasts in growth medium into a 35-mm glass bottom dish, and incubate the cells at 37°C at 8% CO_2 for 1 day.
   b. Transfect each 1 μg/dish of myc-GLUT4-mCherry, HA-sortilin and HaloTag-AS160 plasmid DNAs using Lipofectamine 3000 transfection reagent as follows. The case of transfection into 1 dish is shown.
      i. Dilute 4.5 μL of Lipofectamine 3000 reagent in 125 μL of Opti-MEM medium and mix well.
      ii. Dilute 1 μg each of myc-GLUT4-mCherry, HA-sortilin and HaloTag-AS160 plasmid DNAs in 125 μL of Opti-MEM medium, then add 6 μL of P3000 reagent, and mix well.

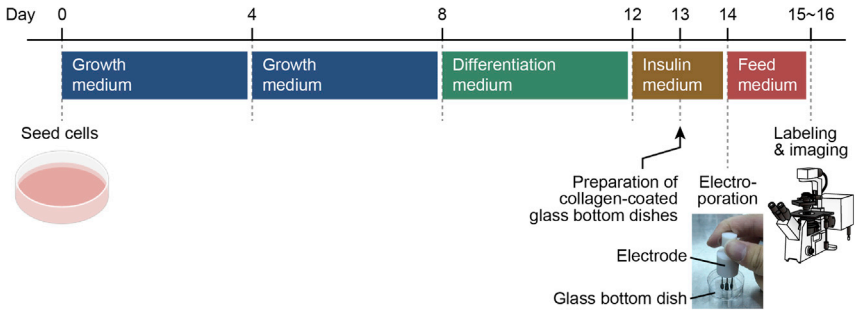

Figure 3. Timeline for differentiation and electroporation in 3T3L1 cells

iii. Add diluted DNA to diluted Lipofectamine 3000 reagent.
iv. Incubate for 5 min at 20°C–25°C.
v. Add the solution to the cells dropwise.
vi. Incubate cells at 37°C at 8% CO_2 for 4 h and change medium.

c. Incubate cells for an additional day.

Preparation of the fusion sensor molecule

Timing: 1 week for antibody purification, 4 h for conjugation reaction

This part of the protocol describes antibody purification and preparation of the fusion sensor molecule anti-myc-SA/BODIPY. Schema for the preparation of the fusion sensor molecule (steps 5–8) are shown in Figure 4. While working with BODIPY fluorescent molecules, minimize light exposure.

4. Purification of anti-myc antibodies from hybridoma cell culture medium.
   a. Maintain MYC 1-9E10.2 hybridoma cells in antibody collection media at 37°C at 5% CO_2 for 4 days.

   Note: Ideally, culture hybridoma cells in vessels that can continuously stir cell suspensions, such as spinner flasks; however, standard culture dishes can also be used. We usually purify anti-myc antibodies using 500 mL of conditioned media per run.

   b. Collect conditioned medium, spin down at 1,000×*g* for 3 min, and keep supernatant.
   c. Remove debris by filtration.
   d. Pour Protein G Sepharose slurry into a column in one continuous motion.
   e. Immediately fill the remainder of the column with PBS and equilibrate the slurry with at least twice column bed volume of PBS.
   f. Apply collected conditioned medium at a flow rate of approx. 2 mL/min.
   g. After applying all conditioned medium, wash the column with at least ten times column bed volume of PBS.
   h. Prepare 6× 1.5 mL tubes containing 50 μL of 1 M Tris-HCl (pH 8.5).
   i. Add 0.2 M glycine buffer (pH 2.5) into the column and collect the eluates (1 mL in each 1.5 mL tube).
   j. Measure absorbance at 280 nm and identify the fractions containing antibodies.
   k. Exchange buffer with PBS-EDTA by ultrafiltration with Amicon Ultra-4 100k MWCO.

   Note: Other standard methods for buffer exchange, such as dialysis or desalting columns, can also be used. However, it may be necessary to concentrate the antibodies after buffer exchange by dialysis or with desalting column; the same applies hereafter.

