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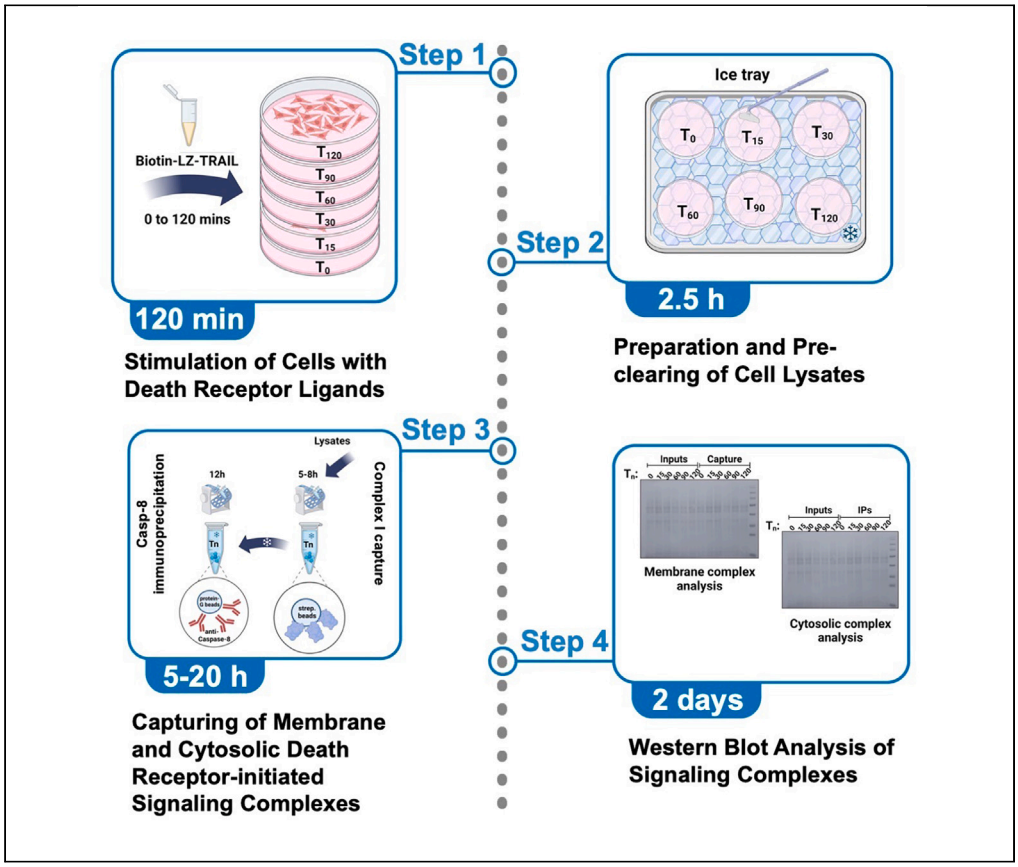
Protocol for analyzing TRAIL- and Fas-induced signaling complexes by immunoprecipitation from human cells



Engagement of TRAIL or Fas death receptors can trigger the assembly of cytoplasmic caspase-8/FADD/RIPK1 (FADDosome) signaling complexes that promote nuclear factor κB (NF-κB) activation. Here, we present a protocol for immunoprecipitation of TRAIL- or Fas-induced FADDosomes from human cell lines. We describe steps for stimulating human cells with TRAIL or Fas ligand, followed by preparation of membrane death receptor-associated, as well as cytoplasmic FADDosome, signaling complexes. This protocol has application in the analysis of death receptor-induced signaling complex formation.

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

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Highlights

Step-by-step guide to isolation of TRAIL- or Fas-induced signaling complexes

Protocol is applicable to adherent or suspension human cell lines and primary cells

Determining optimal conditions for assembly of TRAIL/Fas-induced signaling complexes

Optimal detection of TRAIL/Fas receptor-associated and cytoplasmic complex constituents

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Protocol for analyzing TRAIL- and Fas-induced signaling complexes by immunoprecipitation from human cells

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SUMMARY

Engagement of TRAIL or Fas death receptors can trigger the assembly of cytoplasmic caspase-8/FADD/RIPK1 (FADDosome) signaling complexes that promote nuclear factor κB (NF-κB) activation. Here, we present a protocol for immunoprecipitation of TRAIL- or Fas-induced FADDosomes from human cell lines. We describe steps for stimulating human cells with TRAIL or Fas ligand, followed by preparation of membrane death receptor-associated, as well as cytoplasmic FADDosome, signaling complexes. This protocol has application in the analysis of death receptor-induced signaling complex formation.

For complete details on the use and execution of this protocol, please refer to Davidovich et al.[1]

BEFORE YOU BEGIN

This protocol describes procedures for the stimulation of cells with ligands specific for TRAIL or Fas 'death receptors', followed by ligand-based affinity capture of TRAIL or Fas receptor membrane-associated signaling complexes (called Complex I), which is then followed by immunoprecipitation (IP) of cytoplasmic caspase-8-associated cytosolic signaling complexes (Figure 1), termed FADDosomes (or Complex II), that promote NF-κB activation.[1–3] In addition to caspase-8, the latter complexes typically contain the adaptor protein FADD, RIPK1, $cFLIP_L$, Caspase-10 and a panoply of RIPK1 associated signaling constituents (A20, CYLD, HOIL, HOIP, NEMO and others) that bind to ubiquitin chains attached to the latter kinase. The protocol can also be used to analyze signaling constituents from cells stimulated with TNF, although in this situation the cytosolic Caspase-8/FADD/TRADD/RIPK1 complex formed is termed Complex II and is predominantly involved in promoting apoptosis,[4,5] with the TNFR membrane-associated Complex I primarily involved in promoting NF-κB activation.

The protocol below describes the specific steps involved in the preparation of Fas or TRAIL-induced signaling complexes using HeLa cells. However, this protocol can be used for a wide range of cell types, such as HT-29, HCT-116 and U937 cells that are responsive to Fas- or TRAIL receptor stimulation.

A wide range of cell types are responsive to TRAIL or Fas ligand (FasL) stimulation, but it is advisable to conduct pilot experiments on your cell line of choice to determine optimal concentrations of TRAIL or FasL that are required to achieve optimum stimulation conditions.

