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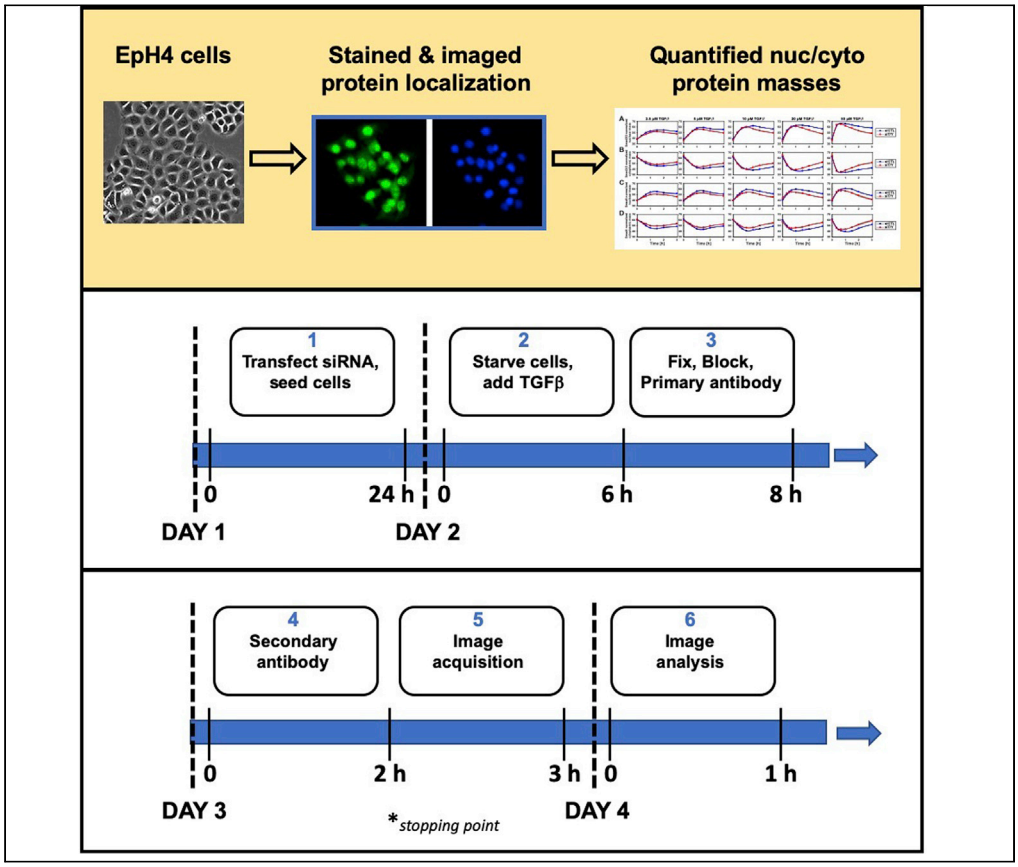
High-content imaging and analysis to quantify the nuclear to cytoplasmic ratio of TGFβ and hippo effectors in mammalian cells



Automated high-content immunofluorescence (IF) microscopy is used to monitor and quantify localization of the TGFβ/Smads and Taz/Yap Hippo effectors in mouse epithelial EpH4 cells transfected with Taz/Yap siRNAs. The nuclear-to-cytoplasmic protein ratios obtained by IF are converted into normalized masses by estimating the ratio of the compartment volumes. This method has the advantage that endogenous rather than tagged proteins are tracked and that knockdown of Taz/Yap can be simultaneously monitored at the single-cell level.

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Highlights

Automated immunofluorescence microscopy to analyze protein subcellular localization

Time- and dose-dependent tracking of TGFβ-stimulated Smad2/3 and Smad4 localization

Efficient siRNA-mediated knockdown of Hippo mediators, TAZ and YAP in epithelial cells

Analysis of TGFβ and Hippo cross talk by monitoring localization of endogenous effectors

Labibi et al., STAR Protocols 2, 100632
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High-content imaging and analysis to quantify the nuclear to cytoplasmic ratio of TGFβ and hippo effectors in mammalian cells

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SUMMARY

Automated high-content immunofluorescence (IF) microscopy is used to monitor and quantify localization of the TGFβ/Smads and Taz/Yap Hippo effectors in mouse epithelial EpH4 cells transfected with Taz/Yap siRNAs. The nuclear-to-cytoplasmic protein ratios obtained by IF are converted into normalized masses by estimating the ratio of the compartment volumes. This method has the advantage that endogenous rather than tagged proteins are tracked and that knockdown of Taz/Yap can be simultaneously monitored at the single-cell level.

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

BEFORE YOU BEGIN

Culturing of EpH4 cells

EpH4 cells, obtained from Dr. Martin Oft (Oft et al., 1996) are cultured in DMEM supplemented with 10% FBS and are split regularly to ensure cells are always maintained at subconfluency. Always bring media, trypsin and PBS to room temperature (RT) (20°C–25°C) before starting to culture cells.

