

A genetically encoded sensor for visualizing leukotriene B4 gradients *in vivo*

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1 Summary:

2 **Leukotriene B₄ (LTB₄) is a potent lipid chemoattractant driving inflammatory responses during**
3 **host defense, allergy, autoimmune and metabolic diseases. Gradients of LTB₄ orchestrate**
4 **leukocyte recruitment and swarming to sites of tissue damage and infection. How LTB₄ gradients**
5 **form and spread in live tissues to regulate these processes remains largely elusive due to the**
6 **lack of suitable tools for monitoring LTB₄ levels *in vivo*. Here, we developed GEM-LTB₄, a**
7 **genetically encoded green fluorescent LTB₄ biosensor based on the human G-protein-coupled**
8 **receptor BLT1. GEM-LTB₄ shows high sensitivity, specificity and a robust fluorescence increase**
9 **in response to LTB₄ without affecting downstream signaling pathways. We used GEM-LTB₄ to**
10 **measure *ex vivo* LTB₄ production of murine neutrophils. Transgenic expression of GEM-LTB₄ in**
11 **zebrafish allowed the real-time visualization of both exogenously applied and endogenously**
12 **produced LTB₄ gradients. GEM-LTB₄ thus serves as a broadly applicable tool for analyzing LTB₄**
13 **dynamics in various experimental systems and model organisms.**

14 Introduction:

15 Gradients of chemoattractants guide leukocyte migration during immune surveillance. Neutrophils as first
16 responders during inflammation are remarkably efficient in interpreting directional cues which recruit them
17 through the vessel wall to sites of tissue damage and infection^{1,2}. Their migration is guided by a vast array
18 of chemically diverse chemoattractants including bacterial peptides, complement fragments, lipid
19 mediators and various chemokines³. Gradients of primary chemoattractants such as N-formylated
20 peptides (fMLP) and complement 5a attract the first neutrophils to inflammation sites, where their
21 activation contributes to a cascade of secondary chemoattractant production⁴. Among these
22 chemoattractants, leukotriene B₄ (LTB₄) acts as a central signal relay molecule, self-amplifying its
23 production to increase the detection range and enhance the robustness and persistence of leukocyte
24 migration during host defense⁵.

25 LTB₄ is synthesized on the nuclear envelope by the sequential action of 5-lipoxygenase (5-LOX) and
26 leukotriene A₄ hydrolase from arachidonic acid, which is released from phospholipids by cytosolic
27 phospholipase A₂ (cPLA₂)⁶. Calcium transients and mechanical stretch on the nuclear membrane jointly
28 contribute to the activation of cPLA₂ and 5-LOX by translocating them to the nuclear membrane⁷. LTB₄
29 is then packaged along with its synthesizing enzymes into vesicles, which bud off from the nuclear
30 envelope, and are ultimately secreted as exosomes from activated neutrophils, macrophages and
31 dendritic cells^{8,9}. Although its secretion and distribution have not been directly visualized, LTB₄ has been
32 proposed to form local and long-range chemoattractant gradients to drive directional migration through

its G protein-coupled receptor, BLT1^{5,10,11}. LTB₄ has also been established as a main driver of neutrophil swarming. This emergent behavior driving collective neutrophil migration to large targets is dependent on the combination of transcellular LTB₄ production and an LTB₄-BLT1 axis dependent self-amplification^{12–15}. A major obstacle to gain further insight into the regulation of these processes has been the lack of tools to directly measure the real-time production of LTB₄.

Measuring LTB₄ and other chemoattractant levels with high spatiotemporal resolution requires novel methods beyond standard biochemical assays. Previous approaches to assess the tissue distribution of chemokines have been based on immunofluorescence assays or transgenic labeling of endogenous chemokines with fluorescent proteins^{16–19}. Alternatively, live measurements of receptor internalization have been used to approximate endogenous chemokine gradients²⁰. However, this method is insufficient to follow the events in real time. In addition, it is not amenable to ligands inducing weak receptor internalization such as LTB₄²¹. To overcome these limitations of detection, we took an approach inspired by recent advances in the neuroscience field, which have led to the development of GPCR-based fluorescent biosensors for the live imaging of neurotransmitters^{22–25}.

Here, we developed the first genetically encoded fluorescent reporter for the direct, rapid and sensitive measurement of extracellular LTB₄ levels. The sensor called GEM-LTB₄ is structurally based on the human BLT1 receptor with a circularly permuted EGFP (cpEGFP) inserted into the third intracellular loop of the GPCR. GEM-LTB₄ shows a robust fluorescence response that allowed us to visualize not only exogenously applied but also endogenous gradients of LTB₄ both *in vitro* and *in vivo*.

Results:

Development and Characterization of GEM-LTB₄ in HEK293A cells

To develop an LTB₄ sensor, we inserted a cpEGFP module with linkers into the third intracellular loop of the high affinity LTB₄ receptor, BLT1 between R212 and F213 (Fig. 1a-b). Linker sequences on the N- and C-termini of cpEGFP were designed based on the previously published GPCR-based dLight sensors²². In our prototype sensors, combinations of long and short linkers were tested (Extended Data Fig. 1a). When expressed in HEK293A cells, these sensors showed a modest fluorescence response upon stimulation with LTB₄ (Extended Data Fig. 1b). Based on the linker sequences of the sensor exhibiting the largest response (linker NL-CS: $\Delta F/F_0 = 35.9 \pm 2.5\%$, mean $\pm$ s.e.m.), we performed a second round of screening by linker length optimization. This screening step identified that the four amino acid long NHDQ linker on the C-terminal end of cpEGFP gives rise to sensors with enhanced dynamic range (Extended Data Fig. 1c). In parallel measurements we evaluated the plasma membrane localization of the sensors exhibiting the highest $\Delta F/F_0$ and identified the version (N4-C4) which shows

the best combination of dynamic range ($\Delta F/F_0=103\pm 1\%$, mean $\pm$ s.e.m.) and membrane targeting (Extended Data Fig. 1d-e). To further enhance its membrane trafficking²³, we attached the IgK leader sequence to our final sensor which we called GEM-LTB₄ (Fig. 1c).

To create a control sensor variant (GEM-LTB₄mut) we introduced a point mutation corresponding to R156A in BLT1, which abolishes LTB₄ binding²⁶. While this mutant sensor localizes well to the plasma membrane, it does not change its fluorescence upon stimulation with LTB₄ (Fig. 1c). For signal normalization we co-expressed the plasma membrane targeted red fluorescent mKate2 protein beside GEM-LTB₄ using the self-cleaving viral P2A peptide²⁷. This resulted in significantly better expression levels than direct mKate2 fusion of the sensor (unpaired t-test, $p=0.003331$, Extended Data Fig. 1f-g).

Spectral characterization of GEM-LTB₄ showed an LTB₄-dependent excitation peak between 450-500 nm with an isosbestic point at 425 nm (Fig. 1d). The apparent affinity of GEM-LTB₄ in HEK293A cells is in the low nanomolar concentration range with an EC₅₀ of ~ 19.8 nM (Fig. 1e-f). LTB₄-induced fluorescence increase was stable over 45 min, and was reversible by BLT1 inhibitors or by removal of LTB₄ from the media (Fig. 1g, Extended Data Fig. 2a-d, Supplementary Video 1). We measured activation kinetics of GEM-LTB₄ using rapid local perfusion combined with high-speed imaging and calculated an average activation time constant of 770 ± 37 ms (Fig. 1h). Next, we applied a series of eicosanoid ligands to reveal that the specificity profile of GEM-LTB₄ is similar to its parent receptor, BLT1²⁸. Of the compounds tested, as expected, only the BLT1 agonists LTB₄ and 20-OH-LTB₄ induced significant changes in fluorescence (One way ANOVA, $p=2.0\cdot 10^{-181}$, Fig. 1i).

We then assessed the coupling of GEM-LTB₄ to downstream cellular signaling pathways. While LTB₄ induces intracellular Ca²⁺ signals in BLT1-expressing HEK293A cells due to known G-protein coupling²⁸, no Ca²⁺ transients were seen in GEM-LTB₄ expressing cells after stimulation (Extended Data Fig. 2e-f, Supplementary Video 1). We also quantified the plasma membrane recruitment of β -arrestin-2, which was only significant in LTB₄ stimulated BLT1 expressing cells (Pairwise t-test, $p=3.0\cdot 10^{-6}$, Extended Data Fig. 2g-h). GEM-LTB₄ did not recruit β -arrestin-2 despite prolonged incubation with LTB₄, which is consistent with the lack of internalization and the stable fluorescence of the sensor seen in cells during long exposure to LTB₄ (Extended Data Fig. 2a-b). These results indicate that GEM-LTB₄ has a minimal potential to interfere with endogenous signal transduction pathways and allows long-term direct measurements of LTB₄ levels without desensitization.

Imaging LTB₄ release from murine neutrophils with GEM-LTB₄

We next evaluated the performance of GEM-LTB₄ in detecting neutrophil-derived endogenous LTB₄ production. Isolated murine neutrophils were stimulated with fMLP and seeded on top of stable GEM-

1 LTB₄-expressing cells. Applying neutrophils prestimulated for 30 min with fMLP to cells expressing the
2 sensor gave rise to an instantaneous fluorescence signal increase (Extended Data Fig. 3a-d) indicating
3 the presence of LTB₄ in the stimulating solution. This was verified by ELISA, showing LTB₄ secretion by
4 neutrophils similar to previously reported values⁵ (Fig. 2a). Application of fMLP or non-stimulated
5 neutrophils alone did not change GEM-LTB₄ fluorescence (Extended Data Fig. 3b-d). To measure real-
6 time kinetics of LTB₄ production we stimulated neutrophils with fMLP while they were sedimenting on top
7 of sensor-expressing cells. GEM-LTB₄ fluorescence increased in areas densely covered by neutrophils
8 (2000 cells/0.1 mm²) ~5 min after fMLP stimulation (Fig. 2b-c). To confirm that the observed signals were
9 due to LTB₄ release, we verified that fMLP-stimulated neutrophils do not elicit responses in GEM-
10 LTB₄mut-expressing cells (Fig. 2b-c). LTB₄ production starts in foci and spreads over larger fields
11 (Supplementary Video 2). Automatic quantification identified significant GEM-LTB₄ signal increase in 38
12 ± 5.4% (mean ± s.e.m.) of the surface area imaged with the sensor cells (Fig. 2d, Unpaired t-test,
13 p=0.001159). Next, we measured LTB₄ release in areas of lower neutrophil density to potentially capture
14 signals from individual cells. In this experimental setup after fMLP stimulation, we could detect GEM-
15 LTB₄ signals radially emanating from areas where individual neutrophils were residing (Fig. 2e-f,
16 Supplementary Video 2). Supporting that these individual neutrophils are actively producing
17 chemoattractants, pseudopod formation in surrounding neutrophils could be detected towards the
18 producing cells (Supplementary Video 2). These results demonstrate that the sensitivity of GEM-LTB₄ is
19 sufficient to detect endogenous LTB₄ secretion from neutrophils *ex vivo*.