Different forms of TRAIL can be used, such as Leucine Zipper (LZ)-TRAIL or super killer TRAIL, or cross-linking antibodies against TRAIL-R1 (DR4) or TRAIL-R2 (DR5). For optimal TRAIL-R stimulation, we suggest titrating the ligand onto cells at concentrations between 50–250 ng/mL as a starting

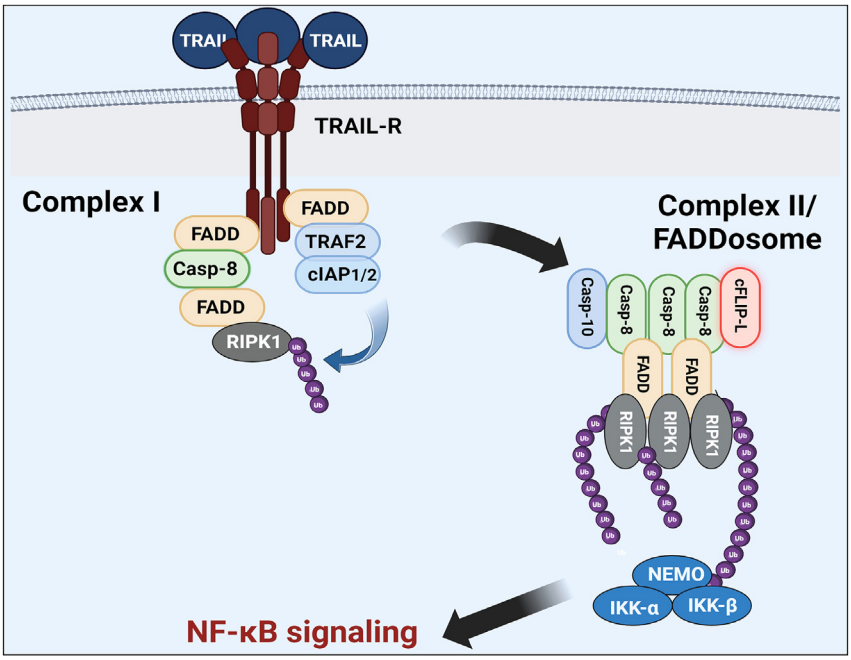

Figure 1. Schematic depiction of TRAIL-induced signaling complexes

TRAIL and FasL engage their cognate membrane receptors to form membrane-associated receptor complexes containing FADD, Caspase-8 and minor amounts of RIPK1 (Complex I) that dissociate to form cytosolic Caspase-8/FADD/RIPK1 'FADDosome' complexes (Complex II), that promote NF-κB activation and inflammatory signaling.

point, and for FasR stimulation we recommend using anti-Fas (CH11) IgM monoclonal antibody at concentrations ranging from 50–250 ng/mL.

Before performing large-scale immunoprecipitations, ensure that the cells under investigation respond to death receptor stimulation by performing small scale pilot experiments. Titrate TRAIL or FasL (or anti-Fas IgM crosslinking antibodies) over a range of concentrations (typically 50 ng/mL to 250 ng/mL) on the cell line under investigation for 18–24 h at 37°C, followed by assessment of apoptosis and/or cytokine production. Check that your cells respond to death receptor stimulation by measuring cell death (apoptosis) or cytokine production (by ELISA) 18–24 h after Fas or TRAIL receptor stimulation (Figure 2). Cells that are FasL or TRAIL responsive will typically undergo apoptosis or secrete cytokines and chemokines (such as IL-6 or IL-8) within 18–24 h of receptor stimulation, depending on the concentration of TRAIL or FasL used. Apoptotic cells can be readily identified by their morphological characteristics (Figure 2A, rounded cells with extensive membrane blebbing that become progressively detached from the plate) and assessed under an inverted phase contrast microscope.[6] In addition, cell lysates can be prepared from apoptotic cells and analyzed by western blotting to confirm processing and activation of caspase proteases that are activated during apoptosis, such as caspase-3 and caspase-7, as well as caspase substrates, such as PARP and Fodrin, that are cleaved in apoptotic cells.[7]

Institutional permissions

The studies described herein use transformed human cell lines that typically do not require institutional approval. Investigators should obtain appropriate ethics committee approvals before working with primary human or murine cells.

Preparation of key buffers and reagents

Timing: 60 min

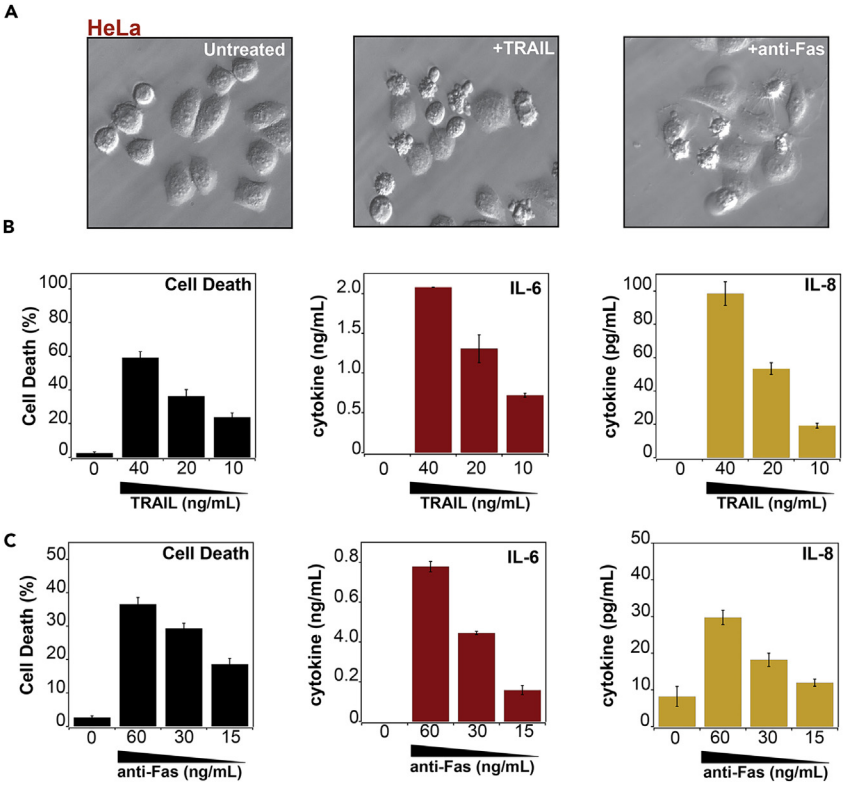

Figure 2. TRAIL and FasL induce cell death and inflammation in HeLa cells

(A) Phase contrast images of untreated HeLa cells, versus HeLa cells treated with either TRAIL- (40 ng/mL) or anti-Fas IgM (40 ng/mL) for 18 h. Cells with classic apoptotic features (retracted from the plate with extensive membrane blebbing) can be seen upon TRAIL or anti-Fas stimulation.