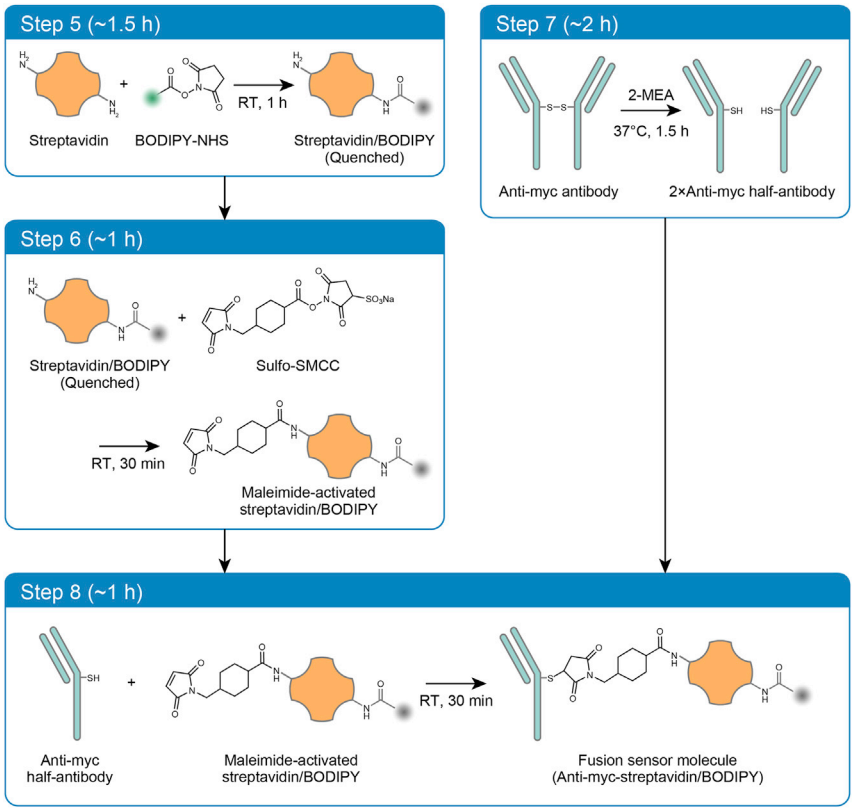

Figure 4. Schema for steps 5–8
Steps 5, 6 and 7 can be carried out concurrently.

⚠ CRITICAL: Presence of EDTA is critical for stabilizing half-antibodies prepared below (step 7) since EDTA prevents oxidation of sulfhydryl groups.

l. Determine antibody concentration, and dilute or concentrate the antibodies to prepare 10 mg/mL solution in PBS-EDTA. Troubleshooting 2.

Note: The specificity and other characteristics of the antibodies produced in this hybridoma are already well established (Evan et al., 1985). Quality can also be checked by western blotting (Nedachi et al., 2009) or immunofluorescence (Hatakeyama and Kanzaki, 2011).

Pause point: The purified antibodies can be used for subsequent reactions immediately or stored short-term at 4°C. Long-term storage at –20°C is also possible if a cryoprotectant (e.g., glycerol) is added to a storage buffer (e.g., PBS), but the cryoprotectant must be removed before subsequent conjugation reactions by ultrafiltration or other standard methods for buffer exchange.

5. Preparation of SA/BODIPY.
   a. Exchange buffer in streptavidin stock solution for sodium bicarbonate buffer through ultrafiltration using Amicon Ultra-4 10k MWCO. After buffer exchange, determine streptavidin concentration using absorption at 280 nm with extinction coefficient $\varepsilon_{280}^{1\ mg/mL} = 3.2\ cm^{-1}$ (Waner et al., 2019) and molecular weight of 52 kDa.
   b. Dilute (or concentrate, if required) this streptavidin to 10 mg/mL in sodium bicarbonate buffer.
   c. Add 200 μL (2 mg) of streptavidin (≈38.5 nmol) into a 1.5 mL tube.

d. Add 3 μL of BODIPY FL-NHS (≈77 nmol) to the contents of the tube.
e. Mix with Eppendorf MixMate (or equivalent) at 800 rpm for 1 h at 20°C–25°C.
f. Exchange buffer for PBS through ultrafiltration using Amicon Ultra-4 10k MWCO and determine protein concentration using absorption at 280 nm (A_{280}) and 504 nm (A_{504}) with

$$[Protein]\ (mg/mL) = \frac{A_{280} - 0.04A_{504}}{\epsilon_{280}^{1\ mg/mL}} \quad \text{(Equation 1)}$$

Note: This equation is derived from manufacturer's instructions.