20 **Visualizing LTB₄ levels in zebrafish larvae with GEM-LTB₄**

21 In order to demonstrate that GEM-LTB₄ is suitable for *in vivo* detection of LTB₄, we created transgenic
22 zebrafish lines expressing the sensor under the control of suprabasal (*krt4*) and basal (*krt19*) epidermal
23 skin layer specific keratin promoters. We measured the penetration of exogenous LTB₄ into the tail fin of
24 amputated zebrafish larvae kept in isotonic embryo media (see Methods for details), which prevents early
25 leukocyte recruitment and wound closure^{29,30}, thereby allowing better penetration of exogenously applied
26 substances through the open wound. By confocal imaging, GEM-LTB₄ showed plasma membrane
27 localization and an LTB₄-dependent fluorescence signal increase close to 100% in both epidermal layers
28 (Fig. 3a-b), which is similar to the $\Delta F/F_0$ response measured in HEK293A cells. We then compared the
29 spatiotemporal LTB₄ signal distribution in the tail fin of intact and amputated zebrafish larvae expressing
30 the sensor in the suprabasal layer. After LTB₄ application, we measured limited GEM-LTB₄ signal in intact
31 fins in contrast to the gradient seen in amputated larvae (Fig. 3a-d, Supplementary Video 3), suggesting
32 that the intact surface epithelium acts as a barrier for LTB₄ penetration. In larvae expressing the control
33 GEM-LTB₄mut sensor, LTB₄ did not alter the fluorescence (Fig. 3d). Corresponding with the visualized

1 spatiotemporal LTB₄ distribution, exogenous LTB₄ triggered neutrophil migration with a time course
2 matching the measured gradient (Fig. 3e).

3 **Measuring endogenous LTB₄ production in zebrafish larvae with GEM-LTB₄**

4 LTB₄ regulates neutrophil swarming during tissue damage across species^{12,31,32}. To capture endogenous
5 LTB₄ release in zebrafish, we measured GEM-LTB₄ signals in wounded tail fins closest to the neutrophils,
6 in the basal epithelial layer. We indeed detected LTB₄ production after sterile injury (Supplementary Video
7 4), however, only in rare instances which is entirely consistent with previous reports showing stochastic
8 swarm development upon wounding in zebrafish^{32,33}. To validate the source of GEM-LTB₄ signals and
9 trigger endogenous LTB₄ release consistently from neutrophils, we used an established protocol which
10 relies on recruiting neutrophils to a wound and activating their 5-LOX dependent LTB₄ production with
11 the Ca²⁺ ionophore A23187 at the same time^{7,32} (Fig. 4a). We used arachidonic acid to recruit neutrophils
12 to an open wound under isotonic conditions, which is a precursor readily transformed to epithelial
13 chemoattractants such as 5-oxoETE in cells around the wound margin²⁹. Subsequently stimulating the
14 larvae with A23187 resulted in the generation of real-time endogenous LTB₄ gradients emanating from
15 the wound margin where neutrophils could be detected by brightfield microscopy (Fig. 4b-d,
16 Supplementary Video 5). A signal was not seen in larvae pretreated with the 5-LOX inhibitor zileuton (Fig.
17 4b-d, Supplementary Video 5) or in the GEM-LTB₄mut expressing control larvae (Fig. 4b-d) nor did
18 A23189 trigger responses in GEM-LTB₄ on its own (Extended Data Fig. 4a-b).

19 **Discussion**

20 In summary, we developed and characterized a fluorescent biosensor for the live imaging of LTB₄
21 release. GEM-LTB₄ has the sensitivity, ligand specificity, photo-stability and kinetics suitable to measure
22 physiologically relevant endogenous LTB₄ production. Importantly, GEM-LTB₄ does not activate
23 downstream signaling pathways, nor does it internalize upon long-term stimulation, which are both
24 required for the reliable measurement of extracellular LTB₄ dynamics. Indeed, GEM-LTB₄ allowed us to
25 visualize LTB₄ distribution and secretion in a variety of *in vitro* and *in vivo* experimental setups.

26 Our current understanding of chemotaxis is largely based on tissue culture experiments where cells
27 migrate towards exogenously applied gradients of chemoattractants^{2,34}. Models can estimate the
28 spatiotemporal distribution of chemoattractants, however, tools such as GEM-LTB₄ will be required to
29 precisely map the diffusion and degradation of chemoattractants around migrating cells¹¹. Methods to
30 determine accurate parameters of diffusion coefficients or dissipation rates of chemoattractants in tissues
31 have been limited, however, they are fundamental to build reliable mathematical models for describing

cell migration. Measurements of exogenously applied chemoattractants in live tissues, such as we showed here for LTB₄ in zebrafish, will be the basis to determine these parameters.

The power of measuring LTB₄ release on the single cell level will also aid our understanding of neutrophil heterogeneity³⁵. Our results on bone marrow derived neutrophils indeed indicate that the LTB₄ producing capacity is variable among these cells, which would not have been possible to determine from bulk ELISA measurements. How heterogeneity contributes to emergent neutrophil behaviors such as swarming, is a question that can be answered in the future with GEM-LTB₄. Just as how GPCR-based biosensors became a key in recent years to understand the spatiotemporal coding in neuronal circuits^{22–24}, we expect novel fluorescent tools such as GEM-LTB₄ to expand the horizons of immuno-imaging. We anticipate that our study will prime future developments of similar sensors in the inflammation biology field to facilitate the long-sought live visualization of chemokines and chemoattractants^{1,34}.

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Author Contributions

B.E. conceived the project and designed the experiments. Sz.T., B.V. and B.R. performed the experiments. B.R. and Sz.T. developed computational tools and wrote computer code to analyze the data. All authors participated in writing the manuscript.

Competing interests

The authors declare no competing financial interests.

1 **Methods**

2 **Cell Lines**

3 HEK293A cells were obtained from ThermoFisher (CAT#: R70507) and maintained in DMEM
4 supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin in a 5%
5 humidified CO₂ incubator at 37°C.

6 For establishing stable HEK293A cell lines expressing our sensors, cells were co-transfected (see
7 Methods Transfection) in a 1:1 ratio with *SB100x Sleeping Beauty* transposase and the SB transposon
8 plasmids encoding the sensors. Cells were selected for at least two weeks using 2 µg/ml puromycin, and
9 positive cells were then isolated by flow cytometry on a BD FACSAria™ III, using 488 and 561nm
10 excitation and 530/30 and 670/30 nm emission wavelengths. These cells were then maintained in
11 complete DMEM as described above, supplemented with 0.5 µg/ml of puromycin.

12 **Zebrafish**

13 Wild-type (AB) and Casper³⁶ strains were used for experimentation and the generation of transgenic
14 lines.

15 To generate our in-house transgenic lines, fresh fertilized embryos (30-60 min post-fertilized) were
16 microinjected with transgenesis plasmids as described³⁷. Positive embryos were selected according to
17 their cardiac marker expression, and raised until sexual maturity to identify founder fish and establish F1
18 generations. We used the QF2/QUAS system³⁸, which in brief consists of a target promoter expressing
19 the transcription factor QF2 and the QUAS site (QF2 DNA binding site) introduced upstream of a target
20 protein. QF2 and QUAS lines are generated separately, and when they are crossed together, the protein
21 of interest is expressed in target cells.

22 The zebrafish lines used for experimentation include: Tg(krt4:QF2), Tg(krt19:QF2), Tg(QUAS:PM-
23 mKate2-P2A-GEM-LTB₄), Tg(QUAS:PM-mKate-P2A-GEM-LTB₄mut) and Tg(mpx:GFP)³⁹.

24 After spawning and microinjection, zebrafish larvae were kept in E3 medium (5 mM NaCl, 0.17 mM KCl,
25 0.33 mM CaCl₂, and 0.33 mM MgSO₄) at 28°C for 5-6 days before being introduced on the main system.
26 Adult fish were maintained as described⁴⁰, at 28°C on a 14/10h light/dark cycle, with the approval of the
27 Institutional Animal Care and Use Committee (IACUC) of Semmelweis University. All experimental
28 procedures were approved by the Hungarian National Food Chain Safety Office (Permit Number:
29 PE/EA/1027-7/2019). Experiments were performed on 3-4 days post-fertilization (dpf) larvae.

30

1 **Plasmid Construction**

2 Human Leukotriene B4 receptor 1 (BLT1, Ensembl: ENSG00000213903) was cloned from
3 complementary DNA derived from human peripheral blood mononuclear cells, and subcloned into the
4 pEGFP-N1 (Clontech) vector backbone between XhoI and HindIII sites using standard molecular biology
5 procedures. To create the GEM-LTB₄ prototypes, we introduced circularly permuted EGFP (cpEGFP)
6 into the third intracellular loop of BLT1 between R212 and F213 using combinations of long (LSSLE) and
7 short (GG) N- and C-terminal linkers. In a second round of screening, various lengths of the N-terminal
8 LSSLI and C-terminal NHDQL linkers were combined. To facilitate the creation of sensor variants with
9 different linkers, we introduced silent mutations resulting in unique restriction sites of PstI and Sall on the
10 N- and C-termini of cpEGFP, respectively, outside of the linker sequences. To generate GEM-LTB₄mut,
11 we introduced the R156A single amino acid mutation²⁶ into the BLT1 coding sequence of the final GEM-
12 LTB₄ sensor.

