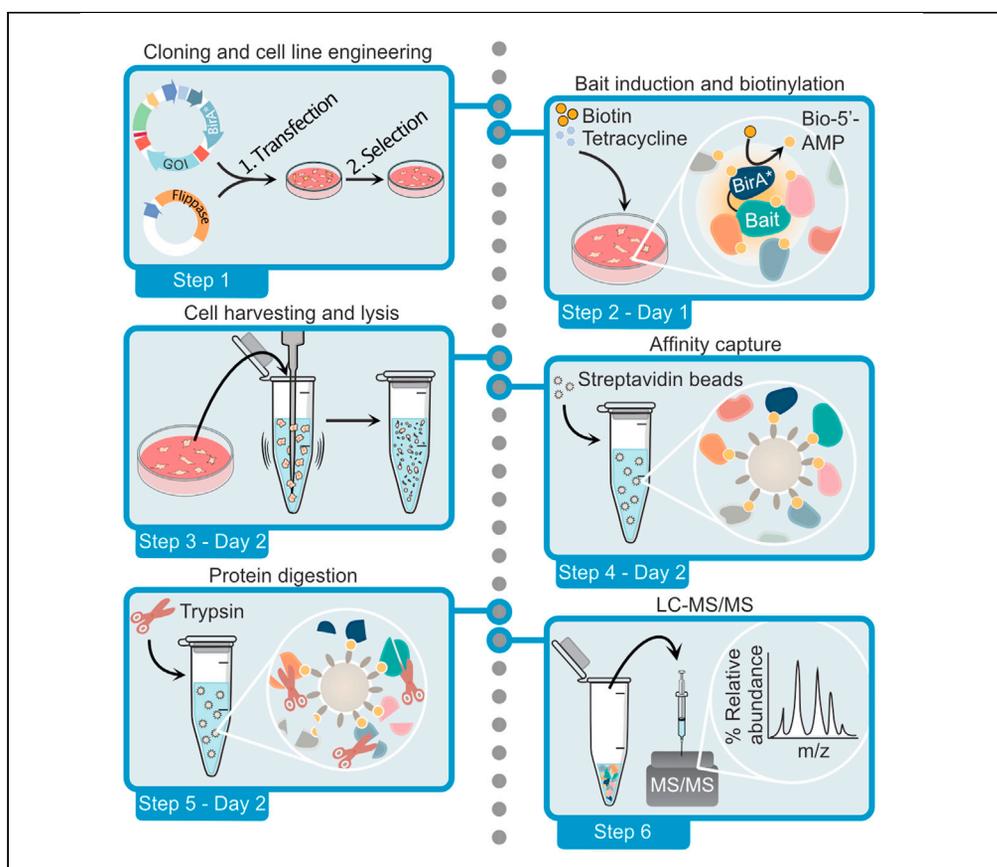


Protocol

Defining the interactomes of proteins involved in cytoskeletal dynamics using high-throughput proximity-dependent biotinylation *in cellulo*



Proximity-dependent biotinylation (BioID) screens are excellent tools to capture *in cellulo* interactomes for a large variety of baits, including transient and weak affinity interactions, as well as localization-specific proximity components, which are much harder to detect with conventional approaches. Here, we describe the major starting steps and a detailed protocol on how to perform BioID in mammalian cells. We also describe the mass spectrometry procedure and the bioinformatics pipeline for the data analysis.

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Highlights

Exploring the proximity interactome of proteins *in cellulo* in their native environment

This BioID protocol is well-suited to determine the interactome of small GTPases

Detailed steps of sample injection into mass spectrometer

In-depth description of the bio-informatics analyses of BioID MS data

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Protocol

Defining the interactomes of proteins involved in cytoskeletal dynamics using high-throughput proximity-dependent biotinylation *in cellulo*

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SUMMARY

Proximity-dependent biotinylation (BioID) screens are excellent tools to capture *in cellulo* interactomes for a large variety of baits, including transient and weak affinity interactions, as well as localization-specific proximity components, which are much harder to detect with conventional approaches. Here, we describe the major starting steps and a detailed protocol on how to perform BioID in mammalian cells. We also describe the mass spectrometry procedure and the bioinformatics pipeline for the data analysis.

For complete details on the use and execution of this profile, please refer to Bagci et al. (2020).

BEFORE YOU BEGIN

This protocol describes the pipeline for proximity-dependent biotinylation assay followed by mass spectrometry (BioID) used to define the proximity interaction networks of the Rho GTPase family (Bagci et al., 2020). To this end, the protein of interest, referred to as the bait, is tagged with an abortive *E. coli* biotin ligase (BirA*). Using biotin as a substrate, BirA* catalyzes the formation of biotinoyl-5'-AMP that reacts with accessible amine groups of exposed lysine residues in an approximately 10 nm radius (Kim et al., 2014). Thus, proteins that are proximal to the bait will be irreversibly biotinylated. These biotinylated proteins in living cells can efficiently be purified, exploiting the high affinity of the biotin/avidin interaction, and subsequently identified by mass spectrometry. This technique has many advantages compared to the standard approaches such as immunoprecipitation (Lambert et al., 2015). First, BioID allows the identification of weak and/or transient interactions that are not detectable by standard affinity purification. Second, it allows the detection of interactions amongst membrane-rich structures and subcellular compartments that are otherwise hard to solubilize, and it maintains the peculiar context of some of the unique protein interactions occurring in living cells (hydrophobicity, membrane curvatures etc.). Rho GTPases interaction with GEFs, GAPs and effectors are transient, and they signal from membrane compartments, due to their membrane



insertion via lipidic modifications (Ridley, 2006), to regulate cytoskeletal organization. Since Rho proteins can be trapped in various activation states, we took advantage of BioID to systematically define the interactome of Rho GTPases using nucleotide-free forms that have higher affinity to GEFs and constitutively active forms to enrich for GAPs and effectors. This experiment was carried out both in HEK293 and in HeLa cells which are compatible with the Flp-In T-Rex system to gain a better view of the conserved and unique interactors in different cellular contexts. However, this protocol can also be successfully used in many other settings, as described in the section below. Other proximity biotinylating variations (TurboID, MiniTurbo, BioID2) and methods (APEX) could also be compatible with this protocol. Here, we present a 3-day step-by-step protocol from the induction of bait expression in cells to the sample preparation, mass spectrometry, and data analyses.