6. Preparation of maleimide-activated SA/BODIPY.
   a. Dissolve sulfo-SMCC in Milli-Q water to 5 mg/mL.

   Note: Do not use buffer containing >50 mM of salts since sulfo-SMCC does not dissolve well in such buffers.

   b. Immediately after a, add 10-fold molar amount of sulfo-SMCC to BODIPY-labeled streptavidin.
   c. Rock gently for 30 min at 20°C–25°C.
   d. Exchange buffer for PBS-EDTA through ultrafiltration using Amicon Ultra-4 10k MWCO and determine protein concentration with equation (1).
7. Preparation of functional anti-myc half-antibodies.

   Note: This step can proceed in parallel with steps 5 and 6.

   Note: 2-Mercaptoethylamine is a mild reducing agent and can selectively cleave hinge-region disulfide bonds of IgG heavy chains without loss of selectivity (Makaraviciute et al., 2016; Sharma and Mutharasan, 2013; Yoshitake et al., 1979). The use of whole IgG should be avoided because labeling with whole IgG, which has two antigen-binding sites, may result in the unintended aggregation of labeled molecules.

   a. Dissolve 6 mg of 2-mercaptoethylamine in 100 μL of PBS-EDTA, and immediately add 1 μL of this 2-mercaptoethylamine solution to each 10 μL volume of 10 mg/mL antibodies.
   b. Rock gently for 1.5 h at 37°C.
   c. Exchange buffer for PBS-EDTA to remove excess 2-mercaptoethylamine through ultrafiltration using Amicon Ultra-4 10k MWCO and determine antibody concentration.
8. Conjugation of SA/BODIPY and anti-myc half-antibodies.
   a. Combine anti-myc half-antibodies and maleimide-activated SA/BODIPY at 1:1.2 molar ratio, which has been determined on the basis of Poisson statistics with the expectation of increasing the 1:1 binding probability (Luchowski et al., 2008).
   b. Rock gently for 30 min at 20°C–25°C.
   c. Exchange buffer for PBS through ultrafiltration using Amicon Ultra-4 10k MWCO.
   d. Determine protein concentration with equation (1) and degree of labeling with

$$Degree\ of\ labeling = \frac{A_{504} \times 52000}{[Protein] \times 68000} \quad \text{(Equation 2)}$$

9. Validation of transferrin-biotin-dependent enhancement of anti-myc-SA/BODIPY *in vitro*.
   a. Prepare 100 nM of anti-myc-SA/BODIPY in PBS.
   b. Prepare 1 μM of Tf-biotin in PBS.
   c. Mix a and b to prepare anti-myc-SA/BODIPY: Tf-biotin = 1:0–32 mixtures as follows.
   d. Rock gently for 10 min at 20°C–25°C.

e. Measure fluorescence intensity with spectrofluorometer (Excitation at 488 nm, Emission at 515 nm). Troubleshooting 3.

Anti-myc-SA/BODIPY and Tf-biotin mixtures for *in vitro* analysis

Reagent	1:0	1:0.3	1:1	1:2	1:3	1:4	1:10	1:20
PBS	90 μL	89.7 μL	89 μL	88 μL	87 μL	86 μL	80 μL	70 μL
100 nM Anti-myc-SA/BODIPY	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL	10 μL
1 μM Tf-biotin	0 μL	0.3 μL	1 μL	2 μL	3 μL	4 μL	10 μL	20 μL
Total	**100 μL**	**100 μL**	**100 μL**	**100 μL**	**100 μL**	**100 μL**	**100 μL**	**100 μL**

⚠ CRITICAL: Streptavidin labeling with BODIPY and antibody conjugation in this order is critical for proper fusion sensor preparation. When antibody conjugation to streptavidin precedes BODIPY labeling, there will be no increases in BODIPY fluorescence in response to the addition of biotin due to insufficient quenching of BODIPY (see expected outcomes).

Pause point: The prepared anti-myc-SA/BODIPY can be used immediately, stored short-term at 4°C, or stored long-term at –20°C with addition of a cryoprotectant (e.g., glycerol).