13 To enhance the plasma membrane localization of the sensors, the mouse IgK leader sequence
14 (METDTLLLWVLLLWVPGSTGD) was inserted upstream of the coding region. To coexpress a
15 membrane targeted red fluorescent protein beside GEM-LTB₄, we fused it N-terminally through a viral
16 self-cleaving P2A peptide⁴¹ with PM-mKate2, which uses the membrane localization sequence
17 MGCVCSSNPENNNN, derived from the Lck protein.

18 To establish stable human embryonal kidney (HEK293A) cell lines, the sensors were subcloned into the
19 “Sleeping Beauty” (SB) transposon plasmid allowing for puromycin-based selection^{42,43}.

20 To create transgenic zebrafish lines, we relied on the Tol2kit system³⁷ combined with the QF2/QUAS
21 system⁴⁴ (see below) to express our sensors. To create plasmids for transgenesis, DNA fragments
22 encoding PM-mKate2-P2A-GEM-LTB₄ and PM-mKate2-P2A-GEM-LTB₄mut were first subcloned into the
23 pME backbone as entry clones, and recombined with the QUAS promoter and SV40 polyadenylation
24 sequence into the pDestTol2CR vector backbone. This backbone contains minimal tol2 elements and a
25 cardiac promoter expressing the red fluorescent mKate2 marker for selection. To express QF2 in
26 epithelial cells, we used the krt4⁴⁵ and krtt1c19e⁴⁶ (referred to as krt19) promoters driving expression in
27 the suprabasal and basal epithelial cells, respectively, from a pDestTol2CG2 backbone harboring a
28 cardiac green selection marker. The p5E-krt4, p5E-krtt1c19e, p5E-QUAS and pME-QF2 plasmids were
29 kind gifts from Philipp Niethammer.

30

31

1 **Cell transfection**

2 For transient DNA expression, HEK293A cells were seeded and transfected with lipofectamine 2000
3 (ThermoFisher), according to manufacturer's guidelines. In brief, we used 1 μ l of lipofectamine with 500
4 ng of DNA for 2.5 cm² of culture surface area. These numbers were then scaled up or down according
5 to the surface area. Cells were then further incubated for 16-24h before experiments.

6 To determine GEM-LTB₄ spectra, cells were electroporated using the Neon Transfection System
7 (ThermoFisher), according to manufacturer's guidelines. In brief, we used 2 pulses at 1005 V for 35 ms
8 on 1×10^6 cells in suspension. Cells were then further incubated for 16-24h before experiments.

9 **Spinning disk confocal microscopy**

10 All imaging was performed on an inverted Nikon Eclipse Ti2 microscope with a motorized piezo stage,
11 perfect focus system and 488 nm and 514 nm laser lines for the Yokogawa CSU-W1 spinning disk scan
12 head combined with two back illuminated Photometrics Prime BSI scientific CMOS cameras for detection.
13 Green and red fluorescence were recorded using 525/50 nm and 600/30 nm emission filters, respectively.

14 Unless otherwise stated, images were recorded with a dimension of 1024x1024 pixels with 2x2 binning
15 and bit depth of 16 bits. For acquisition, we used 40x ApoLambda/NA1.15 water-dipping and 20x
16 ApoLambda/NA0.95 water-dipping objectives.

17 To determine τ_{on} value of GEM-LTB₄, images were recorded with a dimension of 128x128 pixels with a
18 frequency of 50 frames per second.

19 **Fluorescence imaging and treatment of cultured cells**

20 HEK293A, PM-mKate2-P2A-GEM-LTB₄- or PM-mKate2-P2A-GEM-LTB₄mut-expressing stable
21 HEK293A cells were seeded on poly-D-lysine-coated μ -slide 8-well plates (Ibidi) at a density of 30,000
22 cells/well. After 16-24h, cells were transfected with plasmid DNA of interest and further incubated for 16-
23 24h, while stable sensor-expressing cells were directly incubated for 36-48h. Before imaging, growth
24 medium was replaced with transparent extracellular media (EC) consisting of: 3.1 mM KCl, 133.2 mM
25 NaCl, 0.5 mM KH₂PO₄, 0.5 mM MgSO₄, 5 mM Na-Hepes, 2 mM NaHCO₃, 1.2 mM CaCl₂ and 2.5 mM
26 Glucose.

27 During cell experimentation, unless otherwise stated, a 2-3 min baseline was recorded before adding
28 different ligands onto the cells. All eicosanoid ligands were obtained from Cayman Chemicals and
29 include: LTB₄ (CAT#: 20110; CAS: 71160-24-2), Arachidonic Acid (CAT#: 90010; CAS: 506-32-1), 5-
30 oxoETE (CAT#: 34250; CAS: 106154-18-1), 5(S)-HETE (CAT#: 34230; CAS: 70608-72-9), 12(S)-HETE

1 (CAT#: 34570; CAS: 54397-83-0) and 20-Hydroxy LTB₄ (CAT#: 20190; CAS: 79516-82-8). Apart from
2 the dose-response assay, all ligands were used at a final concentration (FC) of 100 nM. Normalized
3 intensity changes were then measured and calculated (see below).

4 For establishing a dose-response curve, increasing LTB₄ concentrations were used, either a single
5 concentration ranging from 1 pM to 1 μM or sequential concentrations of 1, 10 and 100 nM, given at 3
6 min intervals.

7 To inhibit GEM-LTB₄, we used the competitive antagonists of BLT1, CP-105,696 (Sigma-Aldrich, CAT#:
8 PZ0363; CAS: 158081-99-3) and BIIL260 hydrochloride (Sigma-Aldrich, CAT#: SML2286; CAS: 204974-
9 93-6). LTB₄ was added at a FC of 100 nM onto the cells after a 2 min baseline, then the inhibitors were
10 applied either directly or perfused onto the cells using a custom-made perfusion system.

11 Ionophores Ionomycin (Cayman Chemical, CAT#: 10004974; CAS: 56092-81-0) and A23187 (Sigma-
12 Aldrich, CAT#: C9275; CAS: 52665-69-7) were directly added onto the cells at a FC of 10 and 1 μM,
13 respectively.

14 ***Spectral scan***

15 HEK293A cells were transfected with GEM-LTB₄. Before experimentation, cells were lifted and 1*10⁶
16 cells were re-suspended in 100 μl EC media and either stimulated with 100 nM of LTB₄ or not.
17 Fluorescence was measured using a CLARIOstar (BMG Labtech) plate reader. The excitation scan was
18 performed by exciting from 340 to 520 nm with a step width of 2 nm, and collecting emission at 560/20
19 nm. The emission scan was determined by excitation at 470/20 nm and collecting emission from 490 to
20 650 nm with a step width of 2 nm.

21 ***Isolation and stimulation of mouse neutrophils***

22 Mouse neutrophils were isolated from the bone marrow of femurs and tibias of intact mice using sterile
23 and endotoxin-free reagents by the hypotonic lysis of red blood cells followed by centrifugation based
24 Percoll (GE Healthcare) gradient separation of neutrophils as previously described⁴⁷. Finally, neutrophils
25 were re-suspended in EC media and kept at room temperature (RT) until use.

26 To record endogenous LTB₄ release from activated neutrophils, after establishing a baseline of 2 min,
27 2*10⁶ neutrophils/cm² were added on the top of the sensor-expressing stable HEK293A cells and
28 immediately stimulated with 2 μM fMLP (Sigma-Aldrich, CAT#: F3506; CAS: 59880-97-6), or plain EC
29 media. The experiment was recorded for an additional 28 min, in a total volume of 300 μl. Exogenous
30 LTB₄ was then directly added into the wells 5 min before the end of the experiments.

1 To quantify LTB₄ levels from activated neutrophils, 2*10⁶ neutrophils were stimulated with 2 μM fMLP or
2 EC media, and incubated at RT for 28 min. in 300 μl. An LTB₄ Elisa assay (ThermoFischer Scientific)
3 was performed using the supernatant of the cells according to the manufacturer's instructions.

4 ***Fluorescence imaging and treatment of transgenic zebrafish***

5 *In vivo* experiments were conducted on 3-4 days post fertilization (dpf) old larvae. Before experiments,
6 larvae were anesthetized using 0.2 mg/ml Tricaine (Sigma-Aldrich, CAT#: 10521; CAS: 886-86-2) in
7 isotonic E3 (standard E3 prepared with the additional 140 mM NaCl). Larvae were maintained and
8 measured in isotonic solution to prevent early leukocyte recruitment²⁹ and wound closure³⁰, thereby
9 allowing better penetration of exogenously applied substances. If needed, larvae were wounded (ventral
10 nick-wound or tail fin amputation) using a 4 mm carbon steel needle blade micro knife (Fine Science
11 Tools). Afterwards, unless otherwise stated, larvae were mounted in isotonic E3-based 1% low melting
12 agarose (Gold biotechnology).

13 To test GEM-LTB₄ *in vivo*, exogenous LTB₄ was directly added onto intact or wounded larvae at a FC of
14 1 μM after recording a 5 min baseline. To measure endogenous LTB₄ production, larvae were wounded
15 on the ventral tail fin and incubated for 90 min in isotonic E3 medium supplemented with arachidonic acid
16 (FC=20 μM) to attract the neutrophils to the wound^{29,48}. E3 or 20 μM zileuton (Sigma-Aldrich, CAT#:
17 1724656; CAS: 111406-87-2), a 5-lipoxygenase inhibitor, was added 40 min before the end of the
18 incubation. The larvae were then mounted in hypotonic (standard E3 embryo medium) 1% low melting
19 agarose. After a 3 min baseline, A23187 (FC=100 μM) was directly added onto the larvae to activate the
20 cPLA₂ and 5-lipoxygenase enzymes. Endogenous LTB₄ production was also measured in untreated
21 larvae which were wounded, mounted and imaged in normal E3 embryo medium.