Plasmid engineering, cell line generation, and validation of the bait's expression

This section presents an overview of the 3 steps necessary to set up a successful BioID experiment. The plasmids containing the genes of interest are first cloned (Michaud et al., 1997) and are used to generate the inducible stable cell lines followed by validation tests to ensure the expression of the proteins of interest and the functionality of the system. In a context where the goal is to perform a high-throughput screen to gain insights into the close proximity interactors of multiple baits, the results are obtained from protein overexpression in the presence of the endogenous protein counterparts. Ideally, the endogenous levels could be knocked out using the CRISPR-Cas9 technology and the cells could be rescued by the expression of the BirA*-tagged protein. However, in Bagci et al., we have observed that the overexpression of the BirA*-Flag-tagged bait led to a significant decrease of the endogenous levels of the Rho proteins tested (Bagci et al., 2020).

1. The Gateway cloning strategy (Invitrogen) is used as a fast and easy way to insert the gene of interest (GOI) into a desired destination vector (Hartley et al., 2000). Here, we used a plasmid containing a BirA*-Flag sequence under a tetracycline (TET) inducible promoter system and a selection cassette flanked by AttR1/2 sites compatible with Gateway cloning that we engineered (pcDNA5-pDEST-FRT-BirA*-Flag) (Figure 1A) (Couzens et al., 2013). It is important to carefully choose whether to tag the protein of interest in N- or C-terminal, depending on the functional domains, the presence of a peptide signal or other types of modifications in the protein. Generating a N- and C-terminally tagged bait can be done when it is possible without affecting protein function to identify a maximum of proximity interactors (Youn et al., 2018). For Rho proteins, we opted to tag them at the N-terminus since the C-terminus of these proteins is lipidated and essential for membrane insertion and biological functions.
2. Cell lines engineered with the Flp-In T-REX expression system allow for rapid and efficient generation of derived cell lines expressing various baits, from the same genomic locus, in a TET-inducible fashion. To generate cell lines expressing the protein of interest (POI), the resulting pDEST-FRT vector along with the Flp recombinase containing vector (pOG44) are transfected into the Flp-In T-Rex cells lines (HEK293 or HeLa) at around 90% confluence with Lipofectamine 2000. Cells are transfected overnight (16 h) in a 6-well dish with 2 μ g of pOG44 and 1 μ g of pDEST-FRT using 6 μ L of Lipofectamine 2000. HEK293 cells are commonly used in proteomics while HeLa cells are a good model to study GTPase signaling and for microscopy. Once expressed, the recombinase mediates the integration of the pDEST-FRT into the cell genome. The cells are selected with hygromycin according to the selection cassette of the pDEST-FRT vector, for 2–3 weeks (Figure 1A). Alternatively, cell lines that are compatible with Flp-In T-Rex, or even primary cells, can also be transiently transfected or transduced with lentivirus to express the POI provided that the bait expression is similar (Roux et al., 2013; Samavarchi-Tehrani et al., 2018).
3. To validate each bait expression and the biotinylation of the proximal preys, cells are treated for 24 h with TET (1 mg/mL) to induce expression of the bait and with the BirA* substrate, biotin (B) (50 μ M), to induce biotinylation of the preys. The treatment time needs to be adjusted depending on the biotinylating enzyme (TurboID: 10 min, miniTurbo: 10 min, AirID: 3 h) (Branon et al., 2018; Kido et al., 2020). Validate the expression of the bait (anti-flag-HRP antibody) and the biotinylation profile (anti-streptavidin-HRP antibody) by Western-Blotting and verify their

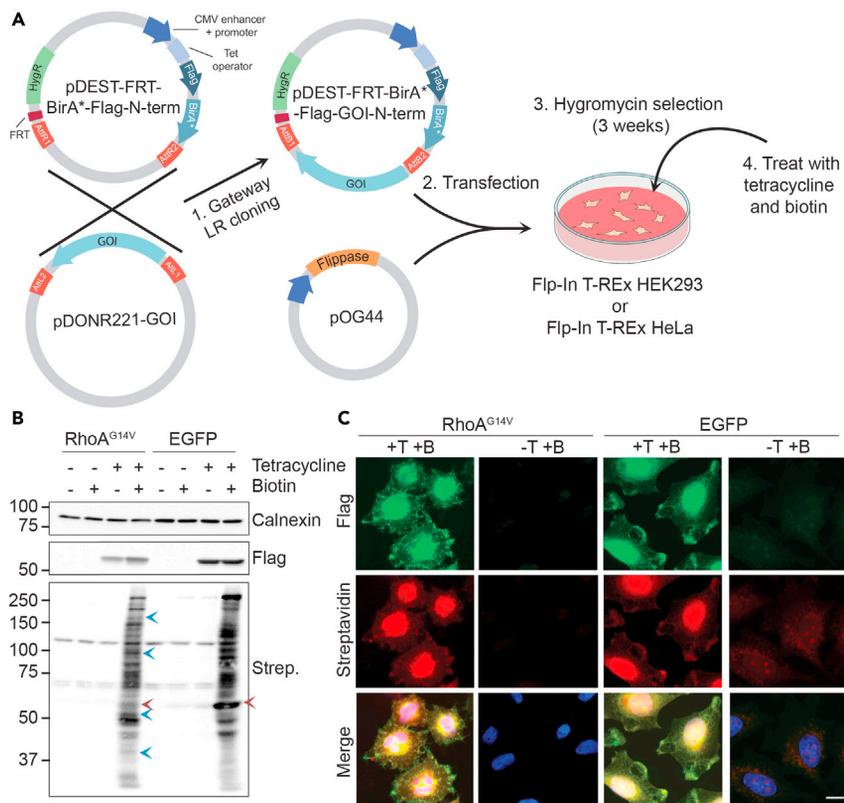


Figure 1. Cell lines engineering: From plasmid generation to bait expression and validation

(A) Schematic representation of how the cell lines were engineered: 1. The gateway cloning strategy is used to insert the gene of interest (GOI) into the pDEST-FRT-Flag-BirA*-N-term plasmid via recombination of the Att sequences. In the resulting plasmid, the GOI is under the control of the CMV promoter and the tetracycline (Tet) operator. 2. The resulting plasmid is transfected along with a plasmid coding for a flippase (pOG44) into a Flp-In T-REx compatible cell line. Once expressed, the flippase enzyme will induce the recombination of the pDEST plasmid via the FRT site into the cell's genome. 3. The transfected cells are positively selected using hygromycin for approximately 3 weeks. (HygR: Hygromycin resistance gene; FRT: Flippase recognition target site). 4. Cells are treated with Tet (1 μg/mL) to induce the expression of BirA*-Flag-RHOA^{G14V} and with biotin (50 μM) to induce the biotinylation of the preys.