(B and C) Cell death and cytokine production by HeLa cells treated for 18 h with the indicated amounts of recombinant LZ-TRAIL- or anti-Fas IgM antibody. Error bars represent the mean of triplicates +/- SEM from a representative experiment.

1. Preparation of key buffers.
   a. IP Lysis buffer (see buffer table for composition). You will need ~100 mL of IP lysis buffer for each experiment, with protease inhibitors added just prior to use.

IP wash buffer (see buffer table for composition). You will need ~100 mL of IP wash buffer for each experiment with protease inhibitors added just prior to use.

2. Key reagents for Fas and TRAIL receptor stimulation and immunoprecipitation.
   a. Biotinylated TRAIL or FasL for receptor stimulation. You will need 1–10 μg of TRAIL or FasL per 10 cm tissue culture plate to trigger receptor activation on a scale need to perform IPs.

   Note: Typically, you will require 10 mL of a 0.2–1 μg/mL solution of death ligand per 10 cm tissue culture plate (i.e. between 1–10 μg of death ligand per dish). Given that a typical experiment will involve conducting a time course containing 5–10 × 10 cm plates, each experiment will require 10–100 μg of death ligand. In these cases, it can be useful to express your own recombinant TRAIL or FasL. Biotinylation of TRAIL or FasL can be achieved using standard biotinylation procedures as described in Davidovich et al.[1] As an alternative to biotinylated-TRAIL or FasL, anti-TRAIL or anti-Fas crosslinking antibodies can be used for receptor stimulation.

   b. Protein A/G agarose or Streptavidin Sepharose pre-equilibrated in **IP Lysis** buffer.

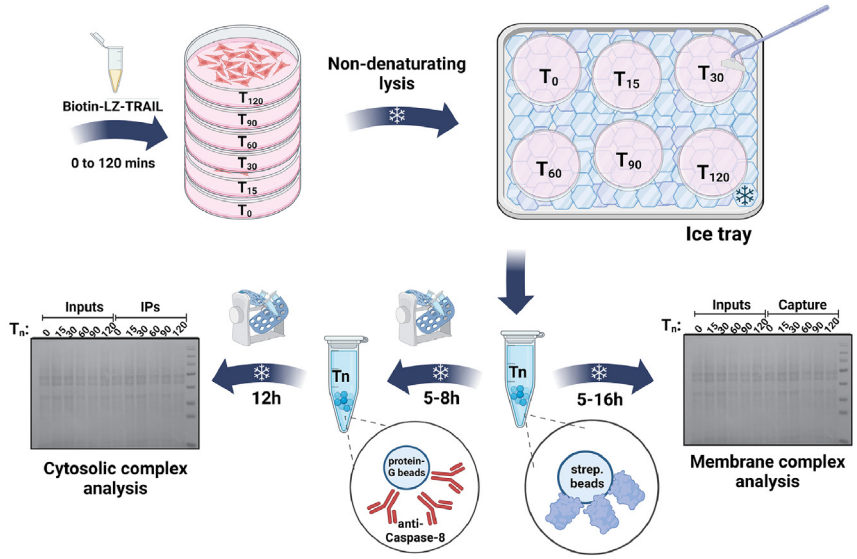

Figure 3. Schematic overview of the procedure used to prepare membrane-associated as well as cytoplasmic FasL or TRAIL-induced signaling complexes

c. Anti-caspase-8 antibodies suitable for IP analysis.

Preparation of cells

Timing: 2–3 days

3. Seed HeLa cells at a density 2 × 10^6 cells per 10 cm plate. Use 8–10 mL of RPMI medium supplemented with 5% FBS and 2 mM glutamine per 10 cm plate.

Note: You will typically need 5–6 × 10 cm plates of cells to perform a time course of stimulation with FasL or TRAIL, with one plate being used for each time point.

4. Incubate cells at 37°C in a standard tissue culture incubator (5% CO_2 and 95% humidity) for 24–48 h before carrying out the next step.

Note: You may wish to silence the expression of specific genes before carrying out the IP analysis. In this situation, plate siRNA-transfected cells at 2 × 10^6 cells per 10 cm plate and incubate for 24 or 48 h to allow gene silencing prior to conducting IP analysis. See Ref. 1 for further details.

5. 24–48 h after plating, treat HeLa cells with 0.2–1 μg/mL of Biotin-LZ-TRAIL (or 0.2–1 μg/mL anti-Fas CH-11 mAb) at 37°C. Ideally, cells should be close to 100% confluency when stimulating with TRAIL or anti-Fas to maximize the biomass available when preparing immunocomplexes at the next step.

Note: FasL or TRAIL stimulations should be carried out in the presence and absence of a poly-caspase inhibitor, such as Z-VAD-FMK (or emricasan) at 5–10 μM, as this can stabilize FADDosomes and lead to improved detection of complex constituents and post-translational modifications.