22 ***Computational analysis and quantification***

23 All image analysis was processed using in-house programming pipelines in Python. Prior to analysis, the
24 background intensity was subtracted automatically using the SMO software⁴⁹ and images were registered
25 using pyStackReg⁵⁰.

26 In experiments on HEK293A cells, GEM-LTB₄- and GEM-LTB₄mut-expressing cells were segmented in
27 the red channel (mKate2) using the Cellpose software⁵¹ and generated masks were tracked using a
28 software developed by Löffler et al.⁵². If needed, full tracked-masks were transformed to only include cell
29 membrane mask. These masks were then used to extract the data, giving the average intensity of each
30 channel (green and red) for each cell at each time point.

1 The extracted data were processed using the pandas Python library. Given that mKate2 (red) and GEM-
2 LTB₄ (green) are expressed in a fixed ratio⁴¹ and both are located in the same cell compartment
3 (membrane), a green/red intensity ratio was calculated as a normalized signal. To express all intensities
4 to a relative baseline of 0, the formula $(F(t)-F_0)/F_0$, named as $\Delta F/F_0$, was applied on the data, where $F(t)$
5 is the intensity at a given time point and F_0 the average intensity of the baseline. In case of the calcium
6 assay, data were normalized between baseline and the ionomycin-induced maximal response values.
7 Therefore, the following formula $(F(t)-F_0)/(F_{\max}-F_0)$ was used, where $F_{\max}$ is the average intensity after
8 ionomycin stimulation.

9 To quantify endogenous LTB₄ secretion from murine neutrophils using sensor-expressing stable
10 HEK293A lines, besides calculating pixelwise $\Delta F/F_0$ values as described above, a threshold value was
11 applied on the red channel of the image to remove the background and create an overall mask of the
12 cells. This mask was then subdivided into region of interests (ROIs) of 32x32 pixels in order to capture
13 local changes in $\Delta F/F_0$ values. The mean intensities of these ROIs were then normalized in the same
14 way as above. ROIs that display a minimum of 50% change in $\Delta F/F_0$ after fMLP stimulation compared to
15 baseline were labelled as positive ROIs. The summed area of all positive ROIs was then compared to
16 the overall area (size of the cell mask) to express the positive ROIs as coverage percentage.

17 To create a radial profile plot expressing the intensity distribution as a function of distance from a center
18 point, the radial profile calculator or the diplib library (<https://diplib.org/>) was used.

19 To quantify relative plasma membrane localization of different GEM-LTB₄ prototypes and β -arrestin2,
20 after generating individual masks for each cell, the masks were separately dilated and eroded and a
21 differential mask was calculated to yield a ring-shaped mask covering the plasma membrane. The eroded
22 mask was considered as the cytoplasm mask. Relative membrane localization was calculated as
23 $F_{\text{membrane}}/F_{\text{cytoplasm}}$ or $F_{\text{membrane}}/F_{\text{cell}}$ after background subtraction.

24 For titration curves, K_d value was obtained by fitting the data on a four-parameter log-logistic function.

25 To determine τ_{on} value of GEM-LTB₄, raw fluorescence intensity data from individual measurements of
26 high-speed acquisition were smoothed by using a 500 msec wide raised cosine Hanning window.
27 Intensity values were then normalized between average baseline (0%) and LTB₄ stimulated (100%)
28 maximum fluorescence values. For each measurement, a horizontal curve was fitted onto the baseline
29 and the maximal values and a rising linear curve on the middle subtriple of the dataset. The latter curve
30 was used to determine the τ_{on} values, the time required to reach 50% of the maximal fluorescence
31 increase. To correct for the msec differences between LTB₄ stimulation times among the experimental
32 samples, a final normalization step was performed along the time axis of the data by setting the starting

point of the rising curves (i.e., time of LTB₄ stimulation) to 0 msec. Finally, a normalized average activation curve was calculated by applying Hanning-smoothing on the combined baseline, the maximal horizontal and the rising linear curves determined above.

For *in vivo* experiments, images were pre-processed similarly as described above. Briefly, a threshold value was applied on the red channel of the image to remove the background and create an overall mask of the expressing cells. A “wound” mask was manually drawn to determine the edges of the wound as a reference. By using the distance transform function from the mahotas library⁵³, a distance gradient map was generated from the wound mask in order to determine intensity values as a function of their distance from the wound margin. Averaged intensities of each gradient layer (i.e. each distance layer from the wound) were binned (by 2x2 pixels) and green/red ratios were calculated. Finally, $\Delta F/F_0$ ratios were calculated based on baseline F_0 values.

For neutrophil migration quantification, the MTrackJ plugin from ImageJ/Fiji⁵⁴ was used to manually track the cells over time. To determine their position relative to the wound, a “wound” mask was also used for each time point.

Quantification and statistical analysis

Normalized and fully processed data were plotted using the seaborn library⁵⁵. Montage images and videos were assembled using ImageJ/Fiji.

Statistical tests were calculated in Python using the Pingouin library⁵⁶. For pairwise analysis, statistical significance was determined by a two-tailed unpaired Student’s t-test with Welch’s correction. For multiple comparison of independent conditions, one-way ANOVA was used with Fisher’s LSD (Least Significant Difference) correction, or with Dunnett’s correction, if a control condition was used. For multiple comparison of the same population over time, one-way repeated measure ANOVA was used with Bonferroni correction. All measurements are expressed as mean $\pm$ s.e.m., with sample size, number of replicates and *P* values indicated in figure legends.

Data and code availability

All data generated in this study are available from the lead contact upon request. All original code has been deposited at www.github.com/XXXX and is publicly available as of the date of publication. The main plasmids generated in this study have been deposited to Addgene (XXXX). Other plasmids and original material are available from the lead contact upon request.

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- 20

1 Figure legends

2 Fig. 1 | Development and characterization of the GEM-LTB₄ sensor in HEK293A cells.

3 **a**, Schematic diagram of LTB₄-sensor design showing fluorescence increase upon ligand binding. The
4 sensor consists of cpEGFP inserted with linkers into the 3rd intracellular loop (ICL3) of BLT1. **b**, Summary
5 of $\Delta F/F_0$ fluorescence responses in all LTB₄-sensor variants tested in this study. Data shown as mean $\pm$
6 s.e.m. for n=17-137 cells from 3 independent experiments. GEM-LTB₄ is shown in blue. **c**, GEM-LTB₄
7 and GEM-LTB₄mut confocal fluorescence and corresponding $\Delta F/F_0$ ratio images in HEK293A cells before
8 and after 100 nM LTB₄ stimulation. Scale bars, 25 μ m. **d**, Excitation and emission spectra of GEM-LTB₄
9 in the absence (dotted lines) and presence (continuous lines) of 100 nM LTB₄. Insert shows ratio of
10 excitation spectra. Each trace is the average of n=3 independent experiments (a.u.=arbitrary units). **e**,
11 Dose-response measurements of GEM-LTB₄ and GEM-LTB₄mut, with the corresponding EC₅₀ value.
12 Data shown as mean $\pm$ s.e.m. for n=66 and 218 cells per condition, respectively, from 3 independent
13 experiments. EC₅₀ value was obtained by fitting the data to a four-parameter log-logistic function. **f**,
14 Average $\Delta F/F_0$ responses of GEM-LTB₄ and GEM-LTB₄mut to sequentially added increasing doses of
15 LTB₄. Data shown as mean $\pm$ s.e.m. for n=113 and 181 cells, respectively, from 3 independent
16 experiments. **g**, GEM-LTB₄ response to 100 nM LTB₄ stimulation followed by treatment with 1 μ M of the
17 BLT1 inhibitor CP-105,696. Data shown as mean $\pm$ s.e.m. for n=85 cells from 3 independent experiments.
18 **h**, Kinetic analysis from high-speed acquisition of GEM-LTB₄ fluorescence in HEK293A cells during 100
19 nM LTB₄ stimulation. All measured normalized data points and the average fitted curve are shown from
20 n=13 cells from 7 independent experiments. **i**, Maximal $\Delta F/F_0$ responses of GEM-LTB₄ and GEM-
21 LTB₄mut to 100 nM of the indicated eicosanoid compounds. Data shown as mean $\pm$ s.e.m. for n=45 and
22 88 cells, respectively, from 3 independent experiments. Statistical analysis were performed with One-
23 way ANOVA ($F=761.3$, $p=2.0 \times 10^{-181}$) with Dunnett's correction (20-OH-LTB₄ and LTB₄ are different from
24 control for GEM-LTB₄).