(B) Western blot validating the expression of the BirA*-Flag-RHOA^{G14V} protein and the biotinylation of the preys in engineered HeLa cells. EGFP-BirA*-Flag is used as a control. Blue arrows indicate RhoA-specific biotinylated prey and red arrows indicate the autobiotinylated baits.

(C) Immunofluorescence validating the localisation of the bait and of the biotinylated prey in engineered HeLa cells. EGFP-BirA*-Flag is used as a control. Scale 10 μm.

localization *in cellulo* by microscopy (Figures 1B and 1C). The following conditions are typically tested: -TET-B, -TET+B, +TET-B and +TET+B to verify the inducibility of the protein expression system and assess biotinylation efficiency. Notably, leaky bait expression can be toxic depending on how the protein affects various cellular processes. This could lead to the loss of cells with high bait overexpression resulting in cultures of cells with very low bait expression ultimately limiting the ideal conditions for BioID. Additionally, further testing can be performed to validate the functionality of the tagged POI if such assays are available.

△ CRITICAL: The cell lines are routinely created and used directly for BioID. Maintaining the cell lines in culture for an extended number of passages risks the decrease in bait expression following induction. We recommend using TET-free FBS to culture the cells to maximally repress bait expression in cell expansion conditions. We also advise freezing multiple aliquots of the created cell lines as soon as the selection is completed.

Choosing the right controls

It is important to choose the right controls for the protein that is being studied. The labeling of the prey depends not only on the distance from the bait but also on the time the bait spends in each subcellular location. For example, nuclear residing proteins will spend more time in the nucleus and will therefore tend to label more non-specific nuclear proteins. Similarly, membrane residing proteins will tend to label more non-specific membrane proteins than cytosolic ones. In each case, it is preferable to choose a control that resides in the same subcellular localization as the bait. Accordingly, it is appropriate to choose an NLS-tagged BirA* control for a nuclear residing bait, an EGFP-CAAX tagged BirA* control for a membrane residing bait, and a cytoplasmic BirA* or and EGFP-BirA* for cytoplasm-soluble baits. Furthermore, using an empty-vector control allows identifying non-specific binding proteins that interact with streptavidin-beads during affinity purification. Ideally, the expression of the controls should be similar to that of the studied bait. If not, adjustments can be done when analyzing the results bioinformatically. In these bioinformatics analyses, the different control datasets can be used in various combinations to optimally filter the bait BioID data (see below). Apart from choosing the controls for the bait, it is also essential to include at least two biological replicates for each condition to ensure the statistical robustness of the results.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-Streptavidin-HRP (1:25000)	BD Biosciences	Cat#554066; RRID:AB_2868972
anti-Flag-HRP (1:8000)	Sigma-Aldrich	Cat#A8592; clone M2
Chemicals, peptides, and recombinant proteins		
Acetonitrile	Fisher Scientific	Cat#A9554; CAS:75-05-8
Ammonium bicarbonate (ABC)	Sigma-Aldrich	Cat#09830; CAS:1066-33-7
Ammonium hydroxide solution (NH ₄ OH)	Sigma-Aldrich	Cat#221228; CAS:1336-21-6
Benzonase	EMD Millipore	Cat#71205-3
Chlorhydrique acide 37% A.C.S. 1L (HCl)	Laboratoire MAT	Cat#CR-0166; CAS:7647-01-0
D-Biotin	Bio Basic	Cat#BB0078; CAS:58-85-5
Dithiothreitol (DTT)	Invitrogen	Cat#15508013; CAS:3483-12-3
DMEM high glucose	WISENT BIOPRODUCTS	Cat#319-005-CL
EDTA	Bio Basic	Cat#EB0185; CAS:6381-92-6
Fetal bovine serum tetracycline free (TET-free FBS)	WISENT BIOPRODUCTS	Cat#081-150
Formic acid	Sigma-Aldrich	Cat#F0507; CAS:64-18-6
Hygromycin B	Calbiochem	Cat#400053; CAS:31282-04-9
Lipofectamine 2000	Life Technologies	Cat#11668-019
Nonidet P40 Substitute (NP-40)	Roche	Cat # 11754599001; CAS:9016-45-9
PBS powder	Life Technologies	Cat#21600044
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich	Cat#P7626; CAS:329-98-6
Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#P8340
SDS	Bio Basic	Cat#SB0485; CAS:151-21-3
Sodium chloride (NaCl)	Bio Basic	Cat#DB0483; CAS:7647-14-5
Sodium deoxycholate (SOD)	Sigma-Aldrich	Cat#30970; CAS:302-95-4
Sodium hydroxide (NaOH)	BioShop	Cat#SHY500.1; CAS:1310-73-2
Streptavidin Sepharose® High Performance beads*	Sigma-Aldrich	Cat#GE17-5113-01
Tetracycline HCl	BioShop	Cat#TET01.10; CAS:64-75-5
Tris-HCl	Bio Basic	Cat#TB0103; CAS:1185-53-1
Trypsin from porcine pancreas	Sigma-Aldrich	Cat#T6567-5X20UG
Water HPLC 4L	Fisher Scientific	Cat#W5-4; CAS:7732-18-5
Experimental models: Cell lines		
Flp-In™ T-REx™ HEK293 (Human)*	Thermo Fisher Scientific	Cat#R78007