6. Take samples at 0, 15, 30, 60, 90 and 120 min after TRAIL or FasL treatment, with one plate being used for each IP (Figure 3).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-A20 (A-12) Use at 1:1,000 dilution	Santa Cruz Biotechnology	#sc-166692, RRID:AB_2204516
Anti-A20 (D13H3) Use at 1:1,000 dilution	Cell Signaling Technology	#5630, RRID:AB_10698880
Anti-caspase-8 (IP) (5D3) Use at 1:1,000 dilution	MBL	#M058-3, RRID:AB_590761
Anti-caspase-10 (4C1) Use at 1:1,000 dilution	MBL	#M059-3, RRID:AB_590721
Anti-cFLIP (7F10) Use at 1:1,000 dilution	Enzo	#ALX-804-961-0100, RRID:AB_2713915
Anti-DR4 Use at 1:1,000 dilution	Cell Signaling Technology	#42533S, RRID:AB_2799223
Anti-DR5 (WB) Use at 1:1,000 dilution	Cell Signaling Technology	#8074s, RRID:AB_10950817
Anti-FADD Use at 1:1,000 dilution	BD Transduction Laboratories	#610400, RRID:AB_397783
Anti-FADD (A66-2) Use at 1:1,000 dilution	BD Transduction Laboratories	#556402, RRID:AB_396409
Anti-Fas (CH11) Use at 25–200 ng/mL	Merck Sigma	#05-201, RRID:AB_309653
Anti-RIPK1 (D94C12) Use at 1:1,000 dilution	Cell Signaling Technology	#3493S, RRID:AB_2305314
Anti-TRAF2 (C-20) Use at 1:1,000 dilution	Santa Cruz Biotechnology	#sc-876, RRID:AB_632533
Peroxidase conjugated AffiniPure goat anti-mouse IgG (H + L) Use at 1:2,500 dilution	Jackson ImmunoResearch	#115-035-003, RRID:AB_10015289
Peroxidase conjugated AffiniPure goat anti-rabbit IgG (H + L) Use at 1:2,500 dilution	Jackson ImmunoResearch	#111-035-003, RRID:AB_2313567
Peroxidase conjugated AffiniPure goat anti-mouse IgG (L) Use at 1:2,500 dilution	Jackson ImmunoResearch	#115-035-174, RRID:AB_2338512
Peroxidase conjugated AffiniPure goat anti-mouse IgG (Fc) Use at 1:2,500 dilution	Jackson ImmunoResearch	#115-035-008, RRID:AB_2313585
Peroxidase conjugated AffiniPure goat anti-rabbit IgG (L) Use at 1:2,500 dilution	Jackson ImmunoResearch	#211-032-171, Lot 145158, RRID:AB_2339149
Chemicals, peptides, and recombinant proteins		
Z-VAD-FMK	Bachem	#N-1510.005
SuperKiller TRAIL	AdipoGen	#AG-40T-0002-C020
Protein G Sepharose fast flow	Sigma-Aldrich	#P3296
Streptavidin Sepharose	GE Healthcare	#17-5113-01
PR619	Sigma-Aldrich	#SML0430
PMSF	Roche	#04693116001
Aprotinin	Roche	#04906845001
Leupeptin	Roche	#11017101001
Chemiluminescent substrate	Thermo Scientific	#15626144
RPMI 1640	Gibco	#31870025
FBS	Gibco	#10270
L-glutamine	Gibco	#25030-024
DPBS	Sigma	#D8537-1L
Non-fat dried milk (NDFM)	Neogen	#NCM0249A
Bromophenol Blue	Sigma	#B0126
β-mercaptoethanol	Sigma	#63689
Tween-20	Sigma	#P1379
EDTA	Sigma	#E4884
Glycerol	Sigma	#G6279
Triton-X 100	Sigma	#T8532

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
NaCl	Sigma	#S3014
Tris	Sigma	#T6066
SDS	Sigma	#L5750
Experimental models: Cell lines		
HeLa human cervical cancer cell line	ATCC	CRM-CCL-2
Other		
Laminar flow hood	MSC-Advantage	51025411
Incubator	Thermo Scientific	3110
Microscope	Olympus	IX71
Scales	KERN	ABS 220-4N
Magnetic stirrer	IKA	Reo basic C
Horizontal rotator	Grant Bio	PTR-35
Hotplate	Techne	DB-3
Power supply	Bio-Rad	PowerPac 300
Orbital rotator	Boekel	260200
Reciprocal shaker	IKA	HS260
Table centrifuge	Eppendorf	5417C

MATERIALS

HeLa cell culture media		
Reagent	Final concentration	Amount
RPMI	N/A	470 mL
FBS	5%	25 mL
L-glutamine (200 mM stock)	2 mM	5 mL
Total	**N/A**	**500 mL**
Note on storage conditions: store at +4°C, no longer than one month.		

IP Lysis buffer for immunoprecipitation		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4, (1 M) stock solution	30 mM	3 mL
Glycerol	10%	10 mL
Triton-X 100	1%	1 mL
NaCl 1 M stock solution	150 mM	15 mL
EDTA (100 mM)	2 mM	2 mL
Protease inhibitor cocktail	As per manufacturer's instructions	1 tablet
PR619 (10 mM)	(10 μM)	100 μL
Phosphatase inhibitor cocktail	As per manufacturer's instructions	1 tablet
H_2O	N/A	38.8 mL
Total	**N/A**	**100 mL**
Note on storage conditions: store at 4°C for up to six months.		

⚠ CRITICAL: IP lysis buffer should be made to pH 7.4 and protease/phosphatase inhibitors added just prior to use.

IP Wash buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4, (1 M) Stock solution	30 mM	3 mL
Glycerol	10%	10 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Triton-X 100	1%	1 mL
NaCl 1 M stock solution	450 mM	45 mL
EDTA (100 mM)	2 mM	2 mL
Protease inhibitor cocktail	As per manufacturer's instructions	1 tablet
PR619 (10 mM) stock solution	(10 μM)	100 μL
Phosphatase inhibitor cocktail	As per manufacturer's instructions	1 tablet
H_2O	N/A	38.9 mL
Total	**N/A**	**100 mL**
Note on storage conditions: store at 4°C for up to six months.		

2X SDS PAGE lysis buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 6.8	0.1 M	1.21 g
SDS (50% stock solution)	4%	4 mL
Glycerol	20%	20 mL
Bromophenol Blue	0.1%	100 mg
β-mercaptoethanol	5%	5 mL
H_2O	N/A	71 mL
Total	**N/A**	**100 mL**
Note on storage conditions: store at 18–22°C up to one year.		

⚠ CRITICAL: Add β-mercaptoethanol just prior to use (50 μL per 1 mL) prior to use.

TBST buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0	10 mM	1.21 g
NaCl	150 mM	8.77 g
Tween-20	0.05%	0.5 mL
H_2O	N/A	1 L approx.
Total	**N/A**	**1 L**
Note on storage conditions: store at 18–22°C up to six months.		

STEP-BY-STEP METHOD DETAILS

Preparation of HeLa cells

Timing: 24–48 h

For the preparation of death receptor immunocomplexes, quite large numbers of cells (approx. 5 × 10^6 per IP) are required. This section outlines the approach used to grow sufficient cells for a typical experiment and how stimulations with death receptor ligands are carried out.

1. Trypsinize HeLa cells from 10 cm tissue culture plates by incubation for 5 min at 37°C with 1.5 mL of trypsin solution per plate.
   a. Examine the cells under an inverted microscope to determine when they have detached from the plate.
   b. When cells have detached from the plate, neutralize the trypsin solution by addition of 4.5 mL of RPMI/5% FBS tissue culture medium per plate.

c. Pellet cells in a bench top centrifuge for 5 min at 250 g.
d. Pour off medium and resuspend the cells in 10 mL of RPMI/5% FBS medium.
e. Count cells using a hemocytometer.