Labeling of myc-GLUT4-mCherry and TfR, and imaging

Timing: 4 h for labeling, 1 h for imaging/sample

This part of the protocol describes labeling myc-GLUT4-mCherry and TfR with anti-myc-SA/BODIPY and Tf-biotin, respectively, and acquiring fluorescent images of BODIPY and mCherry. For fluorescent imaging, 12-bit images were acquired with a Leica SP8 confocal microscope system equipped with an inverted microscope, a 63× oil-immersion objective, a white-light pulsed laser and hybrid detectors as a t-series at intervals of 10 s. BODIPY and mCherry fluorescence are acquired by line-sequential unidirectional scanning at 400 Hz with a 2× line average. Excitations are at 488 nm and 561 nm, and fluorescence values are collected at 495–565 nm and 580–650 nm, respectively. The pixel size is typically set around 0.144 μm/pixel.

10. Incubate the cells with serum-free medium at 37°C at 8% CO_2 for 1 h.
11. Treat the cells with 4 μg/mL anti-myc-SA/BODIPY for 1 h in the presence of 1 nM insulin.
12. Rinse the cells three times with serum-free medium and incubate the cells for at least 3 h with exchange of the medium every hour.
13. Rinse the cells twice with imaging buffer and incubate the cells for 10 min in 1 mL of imaging buffer.
14. During the incubation, place the cells under a confocal microscope and search for the cells displaying proper localization of expressed myc-GLUT4-mCherry, i.e., predominantly localized around perinuclear regions. Troubleshooting 4.
15. Add 10 mL of Imaging buffer containing 5 μg/mL Tf-biotin by perifusion and incubate for 5 min.
16. Wash the cells thoroughly with 30 mL of Imaging buffer by perifusion and incubate for 8 min.
17. Start image acquisition and add 10 mL of Imaging buffer containing 100 nM insulin 2 min after initiation of image acquisition.
18. Continue image acquisition for further 10 min. Troubleshooting 5.
19. Save acquired images as a lif (Leica image format) or tiff files.

Analysis

Timing: depends on number of images

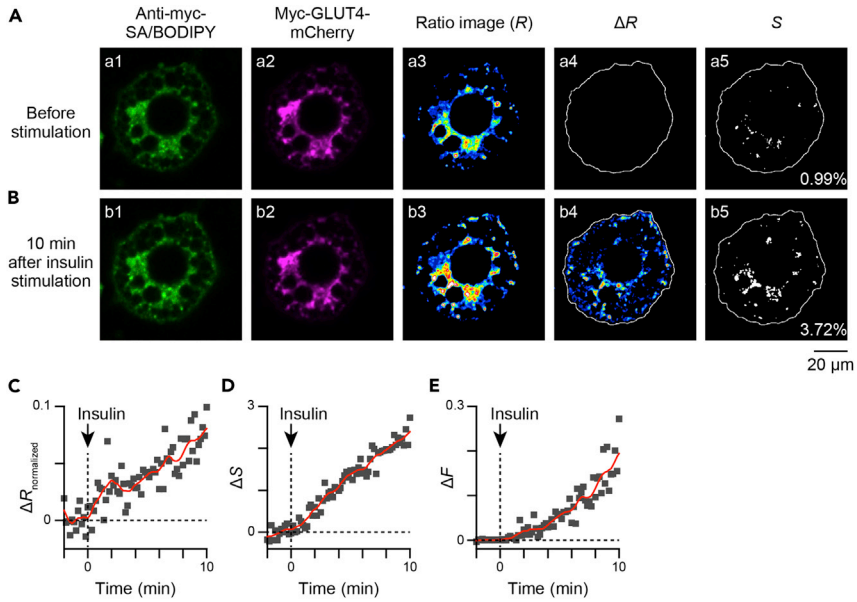

Figure 5. Evaluation of heterotypic endomembrane fusion with ratiometric analysis

(A and B) Fluorescent images of anti-myc-SA/BODIPY (a1 and b1) and myc-GLUT4-mCherry (a2 and b2) in a 3T3L1 adipocyte expressing myc-GLUT4-mCherry and transferrin receptor before (A) and 10 min after (B) insulin stimulation (100 nM). Calculated images, i.e., ratio images (*R*, a3 and b3), images of changes in the ratio values (Δ*R*, a4 and b4) and binary images showing the area for which the ratio value is above the threshold that was set as the top 1% ratio value within the cellular region (*S*, a5 and b5) are also shown. White lines represent the cellular region. In the *S* images, percent areas within cellular regions are also shown. In this case, Δ*S* (changes in *S* from S_0) was 2.73%. Gaussian-filtered images are shown.