25 Fig. 2 | Real-time measurements of LTB₄ release from neutrophils with GEM-LTB₄.

26 **a**, ELISA measurement of LTB₄ secretion by murine neutrophils stimulated with 2 μ M fMLP for 28 min.
27 Data shown as mean $\pm$ s.e.m. for 3 independent experiments, unpaired t-test, $**P=0.0012$. **b**, Brightfield
28 microscopy and corresponding $\Delta F/F_0$ ratio images of murine neutrophils seeded over HEK293A cells
29 expressing GEM-LTB₄ (*left*) and GEM-LTB₄mut (*right*). Scale bars, 100 μ m. Representative images were
30 taken before neutrophil addition (basal), after adding neutrophils and stimulating with 2 μ M fMLP (20 min)
31 and followed by 100 nM LTB₄ stimulation (35 min). **c**, Average traces of $\Delta F/F_0$ responses in GEM-LTB₄
32 and GEM-LTB₄mut expressing cells shown in b. Data are presented as mean $\pm$ s.e.m. for n=600 and 465

1 cells respectively from 3 independent experiments. **d**, Relative surface area of GEM-LTB₄ expressing
2 cells shown in **b**, reacting with over 50% increase in normalized $\Delta F/F_0$ as a response to 2×10^6 neutrophils
3 stimulated with 2 μM fMLP in a 1 cm^2 chamber. Data shown as mean $\pm$ s.e.m. for $n=15$ -15 fields
4 of view from 3-3 independent experiments with unpaired t-test, $***P=0.001159$. **e**, Brightfield microscopy
5 and corresponding $\Delta F/F_0$ ratio images of GEM-LTB₄ expressing HEK293A with neutrophils seeded over
6 them at a $\sim 1/10$ density compared to **b**. Representative $\Delta F/F_0$ images taken before neutrophil addition
7 (basal) and at two time points after stimulation with 2 μM fMLP followed by 100 nM LTB₄. White asterisk
8 (*) refer to the center of the analysis shown in **f**. Scale bars, 20 μm . **f**, Representative spatiotemporal
9 traces of pixelwise $\Delta F/F_0$ GEM-LTB₄ values from **e**. The spatial origo is the center of the marked
10 neutrophil (*) also shown in the inlay image. Similar results were obtained in $n=3$ independent
11 experiments.

12 **Fig. 3 | Expression of GEM-LTB₄ in zebrafish and exogenous LTB₄ penetration detection.**

13 **a**, Representative confocal fluorescence imaging and quantification of $\Delta F/F_0$ responses of GEM-LTB₄ in
14 Tg(krt4:QF2 x QUAS:GEM-LTB₄) (*top*) and Tg(krt19:QF2 x QUAS:GEM-LTB₄) (*bottom*) zebrafish larvae
15 before and after 1 μM LTB₄ stimulation. Enlarged images show the cellular distribution of GEM-LTB₄
16 expression in the respective epithelial layers. Scale bars, 100 μm and data are presented as mean $\pm$
17 s.e.m. **b**, Representative $\Delta F/F_0$ of time-lapse images of amputated zebrafish larvae Tg(krt4:QF2 x
18 QUAS:GEM-LTB₄) after stimulation with 1 μM LTB₄. Scale bar, 100 μm . **c**, Averaged spatiotemporal
19 profile plot of GEM-LTB₄ $\Delta F/F_0$ responses after stimulation of intact tail fins with 1 μM LTB₄ in Tg(krt4:QF2
20 x QUAS:GEM-LTB₄) larvae. $n=3$ larvae. **d**, Averaged spatiotemporal profile plot of GEM-LTB₄ (*left*) and
21 GEM-LTB₄mut (*right*) $\Delta F/F_0$ responses after stimulation of amputated tail fins with 1 μM LTB₄ in
22 Tg(krt4:QF2 x QUAS:GEM-LTB₄) and (krt4:QF2 x QUAS:GEM-LTB₄mut) larvae. The amputation and
23 stimulation were performed under isotonic conditions (see Methods for details). $n=3$ larvae. **e**,
24 Measurement of neutrophil movement triggered by control or LTB₄ towards amputational tail fin wounds
25 imaged in Tg(mpx:GFP) zebrafish larvae by light transmission and fluorescence microscopy. *Top left*:
26 scheme of neutrophil movement quantification towards the wound. *Left*: representative leukocyte tracks
27 capturing all visible cell movements during imaging in control and 1 μM LTB₄ treated samples. *Right*:
28 Time course of average neutrophil movement towards the wound shown in **b**, in control and in 1 μM LTB₄
29 stimulated larvae. Data are shown as mean $\pm$ s.e.m. for $n=25$ and 38 cells from 5 and 4 independent
30 experiments, respectively, with an unpaired t-test, $****P=1.6 \times 10^{-9}$ performed at the endpoint of the
31 measurement.

32

1 **Fig. 4 | Endogenous LTB₄ release measured by GEM-LTB₄ from neutrophils in zebrafish.**
2 **a**, Schematics of experimental design for measuring endogenous LTB₄ release. Larvae were wounded
3 and pre-incubated in isotonic E3 with 20 μ M arachidonic acid for 90 min alone or in combination with 20
4 μ M of the 5-lipoxygenase inhibitor zileuton, and then stimulated with 100 μ M A23187 after mounting in
5 hypotonic agarose. **b**, Representative pseudo-color of green/red ratio of time-lapse images of zebrafish
6 larvae Tg(krt19:QF2 x QUAS:GEM-LTB₄) (top and bottom) and (krt19:QF2 x QUAS:GEM-LTB₄mut)
7 (middle) in the basal epithelial cells. Scale bars, 50 μ m. **c**, Brightfield images of the corresponding
8 wounded tail fins in b, at 90 min with manually traced leukocyte time lapse tracks shown in color. Scale
9 bars, 50 μ m. **d**, Averaged spatiotemporal profile plots of corresponding experiments shown in b. n=3
10 larvae each.