2. Seed cells at a density of 2 × 10^6 cells per 10 cm plate in 10 mL of RPMI/5% FBS.

Note: You will typically need 5–6 × 10 cm plates of cells to perform a time course of stimulation with FasL or TRAIL, with one plate being used for each time point.

3. Incubate cells at 37°C in 5% CO_2 and 95% humidity conditions for 24–48 h until almost confluent.

Note: Ideally, cells should be close to 100% confluency when stimulating with TRAIL or anti-Fas to maximise the biomass available when preparing immunocomplexes at the next step.

Stimulation of HeLa cells with TRAIL or FasL

Timing: 2.5 h

This section of the protocol describes the steps involved in stimulating cells with TRAIL or ant-Fas antibodies to initiate assembly of downstream signaling complexes.

4. 24–48 h after plating, treat HeLa cells with Biotin-LZ-TRAIL (0.2–1 μg/mL final concentration) or anti-Fas IgM antibody (0.2–1 μg/mL CH11 mAb) in the presence and absence of Z-VAD-FMK (5–10 μM final concentration). You will need one 10 cm plate of cells for each IP.

Note: Non-biotinylated TRAIL/FasL or anti-TRAIL/Fas receptor antibodies may be used instead. In this case, you will need to prepare Complex I immunoprecipitates using anti-FasR or anti-TRAIL-R1 or anti-TRAIL-R2 antibodies.

5. Incubate cells for the desired time at 37°C.

Note: Stimulations can also be performed at 18–22°C, or at 4°C, to slow down complex formation which may be advantageous under certain circumstances, for example, when trying to preserve ubiquitin chains attached to complex constituents.[8]

6. A time course of TRAIL or FasL stimulation should be performed with samples taken at 0, 15, 30, 60, 90 and 120 min after stimulation.

Note: Stimulate cells with TRAIL or Fas in reverse order (i.e. starting with the 120 min time point) to enable all time points to be collected at the same time.

7. Examine the cells under an inverted microscope after 60 min of stimulation. At this point you should start to see cells with apoptotic morphology[6] (i.e., extensive plasma membrane blebbing, rounding up, detachment from the plate; Figure 2A).

Note: After 120 min of TRAIL or Fas stimulation, about 50% of the cells should exhibit morphological changes characteristic of apoptosis (however, the latter morphological changes will not be evident if you have included a caspase inhibitor in the culture medium).

8. After incubating cells with TRAIL or anti-Fas for the desired time periods, proceed to the cell lysis step in the next section.

Note: Ideally, samples should not be frozen and thawed before carrying out cell lysis and IPs, as this risks loss of native protein conformations that may lead to complex disassembly.

Lysis of cells and preclearing of cell lysates

Timing: 2.5 h

This section of the protocol describes the steps involved in lysing cells post-stimulation and pre-clearing of the cell lysates prior to preparation of Complex I and II signaling complexes. Perform all operations at 4°C. This will limit protease activity as well as deubiquitinase activity that can strip off ubiquitin chains and their associated binding proteins (e.g., A20, CYLD, NEMO) and will keep complexes more stable. For preparation of cell lysates. It is convenient to work on a shallow tray filled with ice that can accommodate multiple 10 cm plates at the same time (Figure 3).

9. Gently wash the cell monolayers twice with 2.5 mL of ice-cold PBS, pH 7.2.
   a. Remove all remaining liquid from the 10 cm plates and place the cells on ice.
   b. If some cells have detached because of Fas- or TRAIL-receptor stimulation (this will very likely happen at the 60 and 120 min time points), you will need to recover the detached cells from the PBS solution by centrifugation at 400 × *g* on a bench top centrifuge.
   c. Combine the detached cell pellets with the lysate prepared from the remaining cells in the plate after the next step.
10. Add 1 mL of ice-cold IP lysis buffer per plate.
   a. Ensure that the whole surface of the plate is covered with lysis buffer by distributing the buffer repeatedly across the plate with a 1 mL pipette.
   b. Use a cell scraper to detach cells from the surface of the plate to facilitate complete lysis.
   c. Place all plates on an ice tray and incubate 15 min on ice to permit complete cell lysis.
   d. Collect all cell lysates into 1.5 mL Eppendorf tubes and rotate all samples on a horizontal tube rotator for an additional 45 min at 4°C to achieve complete cell lysis.
11. Centrifuge the cell lysates in a refrigerated benchtop centrifuge (or in the cold room) at 15000 *g* for 10 min to pellet insoluble material.
12. Transfer supernatants to fresh 1.5 mL tubes.
13. Repeat the centrifugation step once more.
14. Pre-clarified cell lysates should be transferred to fresh 1.5 mL tubes, followed by addition of 50 μL of Protein A/G agarose bead slurry to each tube to pre-clear lysates.

Note: This step will remove any proteins that non-specifically bind to the Protein A/G beads.

15. Rotate cell lysates with the Protein A/G beads for 1 h at 4°C under constant rotation.
16. Remove the protein A/G beads by centrifugation at 2000 *g* for 3 min. The clarified cell lysates should then be transferred to fresh Eppendorf tubes for the next step.
17. Take 50 μL samples of all clarified lysates at this stage as "input samples" and freeze at −80°C.
18. Pre-equilibrate 1 mL of protein A/G agarose beads by washing twice in 1 mL of IP lysis buffer for 5 min at 4°C.
19. After the last wash, pellet beads at 1000 *g*, remove wash buffer and resuspend beads in IP lysis buffer to the original bead volume.

Preparation of complex I immunoprecipitates

Timing: 5–6 h

This section of the protocol outlines how capture of the plasma membrane-associated receptor signaling complex (Complex I) is achieved.

20. Where biotinylated TRAIL (or biotinylated FasL) has been used to stimulate cells, add 50 μL of pre-equilibrated streptavidin Sepharose beads to each lysate.

21. Capture TRAIL or Fas receptor complexes on streptavidin beads by rotating tubes for 4–5 h at 4°C.

Note: This step can also be prolonged to 12–14 h if desired, although this will risk losing some complex constituents due to protease and/or deubiquitinase activity.

Note: Where non-biotinylated TRAIL or Fas has been used to stimulate cells, you will need to add anti-TRAIL (or anti-Fas) receptor antibodies (1–2 μg/mL final concentration) along with 50 μL of pre-equilibrated Protein A/G agarose beads to each lysate (instead of streptavidin Sepharose beads).