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Flp-In™ T-REx™ HeLa (Human)*	Kind gift of the laboratory of Dr. Stephen S. Taylor, University of Manchester, UK	N/A
Recombinant DNA		
pDONR221	Thermo Fisher Scientific	Cat#12536017
pcDNA5-pDEST-FRT-BirA*-Flag-NT*	Laboratory of A.-C. Gingras, Lunenfeld Tanenbaum Research Institute, CA	https://gingraslab.org/resources/
pcDNA5-pDEST-FRT-BirA*-Flag-CT*	Laboratory of A.-C. Gingras, Lunenfeld Tanenbaum Research Institute, CA (Couzens et al., 2013)	https://gingraslab.org/resources/
pOG44	Thermo Fisher Scientific	Cat#V600520
Software and algorithms		
ProHits	(Liu et al., 2010)	http://prohitsms.com
MaxQuant	(Cox and Mann, 2008)	https://www.maxquant.org/
Proteome Discoverer	Thermo Fisher Scientific	https://www.thermofisher.com/order/catalog/product/OPTON-30945#/OPTON-30945
R	R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.	https://www.r-project.org/
Python	N/A	https://www.python.org/
Perseus	(Tyanova et al., 2016)	https://maxquant.net/perseus/
CRAPome Contaminant Repository for Affinity Purification (CRAPome)	(Mellacheruvu et al., 2013)	https://reprint-apms.org/
SAINT-APMS	(Choi et al., 2011)	https://sourceforge.net/projects/saint-apms/files/
Comparative Proteomic Analysis Software Suite (COMPASS)	(Sowa et al., 2009)	http://besra.hms.harvard.edu/i/pmsmsdbs/cgi-bin/tutorial.cgi
ProHits-Viz	(Knight et al., 2017)	https://prohits-viz.org
Cytoscape	(Shannon et al., 2003)	https://cytoscape.org/download.html
Other		
LTQ Orbitrap Velos	Thermo Scientific	Cat#0723870
Q Exactive	Thermo Scientific	Cat#0726020
Jupiter 5u C18 50x4,6 mm (stationary phase)	Phenomenex	Cat#00B-4053-E0
Self pack picofrit column no coating 360 µm, 75 µm, tip 15 µm, length 50 cm	New Objective Inc	Cat#PF360-75-15-N5
* Critical reagent		

Note: As an alternative for the trypsin from Sigma presented in the key source table, the sequencing grade modified trypsin from Promega (Cat#V5111) can be used. Be sure to test efficiency.

MATERIALS AND EQUIPMENT

Solutions to be prepared in advance

- 20 mM Biotin; Dissolve in a 30% ammonium hydroxide solution to a concentration of 200 mM. Dilute to 20 mM by slowly adding 1N HCl on ice to prevent heating. The final pH should be around 9–10. Sterile filter the solution, aliquot and store at –20°C for up to a year.
- 1 M DTT; Dissolve in HPLC water, aliquot and store at –20°C for up to a year.
- 0,5 M EDTA pH 8,0; Dissolve in HPLC water, adjust pH with NaOH and store at 20°C–25°C for 3 months.
- 5 M NaCl; Dissolve in HPLC water and store at 20°C–25°C for 3 months.

- 100 mM Phenylmethanesulfonyl fluoride (PMSF); Dissolve in isopropanol, aliquot and store at -20°C for up to a year.
- 10% (w/v) SDS; Dissolve in HPLC water and store at 20°C – 25°C for 3 months.
- 10 mg/mL tetracycline; Dissolve in 80% EtOH, filter with $0,2\ \mu\text{m}$ filter, aliquot and store at -20°C for up to a year.
- 1 M Tris-HCl pH 7,4; Prepare in HPLC water, adjust pH with HCl and store at 20°C – 25°C for 3 months.
- 20 mM Tris-HCl pH 8,0; Prepare in HPLC water, adjust pH with NaOH and filter with $0,2\ \mu\text{m}$ filter. Store at 20°C – 25°C for 3 months.

Radio-immunoprecipitation assay (RIPA) lysis buffer

Reagent	Final concentration	Amount
NP-40	1% (v/v)	2,5 mL
SDS	0,1%	2,5 mL of 10% stock solution
Tris-HCl pH 7,4	50 mM	12,5 mL of 1M stock solution
NaCl	150 mM	7,5 mL of 5M stock solution
Sodium deoxycholate	0,5% (w/v)	1,25 g
EDTA pH 8,0	1 mM	500 μL of 0,5M stock solution
Water HPLC	n/a	Complete to 250 mL
Total	n/a	250 mL

Solution can be stored at 4°C for a week.

Solutions to be prepared the day of the experiment

- 50 mM Ammonium bicarbonate (ABC) pH 8,0; Dissolve in HPLC water and adjust pH with NH_4OH . Filter with $0,2\ \mu\text{m}$ filter and keep at 4°C .
- 50% formic acid; Dilute with HPLC water, filter with $0,2\ \mu\text{m}$ filter and keep at 20°C – 25°C .

RIPA lysis buffer with inhibitors

Solution or reagent	Final concentration	Amount
RIPA lysis buffer	NA	49,35 mL
PMSF	1 mM	500 μL of 100 mM stock solution
DTT	1 mM	50 μL of 1M stock solution
Protease Inhibitor Cocktail	n/a	100 μL
Total	n/a	50 mL

The solution should be filtered with a $0,2\ \mu\text{m}$ filter and kept on ice.

Mass spectrometry setup (LC-MS)

These parameters were used on LTQ-Orbitrap Velos and Q Exactive mass spectrometers (Thermo Scientific).