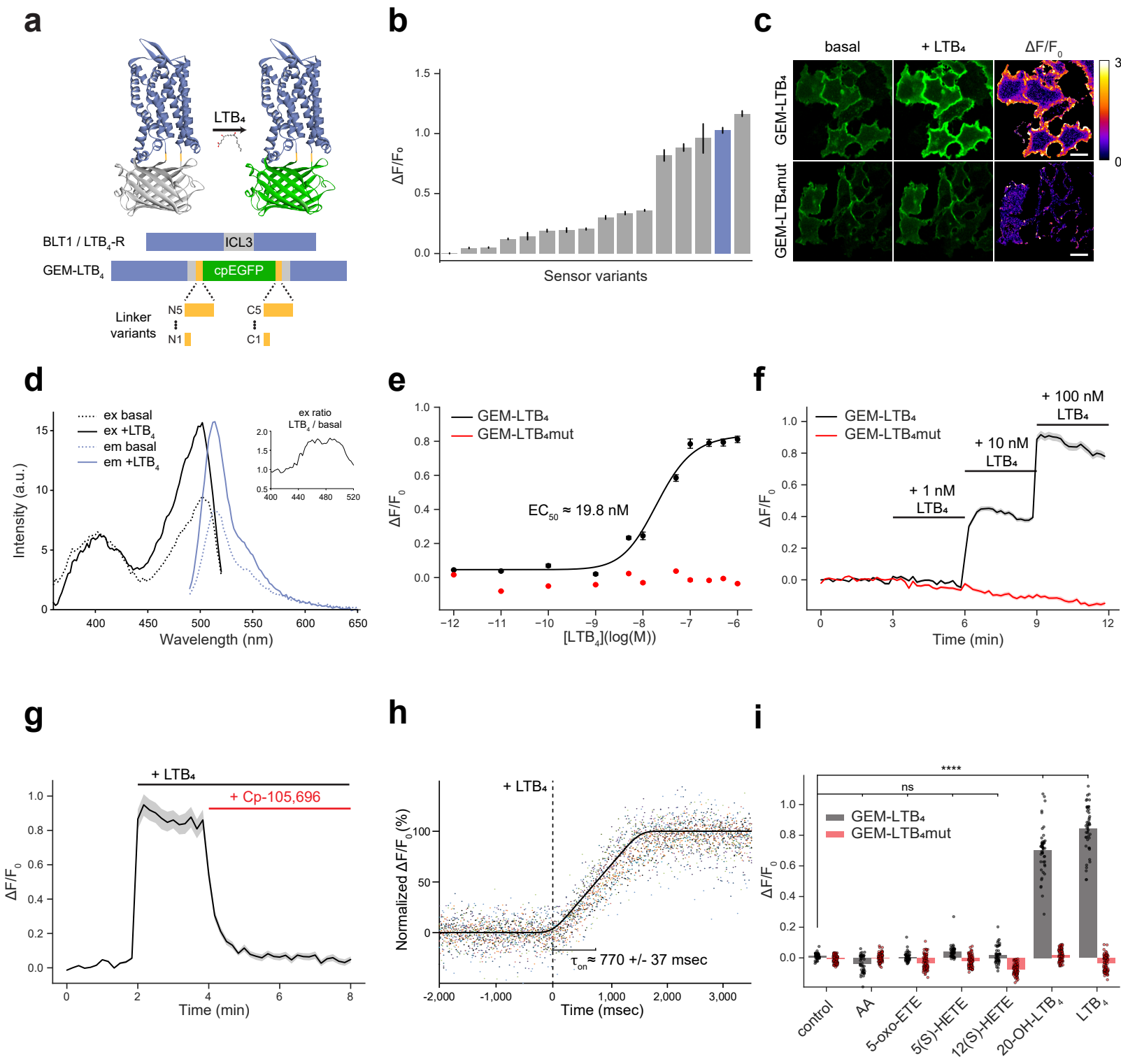
Fig.1

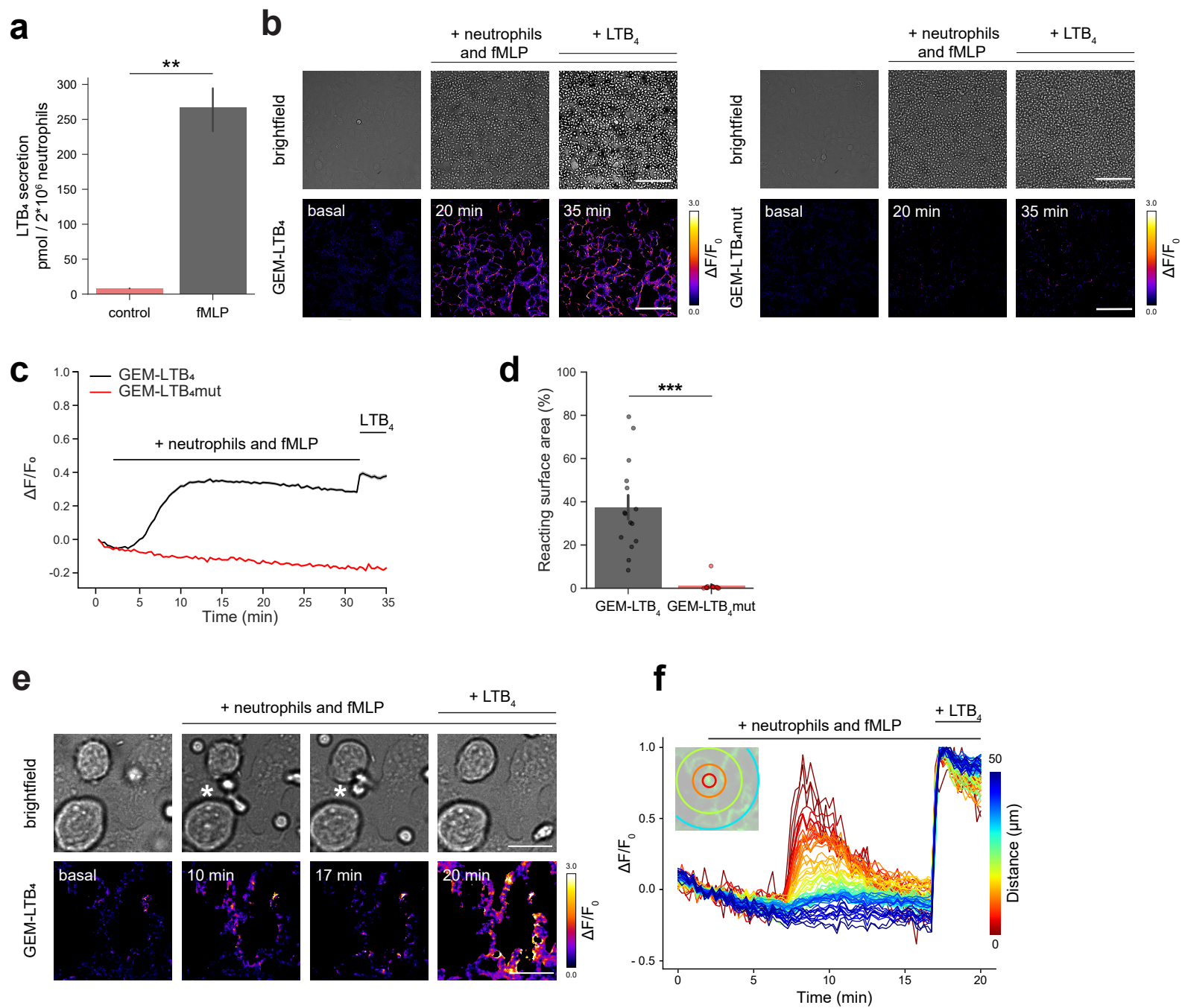
Fig. 2

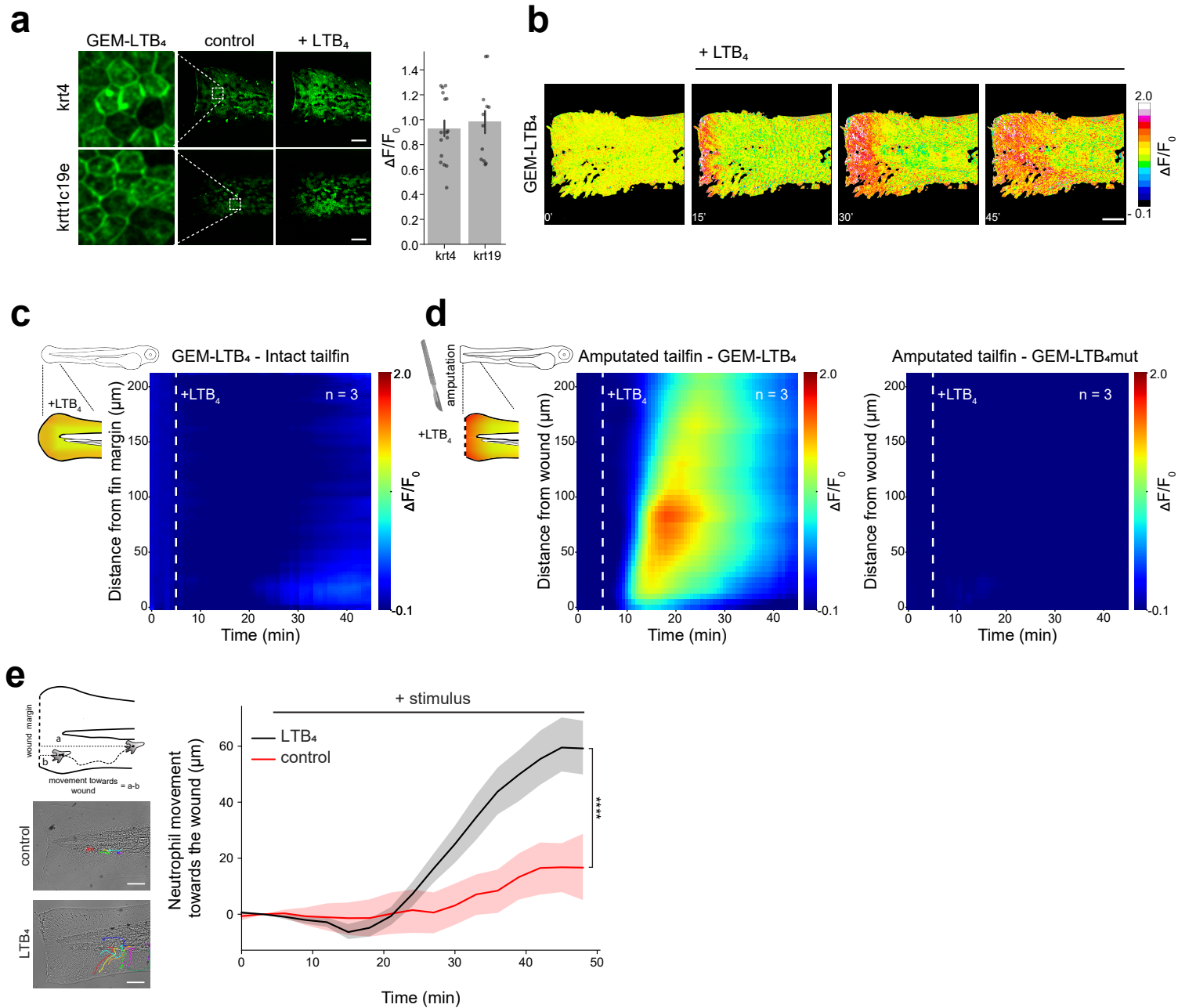
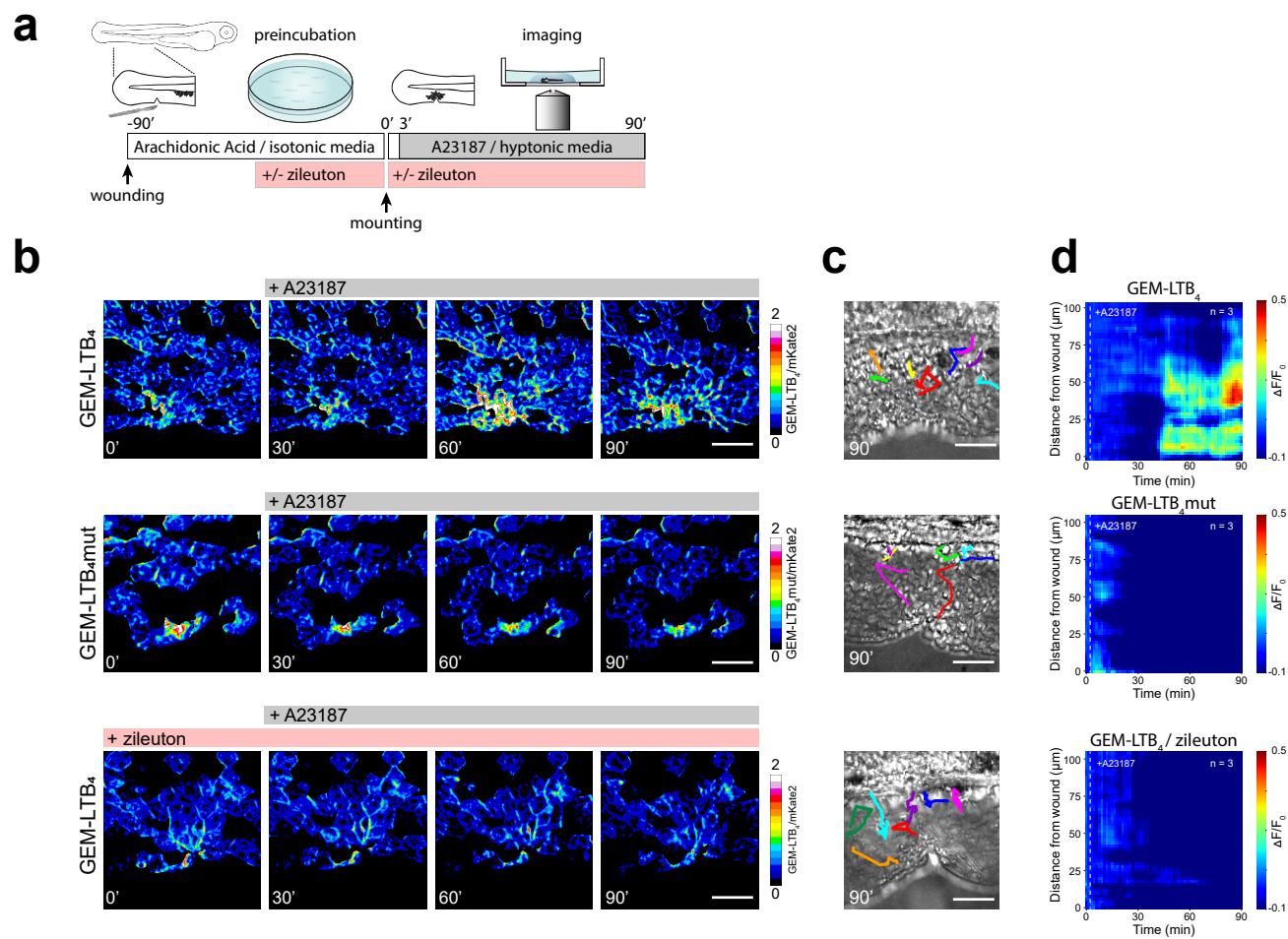
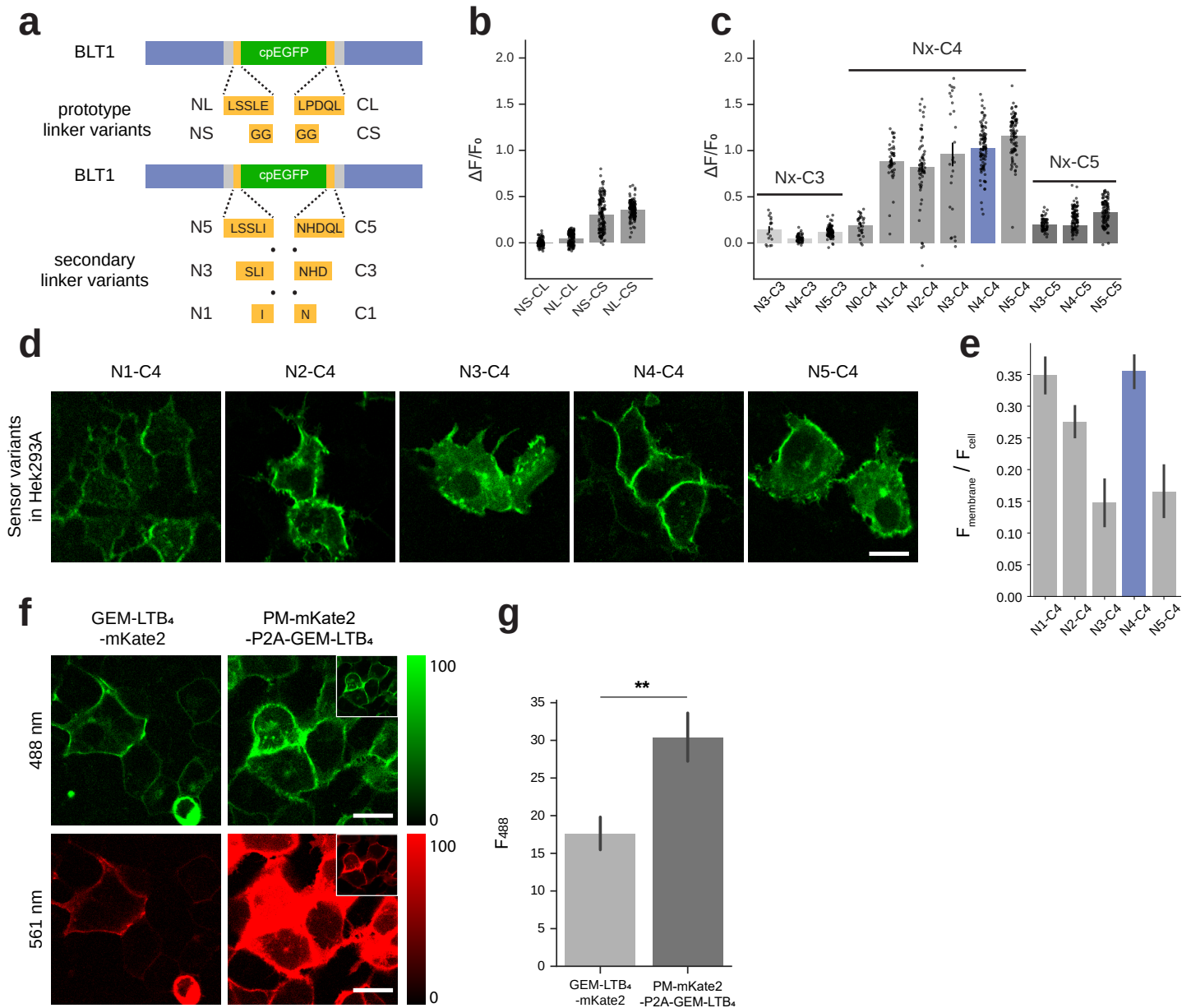
Fig. 3