22. After 4–5 h of rotation, centrifuge tubes for 1 min at 1000 *g* to pellet the streptavidin Sepharose or Protein A/G Agarose beads and transfer the supernatants to fresh tubes and keep on ice.

⚠ CRITICAL: After removal of the Sepharose/Agarose beads at Step 22, keep the cell lysates on ice for preparation of FADDosome/Complex II immunoprecipitates (see below).

23. Wash the streptavidin Sepharose or Protein A/G agarose beads three times with 1 mL of "IP wash buffer" by rotating the beads for 5 min at 4°C in wash buffer.
24. Pellet the beads after each wash step by centrifugation in a benchtop centrifuge for 1 min at 1000 *g*.
25. After the last wash step, carefully remove all wash buffer using a 200 μL pipette.

Note: during the bead washing steps, it is better not to remove all wash buffer after each wash step, as you may lose some of the beads. Only after the last wash should you carefully remove as much of the wash buffer as possible using a 200 μL pipette.

26. Elute the captured **Complex I associated proteins** (Figure 4) from the beads by adding 50 μL of 1X SDS-PAGE loading buffer per sample.

Note: After addition of SDS-PAGE loading buffer, samples can be frozen at −80°C at this stage to await analysis by SDS-PAGE and immunoblotting or can be frozen after the next step.

27. Denature Complex I immunocomplexes by heating tubes for 7 min at 98°C in a hotplate or similar.

Preparation of complex II/FADDosome immunoprecipitates

Timing: 12–18 h

This section of the protocol outlines how capture of the cytoplasmic signaling complex (FADDosome or Complex II), which is formed after death receptor stimulation, is achieved through immunoprecipitation of caspase-8.

28. Prepare Complex II/FADDosome immunoprecipitates from the cell lysates retained after Step 22, by adding 1–2 μg of anti-Caspase-8 antibody per IP tube along with 50 μL of pre-equilibrated Protein A/G-agarose beads.
29. Capture the caspase-8 immunocomplexes on the Protein A/G beads by rotating tubes for 12-18 h at 4°C.

Note: This step can be shortened to 4 h if desired.

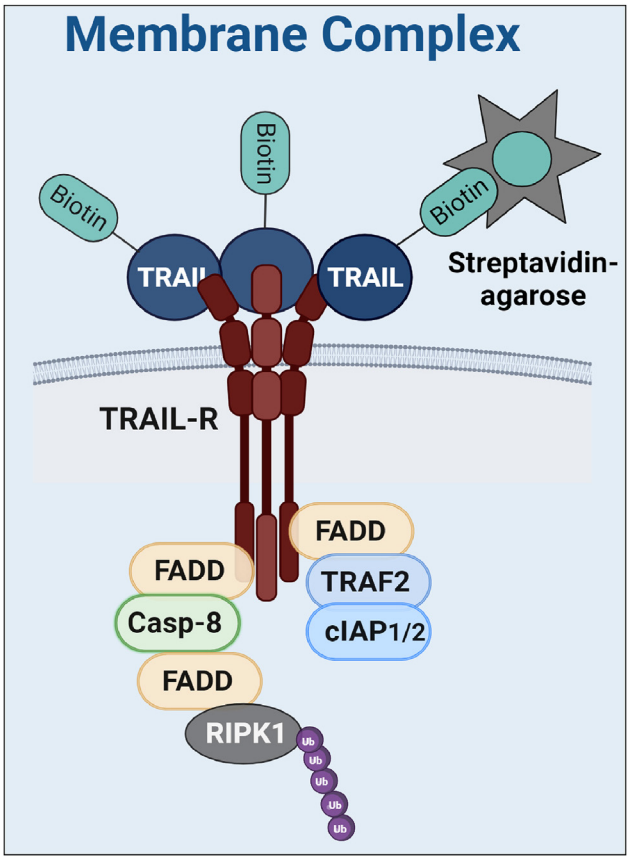

Figure 4. Biotinylated LZ-TRAIL along with streptavidin-agarose beads are used to capture membrane complex I

30. After 12–18 h centrifuge tubes for 1 min at 1000 *g* to pellet the Protein A/G beads containing Complex II/FADDosome immunocomplexes.
31. Transfer cell lysates to fresh 1.5 mL tubes and freeze at −80°C.

Note: you should keep lysates after all capture steps to analyze immunocapture efficiency by comparing these to "input samples".

32. Wash the Protein A/G beads three times with 1 mL of IP wash buffer by rotating the beads for 5 min at 4°C in wash buffer, followed by pelleting the beads after each wash step by centrifugation in a benchtop centrifuge for 1 min at 1000 *g*.
33. After the last wash step, carefully remove all wash buffer using a 200 μL pipette.
34. Elute the captured **Complex II/FADDosome** immunocomplexes (Figure 5) from the beads by adding 50 μL of 1X SDS-PAGE loading buffer per sample.
35. Freeze samples at −70 to −80°C at this stage to await analysis by SDS-PAGE and immunoblotting. Alternatively, samples can be frozen after the next step.
36. Denature proteins by heating tubes for 7 min at 98°C.

SDS-PAGE of complex constituents and immunoblotting

Timing: 20 h

This section of the protocol describes the steps involved in analyzing the captured Complex I and II constituents by SDS-PAGE, followed by Western blot analysis.

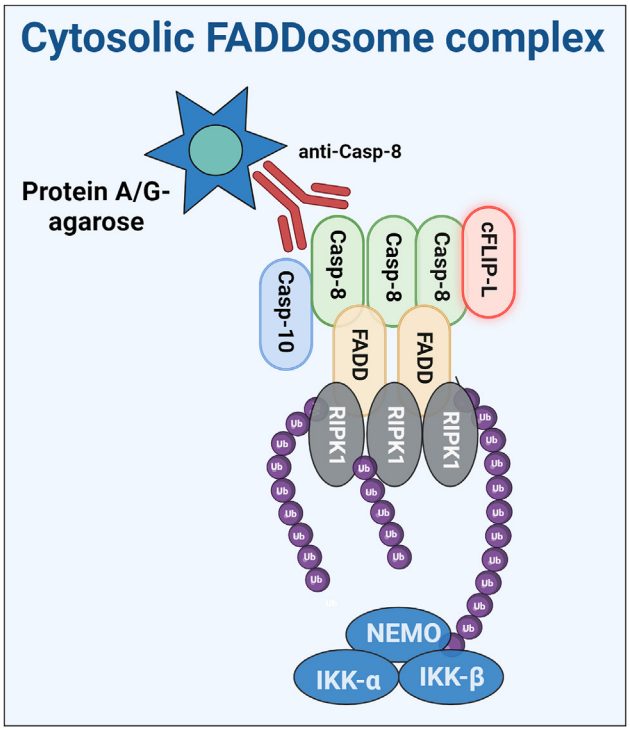

Figure 5. Anti-caspase-8 antibodies immobilized on Protein A/G-sepharose beads are used to capture cytoplasmic FADDosomes (Complex II)

37. Analyze the captured Complex I and Complex II by separating proteins using 8–12% SDS-PAGE gels (e.g., see *Molecular Cloning*, Green and Sambrook[9]).