Main components	Parameters	Values
High-performance liquid chromatography	LC system	Easy-nLC II or Easy-nLC 1000 system (Proxeon Biosystems)
	Column type	75- μm \times 150 mm Self-Pack C18 column (Jupiter 5 μm from Phenomenex)
	Elution	Two-slope gradient: 2%–36% in 100 min 36%–82% in 10 min

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<i>Continued</i>		
Main components	Parameters	Values
	Flow rate	250 nL/min
	Buffer A	0.2% formic acid
	Buffer B	100% acetonitrile/0.2% formic acid
Ionization	Source	Nanospray Flex Ion Source
	Spray voltage	1.3–1.7 kV
	Capillary temperature	250°C
	S-lens voltage	60 V (LTQ Orbitrap Velos) 50 V (Q Exactive)
MS	Type	Orbitrap
	Mode	Positive ion
	Scan range (m/z)	360–2000
	Resolution	60000
	AGC target	1 ^E 6
Fragmentation	Mode	Collision-induced dissociation (LTQ-Orbitrap Velos) Higher-energy Collisional Dissociation (Q Exactive)
	Peptide ions fragmented	11 most intense peptides (LTQ Orbitrap Velos) 16 most intense peptides (Q Exactive)
	Collision gas	Helium (LTQ Orbitrap Velos) Nitrogen (Q Exactive)
	Normalized collision energy	35 (LTQ Orbitrap Velos) 27 (Q Exactive)
	AGC target	1 ^E 4 (LTQ Orbitrap Velos) 1 ^E 5 (Q Exactive)
Detection	Type	Linear ion trap (LTQ Orbitrap Velos) Orbitrap (Q Exactive)

STEP-BY-STEP METHOD DETAILS

Cell plating and treatments

⌚ Timing: 2 days

These steps are required to induce the expression of the POI fused to BirA* in the stable Flp-In T-REx cell line. To robustly biotinylate proximity proteins, BirA* requires exogenous biotin as a substrate.

1. Seed the Flp-In T-REx stable cell line in a 15 cm dish cultured in DMEM supplemented with 10% TET-free FBS and allow them to adhere and proliferate overnight (about 16 h). The number of cells can vary from one cell line to another and should be optimized in each lab.
2. When the confluency reaches 75%–80% treat the cells.
 - a. Aspirate the media.
 - b. Gently replace it with fresh media supplemented with 10% of FBS, 1 ug/mL of tetracycline and 50 μM of biotin for 24 h.

Note: The treatments can otherwise be added directly to the cells.

Cell harvesting

⌚ Timing: 40 min

These steps describe how to properly harvest the cells prior to performing the BioID.

Note: The following steps are done on ice.

3. Aspirate the media and add 5 mL of ice-cold PBS to the dish.
4. Agitate on an orbital shaker at 4°C for 10 min.
5. Scrape the cells using a cell scraper and transfer them to a 15 cm tube.
6. Add 7 mL of cold PBS to the dish.
7. Collect the remaining cells and transfer into the same 15 cm tube.
8. Pellet the cells by centrifuging at 500 g for 5 min at 4°C.
9. Aspirate the supernatant.
10. Wash the pellet with 10 mL of cold PBS.
11. Repeat steps 8–10.
12. Pellet the cells by centrifuging at 500 g for 5 min at 4°C.
13. Aspirate the supernatant.

▮▮ Pause point: At this point, you can freeze the cell pellets at –80°C for several months. This can be useful to synchronize the processing of samples from different experiments.

Affinity capture of biotinylated proteins

⌚ **Timing:** 5–7 h depending on the number of samples

These steps explain how to lyse the cells and how to capture the proteins that were biotinylated by BirA* *in cellulo* using streptavidin Sepharose® beads.

Note: Keep the samples on ice.

14. Thaw frozen cell pellets in 1,5 mL of ice-cold RIPA lysis buffer supplemented with inhibitors (DTT, PMSF and protease cocktail inhibitor).
 - a. Resuspend by pipetting up and down
 - b. Transfer to a 2 mL Eppendorf tube.
15. Add 1 µL of benzonase (250 U/µL) to each sample to digest nucleic acids, prior to the following step.
16. To complete cell lysis, sonicate samples for 30 s at 30% amplitude. Perform 3 rounds of 10 s with 2 s pause in between.

Note: Ideally, the sonication performed at step 16 should be done on ice. If not possible, the sample can be taken from the ice and replaced back after.

17. Centrifuge the lysates for 30 min at 12,000 g at 4°C to pellet the cell debris.
18. Meanwhile wash 70 µL of streptavidin Sepharose® beads slurry in a 2 mL Eppendorf tube.
 - a. Add 1 mL of RIPA buffer (with or without inhibitors) to the 70 µL of beads.
 - b. Spin for 1 min at 375 g at 4°C to pellet the beads.
 - c. Aspirate the wash buffer.
 - d. Repeat step 18 two more times.
19. Collect the clarified cell lysates of step 17 and transfer it to the 2 mL Eppendorf tube containing 70 µL of the pre-washed beads.

Note: A 20 µl or 40 µL aliquot of the supernatant should ideally be stored at –80°C to monitor protein expression, lysis efficiency and solubility.

20. Secure tubes with parafilm to prevent leaking.

21. Incubate with rotation at 4°C for 3 h.
22. Spin down the beads by centrifuging them for 1 min at 375 g at 4°C.
23. Remove the supernatant.

Note: You can keep the supernatant to monitor unbound bait as a control and store it at –80°C.

24. Add 1 mL of RIPA lysis buffer without inhibitors to the beads and transfer to a new 2 mL Eppendorf tube to remove unwanted material that adhered to the tube walls.
25. Spin down the beads by centrifuging them for 1 min at 375 g at 4°C and discard the supernatant.
26. Wash with 1 mL of RIPA lysis buffer.
27. Repeat steps 25–26 one more time.
28. Centrifuge 1 min at 375 g at 4°C and discard the supernatant.
29. Wash the beads with 1 mL of 50 mM cold ABC which provides an optimal pH for the trypsin digestion and is also compatible with the downstream MS analysis.
30. Repeat step 28–29 two times and discard the supernatant.
31. Resuspend beads in 100 µL of 50 mM cold ABC.

Biotinylated proteins digestion

⌚ Timing: 18 h

These steps allow the trypsin-mediated digestion of the bound biotinylated proteins. It is essential to work in a clean environment for steps 32–46.

32. Resuspend trypsin stock in 200 µL of 20 mM Tris-HCl pH 8,0 by vortexing for around one minute and make sure it is well dissolved.

⚠ **CRITICAL:** Wear a mask to perform this step, trypsin powder can be harmful to the respiratory tract.

33. Add 10 µL (1 µg) of resuspended trypsin to each sample.
34. Secure tube with parafilm to prevent leaking.
35. Incubate overnight (about 15 h) at 37°C with rotation to digest peptides. Make sure all samples rotate correctly.
36. The next day, resuspend a new tube of trypsin stock in 200 µL of 20 mM Tris-HCl pH 8,0.