Fig. 4

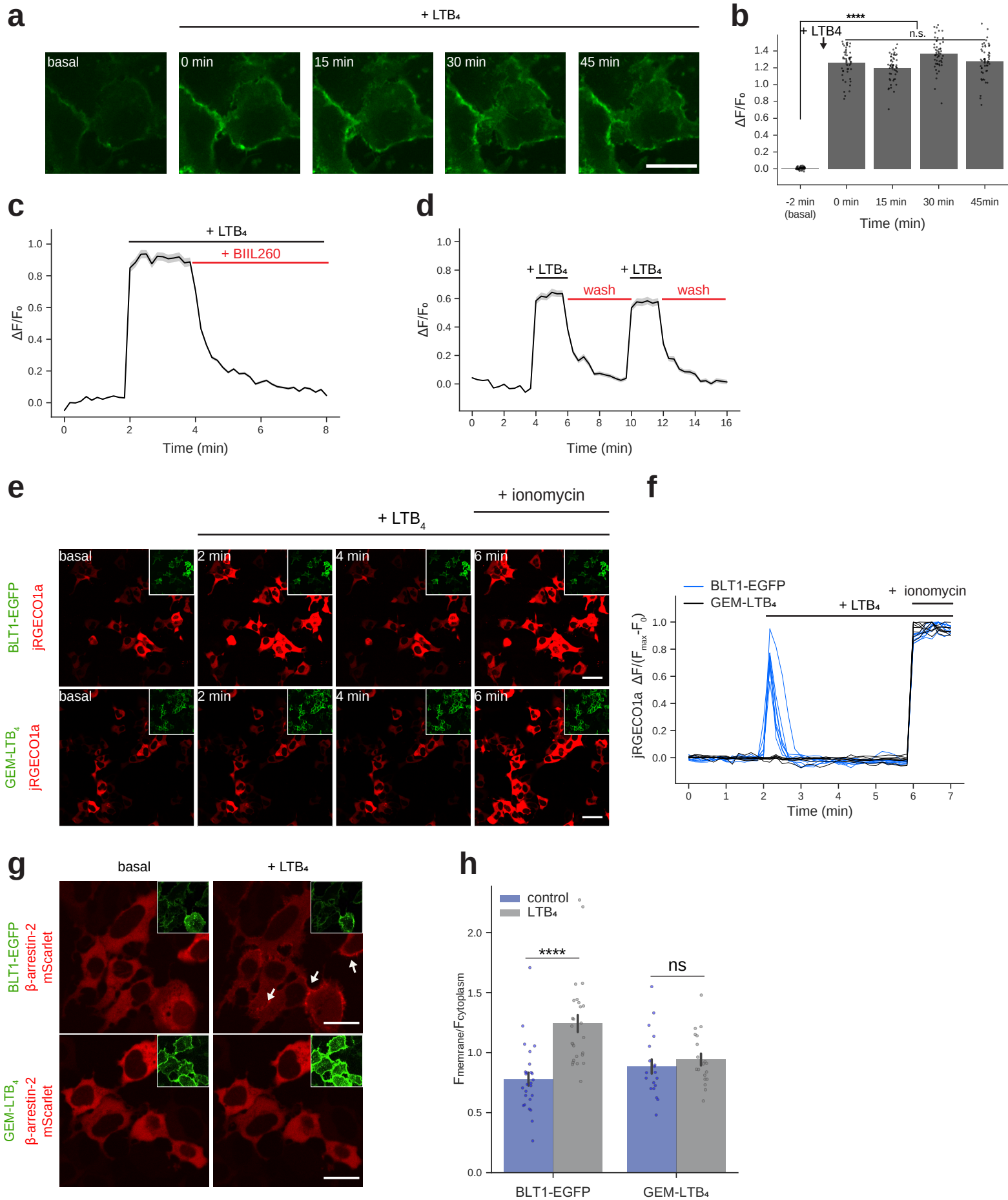
Extended Data Fig.1



Extended Data Fig.1 | Linker length optimization of LTB₄ sensors.

a, Schematic diagram of LTB₄-sensor design showing different N and C terminal linker variants used to create sensor prototypes. **b-c**, Summary of $\Delta F/F_0$ fluorescence responses in sensor variants, shown as mean $\pm$ s.e.m., containing **(b)** prototype linker variants (n=106-139 cells for from 3 independent experiments) and **(c)** secondary linker variants (n=17-105 cells from 3 independent experiments). **d**, Representative confocal fluorescence images of LTB₄-sensor expression in HEK293A cells with various indicated linker combinations. Scale bar, 25 μ m. **e**, Quantification of relative membrane localization of LTB₄-sensors shown in d, as $F_{\text{membrane}}/F_{\text{cell}}$ ratio expressed as mean $\pm$ s.e.m. (n=23-57 cells from 3 independent experiments). GEM-LTB₄ is shown in blue. **f**, Representative confocal fluorescence images of HEK293A cells expressing GEM-LTB₄ with mKate2 directly fused to the C-terminus or co-expressed via the P2A self-cleavable peptide. Scale bars, 25 μ m. **g**, Quantification of average F_{488} intensities of GEM-LTB₄-mKate2 fusion variants shown in a, expressed as mean $\pm$ s.e.m. (n=15 cells from 3 independent experiment, unpaired t-test, ** $P=0.003331$).