Note: Load 5–40 μL of each IP sample per well along with 'input samples' taken at Step 17. It is typical to load 10–20 μL (approx. 10–20 μg of protein) of input samples on the left-hand side of each gel, followed by IP samples, as illustrated in Figure 6.

Note: Before running large volumes of your IP samples, it can be useful to first check the IP efficiency of the complexes by running small (5 μL) samples of the eluted complexes, followed by probing for TRAIL or Fas receptor (in the case of Complex I samples) or caspase-8 (in the case of Complex II samples).

38. Run SDS-PAGE gels at 55 V through the stacking gel, then at 70 V thereafter for 3–4 h.
39. Transfer the separated proteins from the SDS-PAGE gels onto nitrocellulose (or PVDF) membranes for 12–16 h at 40 mA using standard western blotting procedures.[9]
40. After proteins have been transferred to nitrocellulose membranes, block the membranes in 5% non-fat dried milk (or BSA) in TBST buffer by incubating for 1 h at 18–22°C.

Note: transfer efficiency and protein loading can be checked before the blocking step by staining with Ponceau S solution. In this case membranes should be washed for 5 min with distilled H_2O and then incubated with Ponceau S staining solution for 5 min. After staining, excess dye should be washed off with distilled H_2O and blots should be photographed. Ponceau S dye can then be removed by washing membranes in TBST for 10 min followed by blocking as described above.

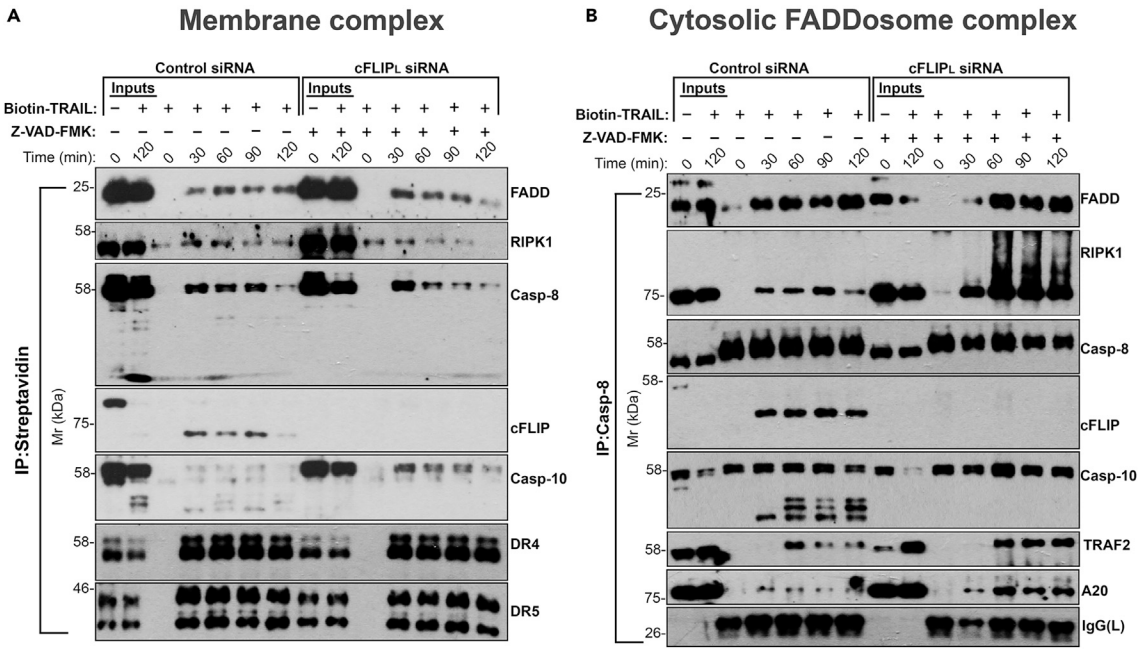

Figure 6. Analysis of TRAIL-induced membrane-associated (Complex I) and cytosolic FADDosome (Complex II) signaling complexes

HeLa cells were stimulated with biotinylated-LZ-TRAIL (1 μg/mL) for the indicated times in the presence or absence of the polycaspase inhibitor Z-VAD-FMK (10 μM) followed by preparation of membrane-associated signaling complexes (Complex I) and cytoplasmic FADDosome signaling complexes (Complex II) as described in the main text.

(A) Western blot analysis of TRAIL-induced membrane-associated signaling complexes.

(B) Western blot analysis of TRAIL-induced FADDosome (Complex II) signaling complexes. Data are reproduced from Davidovich et al., 2023.[1]

Detection of complex I and complex II constituents

Timing: 4–18 h

This section describes how Complex I and II constituents are detected using standard immunoblotting techniques. It is typical that blots will need to be probed with the same antibody several times in succession to obtain optimal signals.

41. Incubate blots with the probe antibody of interest for 2 h at 18–22°C or 12–14 h at 4°C. Use 5 mL of primary Ab at a 1:1000 dilution in 5% non-fat dried milk in TBST.
42. Wash blot membranes twice (10 min per wash) with TBST at 18–22°C.
43. Incubate blots with appropriate peroxidase-conjugated secondary antibody for 1 h at 18–22°C at a 1:2500 dilution in 5% non-fat dried milk in TBST.

⚠ CRITICAL: It is highly advisable to use anti-light chain (κ or λ) anti-Ig antibodies to avoid detection of the heavy chain of the anti-caspase-8 antibody that was used for the IP. Recommended antibodies include: Peroxidase-conjugated AffiniPure Goat anti-Mouse IgG (L); Peroxidase conjugated AffiniPure Goat anti-Rabbit IgG (L); Peroxidase conjugated AffiniPure Goat Anti-Mouse IgG (Fc).