Note: It is important to use a new tube of trypsin each day. Trypsin stored at 4°C overnight can lose its activity.

37. Add 10 µL (1 µg) of resuspended trypsin to each sample.
38. Secure tube with parafilm to prevent leaking.
39. Incubate for 2 h at 37°C with rotation to make sure proteins are maximally digested.

Peptide recovery

⌚ Timing: 4–5 h depending on the number of samples

These steps describe how to collect the released peptides from the digestion and how to concentrate them.

40. Pellet the beads by centrifugation for 1 min at 375 g at room temperature (20°C–25°C).
41. Transfer 100 µL of supernatant which contains peptides to a 1,5 mL Eppendorf tube.

Note: From now on be careful not to carry over any beads. To avoid disturbing the bead pellet, we recommend limiting movement by staying close to the centrifuge and manipulating the samples the least possible.

42. Rinse beads with 100 μL of HPLC grade water.
43. Centrifuge the beads for 1 min at 375 g at room temperature (20°C–25°C).
44. Collect the supernatant containing the remaining peptides in the same tube as step 41 to pool all peptides.
45. Repeat steps 42–44 one more time.
46. Add 30 μL of 50% formic acid stock solution for a final concentration of 5% formic acid to the 1.5 mL Eppendorf tube containing peptide to end digestion and mix by pipetting up and down.
47. Centrifuge for 10 min at 16300 g at room temperature (20°C–25°C) to pellet remaining beads.
48. Transfer supernatant to a new 1.5 mL Eppendorf tube making sure not to carry over any beads by leaving a few μL (5–15) in the tube.
49. Repeat steps 47 and 48 again.
50. Place samples in a SpeedVac for 3 h at maximum speed and 35°C to dry completely.
51. Freeze samples at -80°C .

▮▮ Pause point: Frozen samples can be stored for several months.

52. Before MS injection, resuspend the pellet in 15 μL of 5% formic acid by pipetting up and down.

MS injection

⌚ Timing: 145 min/sample

These steps of the protocol describe how to inject the samples prepared above in the LC-MS/MS system.

53. For each series of samples (duplicates or triplicates) perform an LC-MS/MS analysis using a short concentration gradient on at least one diluted sample (1:20) prior to the full gradient injection.

⚠ CRITICAL: This run is important as it allows to determine the highest volume to be injected for the full gradient analysis. The goal is to reach the maximum sensitivity without saturating the LC-MS/MS system.

54. Load the samples at the previously determined dilution into a 75 μm i.d. \times 150 mm Self-Pack C18 column installed on the Easy-nLC II or the Easy-nLC 1000 system.
55. Elute the peptides with a two-slope gradient at a flow rate of 250 nL/min (The HPLC system is either coupled to a Q Exactive or the LTQ Orbitrap Velos mass spectrometer through a Nanospray Flex Ion Source.)
 - a. Set the Nanospray and S-lens voltages to 1.3–1.7 kV and 50–60 V, respectively.
 - b. Set the capillary temperature to 250°C.
 - c. Increase solvent B from 2 to 36% in 100 min and then from 36 to 82% B in 10 min.
56. Acquire full scan MS survey spectra (m/z 360–2000) in profile mode in the Orbitrap with a resolution of 60,000 and the AGC target at $1^{\text{E}6}$.
57. Depending on the machine available follow the steps (a) or (b) below:
 - a. On the Q Exactive
 - i. Fragment the 16 most intense peptide ions in the collision cell at a normalized collision energy of 27.
 - ii. Analyze MS/MS spectra in the Orbitrap with a resolution of 17,500, the AGC target at $1^{\text{E}5}$, and the dynamical exclusion set to 7 s.
 - b. On the LTQ Orbitrap Velos

- i. Fragment the 11 most intense peptide ions in the high-pressure cell at a normalized collision energy of 35.
- ii. Analyze MS/MS spectra in the linear trap with the AGC target at 1^E4 and the dynamical exclusion set to 30 s.

EXPECTED OUTCOMES

After the acquisition of LC-MS/MS data (Figure 2A – Step 1), the identification and quantification of peptides/proteins are conducted against a species-specific protein sequences database, like RefSeq or UniProt, supplemented with common contaminants and decoy sequences. We usually perform this task in ProHits (Liu et al., 2012) (Figure 2A – Step 2), a laboratory information management system that allows the storage and analysis of MS data with a focus on interaction proteomics. ProHits can run and combine several search engine results, such as Mascot (Perkins et al., 1999), X! Tandem (Craig and Beavis, 2004) and MS-GF+ (Kim and Pevzner, 2014), by using iProphet (Shteynberg et al., 2011). Users can freely download ProHits and get more information by reading the online manual and (Liu et al., 2012). Working outside of ProHits is also possible by using search engines such as MaxQuant and Proteome Discoverer (PD) platform to quantify proteins.

Once the search engines have completed the quantification of proteins, the data is explored, and a quality control investigation is performed. Next, we export the identified proteins and spectral count results to evaluate the levels of Spearman correlations (non-Gaussian distribution), as well as batch effects between the biological replicates by using the Principal Component Analysis (PCA) method. The samples showing low correlation scores (< 0.9) and/or batch effects (e.g., bait A1 clustering with control N1 instead of bait A2) are discarded. If the bait of interest localizes to the plasma membrane, interactions can be prefiltered by calculating enrichment levels against a plasma membrane-targeted BirA*-Flag-EGFP-CAAX bait control. The CAAX ratios are calculated by dividing each prey's average spectral counts by their counterpart from the CAAX control (Preys AvgSpec/CAAX Avg-Spec). True interactions occurring at the plasma membrane should at least display a CAAX ratio ≥ 1.5 . Otherwise, low CAAX ratios can be considered to depict random encounters at the plasma membrane by the bait. These investigations can all be performed in R, Python or Perseus.

Having proper BioID data, the protein-protein interactions can be scored with the Significance Analysis of INTERactome (SAINT or SAINTexpress) software (Teo et al., 2014) (Figure 2A – Step 3). ProHits offers a user-friendly interface that manages and converts the search engine results into the suitable data input format required by SAINT. SAINT analyses can then be performed online on the CRA-Pome (Contaminant Repository for Affinity Purification) website or locally through a command line interface. Once initiated, SAINT calculates confidence scores by comparing prey abundances of a given bait to its negative controls. Since label-free protein quantification varies inevitably between MS runs, SAINT also offers to bypass this issue with the compression parameter. This option sets the number of the most abundant replicates that SAINT will consider for the comparative analysis.