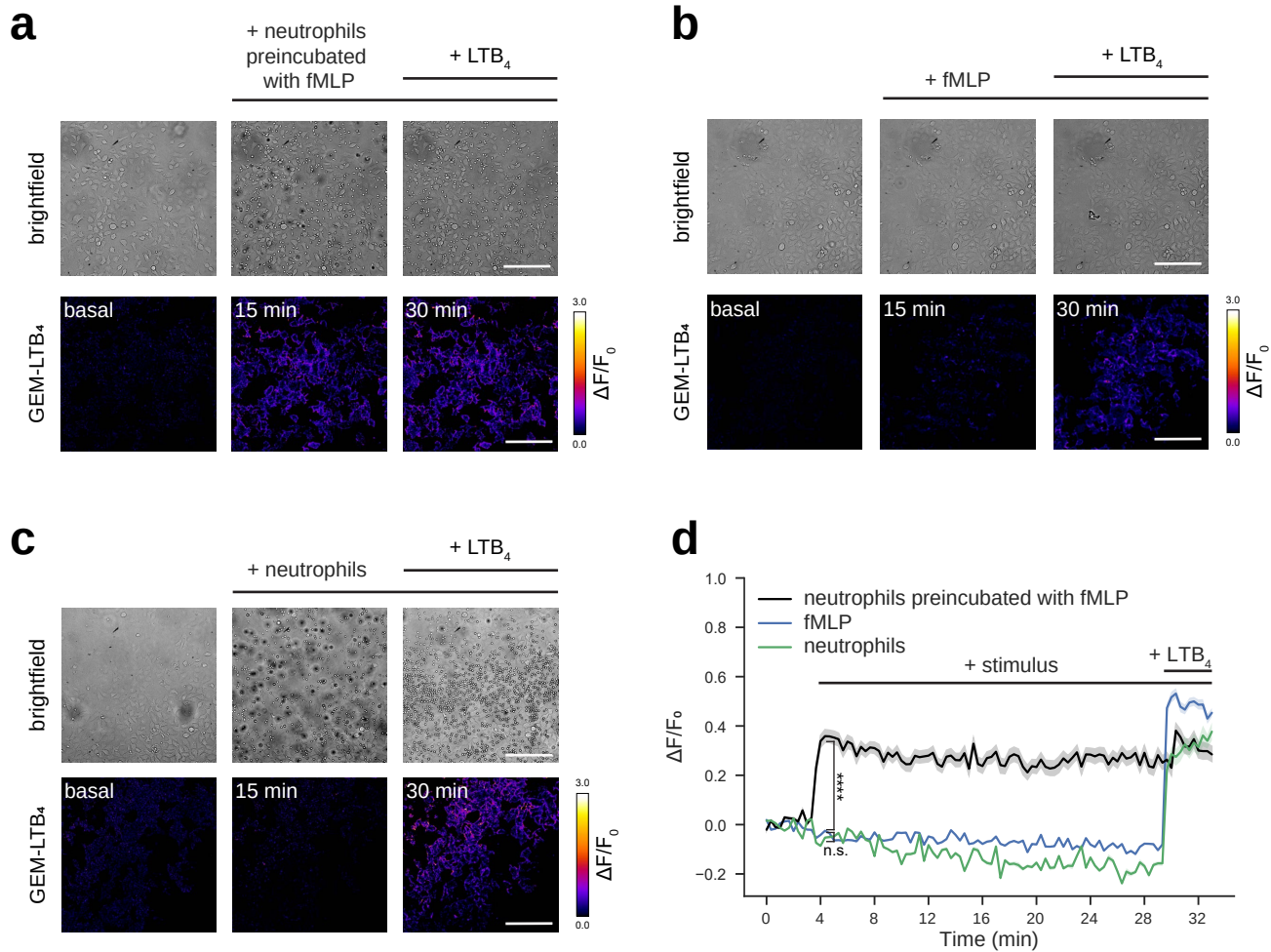
Extended Data Fig. 2



Extended Data Fig.2 | GEM-LTB₄ signals are sustained and reversible, without triggering GPCR signaling

a, Representative GEM-LTB₄ confocal fluorescence time-lapse images of HEK293A cells over the course of a 45 min stimulation with 100 nM LTB₄. Scale bar, 25 μ m. **b**, Average $\Delta F/F_0$ fluorescence response of GEM-LTB₄, shown as mean $\pm$ s.e.m., during a 45 min stimulation with 100 nM LTB₄ (n=49 cells from 3 independent experiments). Statistical analysis were performed with one way repeated measure ANOVA ($F=679.724$, $P=0.0$) with Bonferroni correction (basal vs. 0-45 min: **** $P=5.74 \times 10^{-70}$, 5.13×10^{-73} , 2.68×10^{-71} and 5.91×10^{-66} , respectively; and 0 min vs. 15-45 min: n.s., not significant, $P=0.69$, 0.055 and 1 , respectively). **c**, GEM-LTB₄ response to 100 nM LTB₄ stimulation followed by treatment with 1 μ M of the BLT1 inhibitor BIL-260. Data shown as mean $\pm$ s.e.m. for n=83 cells from 3 independent experiments. **d**, Average $\Delta F/F_0$ fluorescence response of GEM-LTB₄ during repetitive stimulation and wash cycles with 100 nM LTB₄. Data shown as mean $\pm$ s.e.m. for n=344 cells from 3 independent experiments. **e**, Confocal fluorescence images of the calcium sensor jRGECO1a (red) and BLT1-EGFP or GEM-LTB₄ (green) co-expressing HEK293A cells before (basal) and after 100 nM LTB₄ stimulus followed by treatment with 1 μ M of the calcium ionophore ionomycin. Scale bars, 50 μ m. **f**, Individual traces of $\Delta F/(F_{\max} - F_0)$ jRGECO1a responses in BLT1-EGFP or GEM-LTB₄ co-expressing cells, as shown in e, after 100 nM LTB₄ stimulus followed by stimulation with 1 μ M of the calcium ionophore ionomycin. Data shown as mean $\pm$ s.e.m. for n=7 and 8 cells, respectively, from 3 independent experiments. **g**, Confocal fluorescence images of β -arrestin-2-mScarlet (red) and BLT1-EGFP or GEM-LTB₄ (green) co-expressing HEK293A cells before (basal) and after 100 nM LTB₄ stimulus. Translocation of β -arrestin-2-mScarlet to the membrane is indicated by white arrows. Scale bar, 25 μ m. **h**, Quantification of relative β -arrestin-2-mScarlet translocation as a $F_{\text{membrane}}/F_{\text{cytoplasm}}$ ratio before and after 100 nM LTB₄ stimulus in BLT1-EGFP or GEM-LTB₄ co-expressing cells. Data shown as mean $\pm$ s.e.m. for n=26 and 19 respectively from 2 independent experiments, with an unpaired t-test **** $P=0.000003$, n.s., not significant ($P=0.058$).

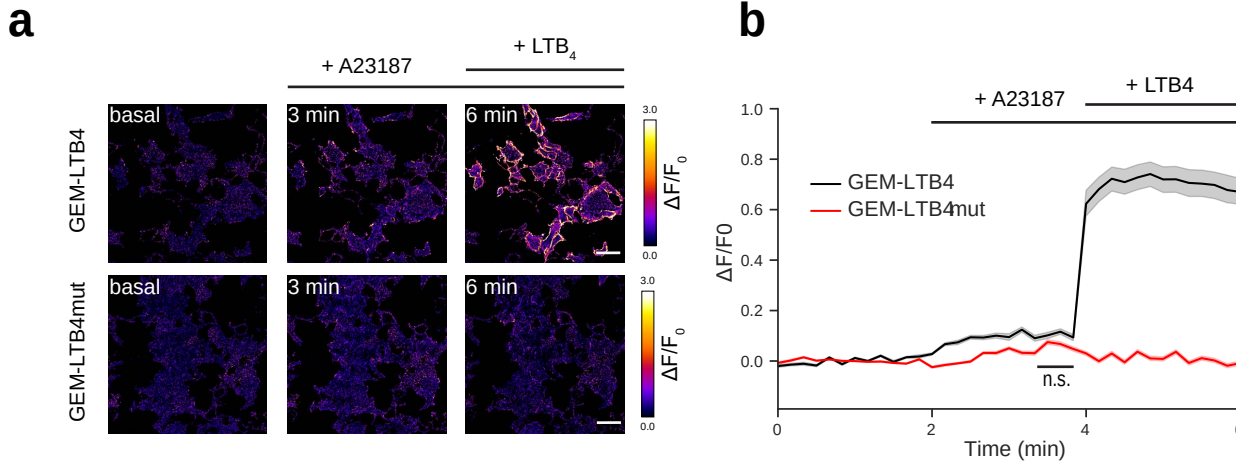
Extended Data Fig.3



Extended Data Fig.3 | Measuring endogenous LTB₄ secretion from neutrophils with GEM-LTB₄

a-c, Brightfield microscopy and corresponding $\Delta F/F_0$ ratio images of GEM-LTB₄ expressing HEK293A cells stimulated at 4 min with murine neutrophils (2×10^6 cells/cm²) pretreated with 1 μ M fMLP for 30 min (**a**), with 1 μ M fMLP alone (**b**), or with non-treated neutrophils (**c**). All treatments were followed by a 100 nM LTB₄ stimulus at 30 min. Scale bars, 100 μ m. **d**, Average traces of $\Delta F/F_0$ responses of GEM-LTB₄ expressing cells shown in a, b and c with respective stimulation at 4 min. Data shown as mean $\pm$ s.e.m. for $n=42$ (a), 44 (b) and 38 (c) cells from 3 independent experiments. Statistical analysis were performed with one-way ANOVA ($F=222.9$, **** $P=2.6 \times 10^{-41}$) with Fisher's LSD correction (fMLP-activated neutrophils being different from the other 2 conditions).

Extended Data Fig. 4



Extended Data Fig.4 | GEM-LTB₄ fluorescence is not affected by A23187

a, Representative $\Delta F/F_0$ images of GEM-LTB₄ and GEM-LTB₄mut expressing HEK293A cells treated with 1 μ M A23187 followed by stimulation with 100 nM LTB₄. Scale bars, 50 μ m. **b**, Average traces of $\Delta F/F_0$ responses in GEM-LTB₄ and GEM-LTB₄mut expressing cells shown in a. Data are presented as mean $\pm$ s.e.m. for n=40 and 79 cells respectively from 3 independent experiments with Unpaired t-test (n.s., not significant, $P=0.09706$) before LTB₄ stimulation.