(C–E) Insulin-responsive changes in $\Delta R_{normalized}$ (C), Δ*S* (D), and Δ*F* (E). A 100 nanomolar quantity of insulin was applied at time 0 (arrow). Red curves represent 10-point Lowess smoothing.

This part of the protocol describes analysis of acquired imaging data for quantifying heterotypic endomembrane fusion. Examples of analysis is shown in Figure 5.

20. Open the acquired BODIPY and mCherry images (e.g., a1/b1 and a2/b2 images in Figures 5A and 5B, respectively) with Fiji ImageJ.
21. Set "Divide by zero value" in Fiji (Edit > Options > Misc…) to 0.
22. Create a new image as a 32-bit floating-point data by dividing each BODIPY frame image by each corresponding mCherry frame image with Image Calculator of Fiji and save it as a "ratio image" (e.g., a3/b3 images in Figures 5A and 5B).

Note: Since the Leica hybrid detectors have very low background signals, it is not necessary to subtract the background. When using other detectors having non-negligible background signals including PMTs and CCDs, it is critical to perform appropriate background subtraction on each image prior to division.

23. Set ROI for analysis by extracting cell contours using the mCherry image (e.g., white lines of a4/b4 and a5/b5 images in Figures 5A and 5B).
    a. Apply Gaussian Blur filter to each mCherry frame image to smooth the contour.
    b. Binarize the images.
    c. Run "Analyze Particles" of Fiji over all frames with turning on "Add to Manager" to add the measured particles to the "ROI Manager".
24. Using the "ratio image" created in step 22 and the ROI obtained in step 23, measure the following two values, *R* and *S*, for each frame.

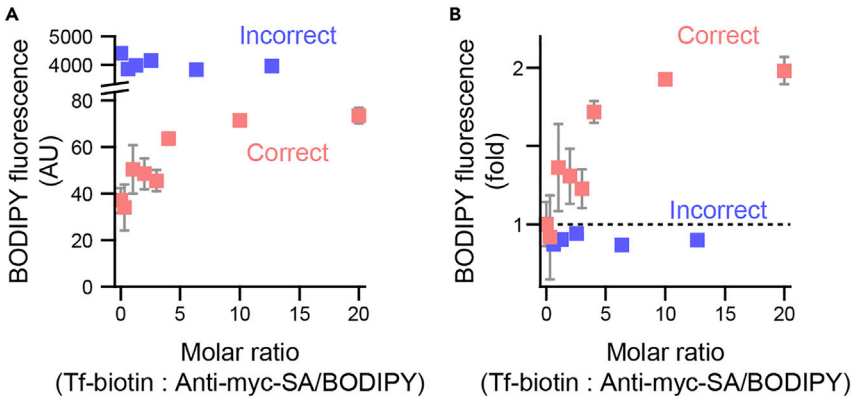

Figure 6. Enhancement of BODIPY fluorescence of the fusion sensor molecule in the presence of transferrin-biotin in vitro

(A and B) Changes in BODIPY fluorescence of anti-myc-SA/BODIPY prepared in the correct order according to in this protocol (red, degree of labeling was 1.4) and that prepared in the incorrect order, i.e., BODIPY labeled after antibody-SA conjugation (blue, degree of labeling was 72), are shown. A 10 nanomolar quantity of anti-myc-SA/BODIPY was mixed with various amounts of transferrin-biotin, and absolute intensity (A) and fold changes with no addition of transferrin-biotin set to 1 (B) are shown. Unlike the sensor molecule prepared in the correct way, anti-myc-SA/BODIPY prepared in the incorrect manner showed bright fluorescence but no increases in fluorescence with the Tf-biotin addition. Data are represented as mean ± SEM (n=3).

a. R: Ratio value within cellular regions.
b. S: Area of which the ratio value above the threshold, set as the top 1% ratio value within the cellular region just prior to insulin stimulation (e.g., a5/b5 images in Figures 5A and 5B).

25. Calculate mean R and S values for the frames imaged during the 2-min period before insulin stimulation (defined as R_0 and S_0, respectively).
26. Calculate normalized changes in R from R_0, i.e., $(R–R_0)/R_0$ (defined as $\Delta R_{normalized}$, e.g., Figure 5C).
27. Calculate changes in S from S_0, i.e., $S–S_0$ (defined as ΔS, e.g., Figure 5D).
28. Calculate index of insulin-responsive heterotypic fusion ΔF as the product obtained by multiplying $\Delta R_{normalized}$ and ΔS (e.g., Figure 5E).