44. Wash membranes three times (10 min per wash) with TBST at 18–22°C.
45. Add western blot substrate (e.g., enhanced chemiluminescence detection reagent; SuperSignal West Pico PLUS) to blot membranes.
46. Incubate blot membranes with substrate for 3–5 min.
47. Take multiple exposures of blots using a digital imager or using standard X-ray film.

Note: When probing immunocomplexes, it is typical that the first probe will not generate the most optimum signals (Figure 6). Sub-optimal signals include detection of very strong bands representing the heavy and light chains of the anti-caspase-8 antibody used to conduct the IP, with only faint signals associated with other bands (see troubleshooting section). In this case, the blot should be erased by incubation in 200 mM NaOH (in H_2O) for 15 min at 18–22°C, followed by washing for 10 min in TBST and re-probing again with the same primary and secondary antibodies (see steps 41–47 above). Frequently two or three cycles of probing, erasing, and re-probing of the blot will be required to generate optimum signals.

EXPECTED OUTCOMES

Successful immunoprecipitation of TRAIL- or Fas-initiated signaling complexes will reveal time-dependent recruitment of the adaptor protein FADD to the receptor signaling complexes (Complex I), along with caspase-8 and cFLIP (Figure 6A). Trace amounts of RIPK1 and other signaling proteins (e.g., cIAP1, cIAP2, TRAF2, A20, CYLD) may also be detected in the receptor signaling complexes, but these are more readily detected in the cytoplasmic signaling complex (Complex II/FADDosome).

Successful immunoprecipitation of cytoplasmic signaling complexes should reveal time-dependent recruitment of caspase-8, FADD, RIPK1 and other downstream signaling components, such as A20, CYLD, TRAF2, LUBAC complex, IKKγ/NEMO and other complex constituents. RIPK1 will frequently exhibit extensive poly-ubiquitination at K63 and M1 ubiquitin linkages, which will be revealed through obtaining prolonged RIPK1 exposures and by re-probing multiple times (Figure 6B).

LIMITATIONS

Insufficient Fas- or TRAIL- receptor stimulation will result in poor formation of receptor complexes; therefore, it is advisable to ensure that optimum amounts of TRAIL or FasL are used to stimulate cells. This should be assessed in advance of performing IPs by titrating biotinylated-TRAIL or -FasL onto the cells under investigation and assessing apoptosis or cytokine production at 18 h after stimulation. Saturating doses of TRAIL or FasL (approx. 0.2–1 μg/mL) will be required to achieve optimum stimulation of cells in 10 cm plates at the cell densities (approx. 5×10^6 cells per plate) required to conduct IPs. It is also critical to ensure that anti-caspase-8 antibodies that are used to perform complex II/FADDosome IPs are suitable for this purpose. It can be quite challenging to detect Complex I and Complex II constituents due to the low abundance of these components when performing endogenous IPs.

TROUBLESHOOTING

Problem 1

Upon probing western blots, protein signals of interest can be located close to very strong signals derived from the detection of Ig heavy or light chains (derived from the anti-caspase-8 antibody used to immunoprecipitate FADDosomes) that can prevent obtaining optimum exposures of the signals of interest (step 47).

Potential solution 1

Use anti-Ig light chain (or heavy chain) specific secondary antibodies to minimize detection of Ig signals.

Conformation-specific secondary antibodies, that do not detect denatured antibodies, can also be used to avoid strong signals from Ig heavy or light chains. Alternatively, the blotting membrane can be cut just above or below the unwanted Ig signal, followed by erasure of the blot in 200 mM NaOH solution for 15 min, followed by re-probing of the blot with fresh primary and secondary antibodies.

Problem 2
Poor immunoprecipitation of the protein(s) of interest (Steps 26, 34).

Potential solution 2
Ensure that the anti-TRAIL or anti-Fas receptor antibodies you are using for the preparation of Complex I immunocomplexes (step 26) are suitable for IP analysis. If biotinylated TRAIL or FasL is used to prepare death receptor complexes, ensure that biotinylation has been successful. Ensure that the anti-caspase-8 antibody that you are using for the preparation of Complex II/FADDosome immunocomplexes (step 34) is suitable for IP analysis. Antibodies used for IP analysis need to bind to a surface epitope on the protein of interest to be capable of recognizing the native conformation of the protein. Caspase-8 should be very easy to detect in FADDosome IPs (step 47), therefore, if there is a problem detecting this protein, it is essential to check that the antibody used for IP is suitable for this. Alternatively, if immunoprecipitation of caspase-8 is inefficient, it may be necessary to increase the amount of anti-caspase-8 antibody used for each IP to 3–4 μg per tube (step 28).

Problem 3
Caspase-8 is immunoprecipitated but few other proteins can be detected in the IPs (Step 47).

Potential solution 3
Sub-optimal amounts of TRAIL or FasL may lead to inefficient assembly of receptor signaling complexes. Ensure that optimal amounts of TRAIL or FasL are used to stimulate cells by performing a dose response analysis of complex assembly (using concentrations of ligand ranging from 2 μg/mL down to 100 ng/mL) followed by IP analysis of complexes to find the optimal ligand concentration for stimulation.

Problem 4
Blot signals are 'dirty' and contain a lot of non-specific smearing (step 47).

Potential solution 4
Non-specific signals can be eliminated or minimized by erasure of the blot in 200 mM NaOH for 15 min, followed by washing blotting membranes twice with TBST for 5 min. Blots should be re-probed again with primary and secondary antibodies and re-developed as normal. Often, erasure of the first signal can lead to much cleaner signals on the second (or even third) probing.

Problem 5
Poor or uneven recovery of Protein A/G agarose beads after washing (steps 25, 33).

Potential solution 5
During the Protein A/G agarose bead washing steps, it's better not to remove all wash buffer after each wash step to avoid losing beads. Only after the last wash should you carefully remove as much of the wash buffer as possible using a 200 μL pipette while holding tubes in your line of vision so that the bead/wash buffer interface can be seen clearly.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Seamus Martin (martinsj@tcd.ie).

Technical contact
Questions regarding technical issues should be directed to Prof. Seamus Martin (martinsj@tcd.ie).

Materials availability
The present study did not produce any novel or distinct reagents.

Data and code availability

No datasets were generated for this protocol.

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AUTHOR CONTRIBUTIONS

Conceptualization and visualization, S.J.M. and P.D.; methodology, data acquisition, and writing, P.D.; funding acquisition and writing, S.J.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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