As an indicator of a successful experiment, it is expected to identify the over-expressed bait and the BirA* at the top of the spectral count list as they will be auto-biotinylated. Streptavidin peptides will also be abundant since this protein was coupled to the streptavidin Sepharose® beads and used to isolate the biotinylated proteins. The spectral counts of each protein identified should be comparable between replicates as it is a good measure of reproducibility (Figure 2B).

Upon SAINT analysis completion, users will now be able to segregate true from false interactions by filtering the SAINT score or the Bayesian FDR (BFDR) (Figure 2A – Step 4) using a threshold. A good way to establish a SAINT score or a BFDR threshold is by plotting a precision-recall curve using known interactions from protein interaction databases, such as BioGrid (Oughtred et al., 2021) or Intact (Orchard et al., 2014). The identification of known and established interactors is also a good indicator of the success of the experiment (Figures 2C and 2D). Note that some of these

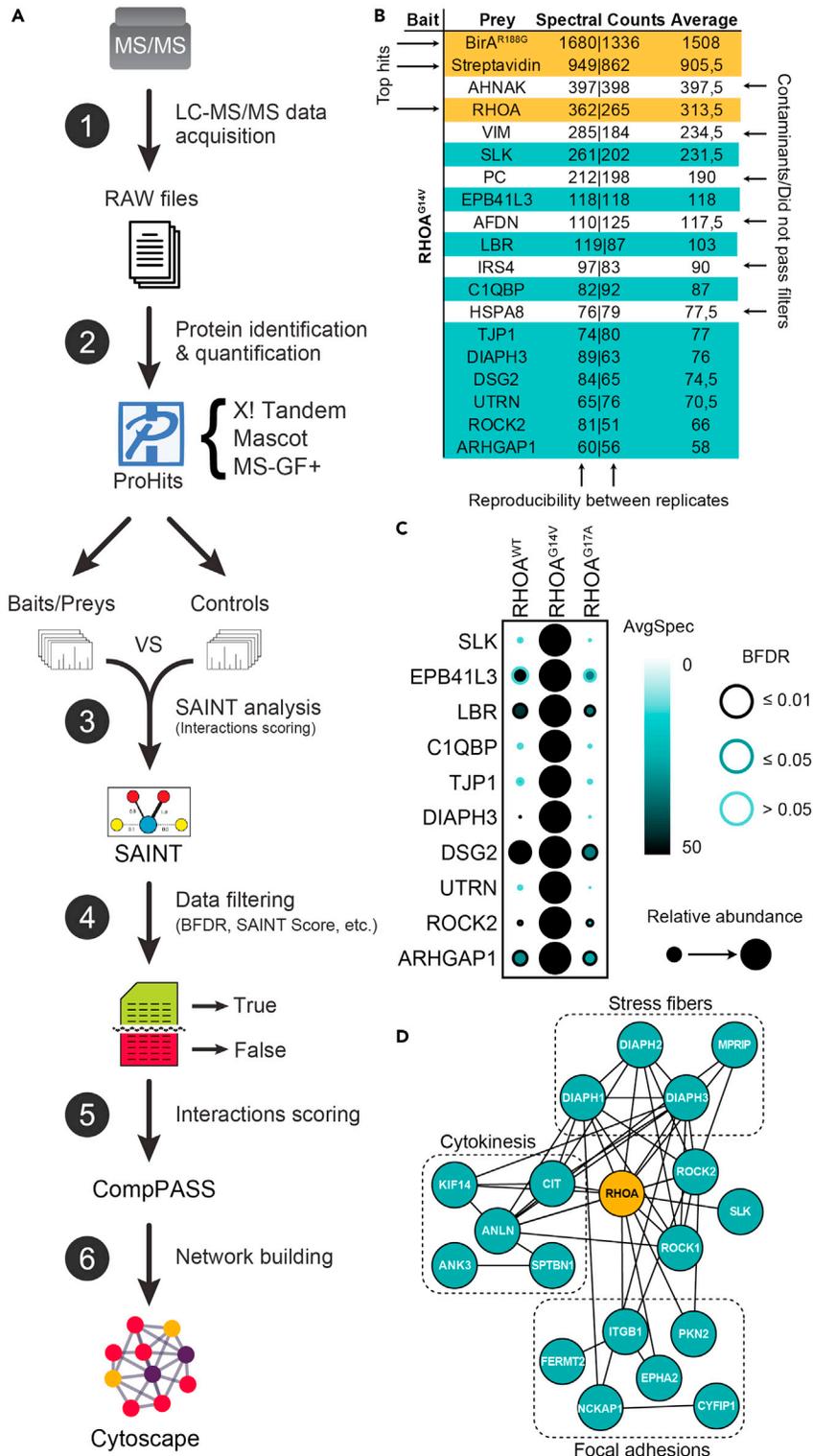


Figure 2. SAINT file representation before and after filtering using different tools

(A) BioID analysis pipeline.

(B) SAINT report top hits of the constitutively active RhoA mutant (RHOA^{G14V}) showing the spectral counts for each replicate.

(C) Comparative dot plot of RhoA wild-type (RHOA^{WT}) with the constitutively active (RHOA^{G14V}) and nucleotide-free (RHOA^{G17A}) mutants generated through the ProHits-viz website.

(D) Network of known RhoA interactors involved in different cellular processes generated with Cytoscape.

protein interactions can be cell context-dependent and it might therefore be impossible to identify them all. Even when performing the BioID on very well documented proteins, most identified prey will probably not be known interactors. These proximity binding partners can be members of a complex, a signaling cascade, or proteins resident of the same cellular compartment as the POI. The latter can be useful to determine the transient localization of the POI since the 24 h biotinylation leaves a permanent trace of the locations visited by the bait (Go et al., 2021). Yet, if the interaction knowledge on the bait is too scarce and prevents the validation of the success of the experiment as well as the plotting of an appropriate precision-recall curve, it is considered that high confidence BioID interactions usually display a SAINT score ≥ 0.95 or a BFDR ≤ 0.01 in most publications. More details on SAINT are available in (Choi et al., 2012).