EXPECTED OUTCOMES

From step 5 to step 8, we obtained anti-myc-SA/BODIPY with a concentration of 5.5 mg/mL and labeling degree of 1.4. Tf-biotin addition obviously increased BODIPY fluorescence in vitro (Figure 6). Preparation of the fusion sensor molecule in the indicated order is critical. It is also important to keep the molar ratio as low as possible in any labeling reaction. Antibody-streptavidin conjugation prior to BODIPY labeling resulted in bright fluorescence but no increases in fluorescence with the Tf-biotin addition. Cellular labeling with this fusion sensor molecule resulted in marked insulin-responsive increases in all three values indicative of heterotypic endomembrane fusion activity, i.e., $\Delta R_{normalized}$, ΔS and ΔF (Figures 5C–5E). This increase was especially pronounced in the perinuclear regions (b3–b5 images in Figure 5B), where the GLUT4 storage compartments are mainly located. For detailed descriptions of physiological analyses with this fusion sensor molecule, see (Hatakeyama et al., 2022).

LIMITATIONS

The fusion sensor molecule presented herein can be used for analyzing endomembrane fusion processes other than heterotypic fusion between GLUT4-containing vesicles and TfR-containing endosomes so long as the vesicles of interest can be labeled with BODIPY/streptavidin and biotin, respectively. However, with this technique post-fusion events cannot be analyzed continuously since this technique is based on the usage of biotin-streptavidin binding. Highly sensitive ways to detect

molecular interactions, based on a precise molecular design, may provide a solution to this challenge. It should be noted that all experiments presented herein were conducted in model cells overexpressing several proteins, including myc-GLUT4-mCherry, TfR, HA-sortilin, and HaloTag-AS160, and not on endogenous proteins. All imaging experiments were performed in cells mildly expressing the proteins (inspected with the fluorescence) rather than in cells displaying extremely high expressions, but the expression levels are still considered to be higher than the endogenous levels. Knock-in experiments employing genome editing technology would allow analysis at the endogenous expression levels. Finally, the present study was conducted in culture cells but not in vivo. However, we previously successfully performed intravital imaging of quadriceps femoris muscles of myc-GLUT4-EGFP transgenic mice (Tsuchiya et al., 2018). Combining this technique with the fusion sensor molecule will provide insights into regulation of GLUT4 trafficking itineraries so long as myc-GLUT4 can be successfully labeled with the fusion sensor molecule in vivo.

TROUBLESHOOTING

Problem 1

3T3L1 cells do not differentiate well.

Potential solution

There are several potential solutions to consider. First, use cells with lower passage numbers. Second, ensure cells have grown to 100% confluence for 2 days prior to replacing the medium with the differentiation medium.

Problem 2

Low antibody yield.

Potential solution

Use hybridomas with lower passage numbers. Let the cells grow until they reach ~90% confluence and the culture medium turns yellow.

Problem 3

Low BODIPY signals.

Potential solution

The fluorescent intensity of BODIPY signals is inevitably dim (especially before insulin stimulation) since BODIPY fluorescence is quenched and the degree of labeling is low. Avoid unnecessarily increasing the laser power. Binning or dilation of the confocal aperture is among the potential solutions, but it should be kept in mind that resolution will be reduced.

Problem 4

Myc-GLUT4-mCherry is mainly localized at the plasma membrane.

Potential solution

Insulin washout (step 12) is insufficient. Carefully remove the insulin-containing medium and rinse the cells with serum-free medium thoroughly.

Problem 5

Bleaching of fluorescent signals.

Potential solution

Decrease laser power to the lowest level possible. Using the Bleach Correction plug-in of Fiji (Edit > Adjust) would theoretically correct signal decay, but we have never attempted this approach.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Makoto Kanzaki (makoto.kanzaki.b1@tohoku.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this submission will be shared by the lead contact upon request. This manuscript does not contain original code.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.K. and H.H.; methodology, H.H. and M.K.; software, H.H.; validation, H.H. and M.K.; format analysis, H.H. and M.K.; investigation, H.H. and M.K.; resources, M.K. and H.H.; data curation, M.K. and H.H.; writing – original draft, H.H. and M.K.; writing – review & editing, M.K. and H.H.; visualization, H.H. and M.K.; supervision, M.K.; project administration, M.K.; funding acquisition, M.K. and H.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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