Although SAINT is a great tool discriminating true from false interactions by using the negative controls as comparatives, scoring the interactions by comparing preys' abundances altogether will greatly enhance the BioID analysis (Figure 2A – Step 5). To achieve this, the Comparative Proteomic Analysis Software Suite (Sowa et al., 2009), also called CompPASS, offers many specificity scoring metrics: The Z-, S-, D-, and WD-scores. From the SAINT file results, any of these specificity scores can be calculated online on the ProHits-viz websites, which can also be used as a tool to build Dot plot (Figure 2C). Keep in mind that CompPASS works optimally with multiple baits. The more baits there are, the better CompPASS will single out redundant interactions, which can be considered as contaminants or non-specific interactions, from highly specific ones.

Now, the protein-protein network can be built by importing your CompPass or SAINT output file in Cytoscape (Figure 2A – Step 6 and 2D), a free software package for visualizing, modeling and analyzing protein interaction networks (Shannon et al., 2003). Through Cytoscape, filtering the interactions will be possible by applying a SAINT metric threshold (AvgP or BFDR), augment your network by supplementing prey-prey interactions from public databases and perform edge-weighted clustering by using the CompPASS metric. For more details on how to use Cytoscape, we strongly advise to read and follow the nicely written protocol by Trey Ideker and Gary Bader groups (Cline et al., 2007).

LIMITATIONS

As much as BioID is powerful to provide an overview of the proteins surrounding a bait of interest, it also comes with limitations. Results are based on proximity and do not confirm any direct interactions. The complexity of the macromolecular interactions identified will have to be confirmed through other methods like co-immunoprecipitation, pull-down assays, bioluminescence resonance energy transfer (BRET), yeast-two-hybrids and split GFP assays. Additionally, BioID allows one to obtain a view of the bait proximity interactors in a 24 h' time frame in which cells undergo a multitude of events such as cell division and environmental stimulation. Therefore, BioID is not very suitable to study the dynamics of proximal interactions in specific biological events and this limitation has led to the development of derived methods such as miniTurbo and TurboID that provide more temporal resolution since the biotinylation is carried out in a shorter time frame.

Regarding the protocol itself, a noteworthy aspect to be aware of is that we are working with overexpression. Therefore, the bait overexpression could create artificial interactions, putting emphasis on the importance of validating these proximal partners and having optimal controls. As an alternative way to avoid bias introduced by overexpression, endogenous proteins can be tagged by BirA* using CRISPR technology (Vandemoortele et al., 2019). Another crucial point is that BioID relies on the number of peptides detected by mass spectrometry for the protein identification. The number of peptides generated can be influenced by the size of a protein or by the number of cleaving sites available for trypsin. Consequently, very small proteins with limited lysine residues exposed to solvent can be harder to identify.

TROUBLESHOOTING

Problem 1

Few biotinylated proteins associated with low bait expression ([before you begin](#) – Step 3).

Potential solution

Verify the bait expression and the biotinylation efficiency of the Flp-In T-REx cell line by performing a western-blot or immunofluorescence staining using an antibody against flag and streptavidin. The low bait expression can be associated with a suboptimal hygromycin selection where cells that did not incorporate the GOI remained and repopulated the culture over time. In such a case, reselect your cells, thaw a lower passage or try regenerating the cell line.

Problem 2

Limited starting material: limited availability of the cells of interest (Step 1).

Potential solution

It is possible to decrease the quantity of the starting material if the quantity of the cells is limited. Therefore, instead of using a confluent 15 cm plate of HeLa cells which contains around 7000 ug of total protein, use a 6 well plate (9,6 cm²) or a 6 cm dish (21,5 cm²). In this case, it is essential to adjust the volume of the streptavidin beads used in the immunoprecipitation step 18. Approximately, 1 uL of beads is used for each 50–100 ug of protein. If this avenue is used, the amount of trypsin can also be reduced.

Problem 3

Samples contain a high background of contaminants such as keratin ([expected outcomes](#)).

Potential solution

Make sure to use all precautions to prevent contaminating the samples. Always use gloves, lab coat, mask and hair net and limit unnecessary sample manipulation. To prevent outside contamination, work on a clean bench and in a quiet and confined environment. If necessary, work in a sterile environment such as a biosafety cabinet. Using more negative experimental controls such empty-vector, BirA*-Flag-EGFP, BirA*-Flag, or subcellular compartment specific BirA*-Flag with NLS, CAAX, etc. will further improve the elimination of non-specific contaminants after data filtering.

Problem 4

Low protein sequence coverage or low number of spectral counts ([expected outcomes](#)).

Potential solution

An alkylation/reduction step can be added following step 31. The reduction and alkylation are generally performed to reduce the disulfide bonds and then alkylate the sulfhydryl groups. In some cases, peptides involved in disulfide bonds might be difficult to identify if this step is omitted from the protocol. If users would like to obtain enhanced protein sequence coverage and high number of spectral counts, this step could be performed by using urea as denaturing agent, (2-carboxyethyl)phosphine (TCEP) for reduction of disulfide bonds, and iodoacetamide for alkylation of sulfhydryl groups.

Problem 5

Very high streptavidin peptide counts compared to other peptides leading to low protein recovery ([expected outcomes](#)).

Potential solution

Eliminate the second trypsin digestion step (steps 36–39) to prevent over-digestion of streptavidin bound to beads. In some cases, the trypsin can have a high activity thereby avoiding the need of a

second digestion. Testing the trypsin efficiency using commercially available kits can be performed if needed.

RESOURCE AVAILABILITY

Lead contact

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Materials availability

The material described here can be requested from the authors.

Data and code availability

Raw mass spectrometry data generated in the original study by Bagci et al. is deposited on ProteomeXchange (dataset # PXD015918).

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AUTHOR CONTRIBUTIONS

S.N. and L.Q. wrote the original draft of the manuscript and prepared the figures. J.B. wrote the section on bioinformatics and prepared the figures. D.F. wrote the section on MS injection. H.B. was involved in setting up the protocol and edited the manuscript. A.C.G. and J.F.C. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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