⚠ CRITICAL: Use low passage EpH4 cells, ideally less than 20 passages.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-Smad2/3	Cell Signaling Technology (CST)	8685
Mouse anti-Smad4	Santa Cruz	sc-7966
Mouse anti-YAP	Santa Cruz	sc-10119
Rabbit anti-Actin	Millipore Sigma	A2066
Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L)	Invitrogen	A11034
Alexa Fluor® 546 Goat Anti-Mouse IgG (H+L)	Invitrogen	A11030
Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)	Invitrogen	A11029
Chemicals, peptides, and recombinant proteins		
DMEM media	Gibco	11995065

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal Bovine Serum (FBS)	Gibco	12103C
Phosphate-buffered saline (PBS)	Wisent Bio Products	311-010-CL
Trypsin	Gibco	25200056
TGFβ1	R&D Systems	P01137
Lipofectamine RNAiMAX	Life Technologies	13778150
Opti-MEM	Life Technologies	31985-070
Tween-20	Millipore Sigma	P9416
Triton-X100	Millipore Sigma	T8787
Paraformaldehyde (PFA)	Sigma-Aldrich	P6148
Bovine Serum Albumin (BSA)	Millipore Sigma	10735086001
Fish gelatin	Millipore Sigma	G7765
Sodium azide (NaN3)	Millipore Sigma	S2002
Oligo (dT) primer	Life Technologies	18418-012
M-MLV Reverse Transcriptase	Life Technologies	28025-013
SYBR Green PCR Master Mix	Applied Biosystems	4309155
DAPI	Millipore Sigma	D9542
SuperSignal™ West Dura Extended Duration Substrate	Thermo Fisher Scientific	PIA34075
Critical commercial assays		
RNA isolation kit: PureLink RNA Mini Kit	Life Technologies	12183025
Experimental models: cell lines		
Mouse: EpH4 cells	Oft et al., 1996	PMID: 8843198
Oligonucleotides		
Primer: Ankrd1 Forward: TGCGATGAGTATAAAC GGACG Reverse: GTGGATTCAAGCATATCTCGGAA	ACGT Corporation	n/a
Primer: Cyr61 Forward: CTGCGCTAAACAACTCA ACGA Reverse: GCAGATCCCTTTCAGAGCGG	ACGT Corporation	n/a
Primer: Hprt Forward: TCAGTCAACGGGGGACA TAAA Reverse: GGGGCTGTACTGCTTAACCAG	ACGT Corporation	n/a
Primer: Taz (gene name Wwtr1) Forward: GAAGGTGATGAATCAGCCTCTG Reverse: GTTCTGAGTCGGGTGGTTCTG	ACGT Corporation	n/a
Primer: Yap1 Forward: CCCTTTCTTAACAGTGGC ACC Reverse: GTTGAGGAAGTCGTCTGGGG	ACGT Corporation	n/a
siRNA siGENOME, set of 4 against Taz (Wwtr1)	Dharmacon	MU-041057-01
siRNA siGENOME, set of 4 against Yap	Dharmacon	MU-046247-01
siRNA siCTL (ON-TARGETplus Non-targeting Control Pool)	Horizon (Dharmacon)	D-001810-03-20
Software and algorithms		
MATLAB	www.Mathworks.com	Release 2019b
Quantity One® software	Bio-Rad	Version 4.6.3
Other		
Hemocytometer	Fisher Scientific	0267110
IN Cell Analyzer 6000	GE Healthcare	28-0433-23
µ-Plate 96 Well	ibidi	89626
MicroAmp™ Optical 8-Tube Strip, 0.2 mL	Thermo Fisher Scientific	4316567
Micro multichannel (BioPette Plus 8 channel 1 to 10 µL)	Labnet International	P4808-10
8-Channel plastic aspirator for disposable tips, with ejection device	LabRepCo	EV520
Labnet International P4812-200 BioPette Plus 12 channel 20 to 200 µL	Labnet International	P4812-200
Thick aluminum foil sealing film (AlumaSeal® 96 film)	Millipore Sigma	Z721549-100EA
Hoefer Red Rotor Lab rotator	Manufacturer Hoefer Inc.	PR70-115v
NanoDrop One-C-	Thermo Fisher	Nd-1000
Nitrocellulose membranes 0.45 µM	Bio-Rad	1620115
4% PFA	This paper	n/a
PBS-T	This paper	n/a
Blocking Buffer for IF Experiment	This paper	n/a

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
10× Transfer buffer	This paper	n/a
1× Transfer buffer	This paper	n/a
1× TBS-T	This paper	n/a

MATERIAL AND EQUIPMENT

Here is a list of reagents needed.

4% Paraformaldehyde (PFA)		
Reagent	Final concentration	Amount
PFA	4%	4 g
NaOH 10 M	2 mM	20 µL
10× PBS	1× PBS	10 mL
Milli-Q water	n/a	90 mL

Note: Pre-warm 90 mL of water in a glass bottle with a lid in a water bath at 65°C–70°C. Add 20 µL of 10 M NaOH and 4 g of PFA powder, close the lid and heat and stir the bottle in the water bath at 65°C–70°C until the PFA powder is almost dissolved (small amounts of precipitates can be ignored). Mix occasionally to aid in dissolving PFA. Chill the solution on ice, add 10 mL of 10× PBS and filter the PFA solution to remove any precipitates.

Note: Keep the PFA solution at 2°C–8°C for up to a week.

⚠ CRITICAL: An important point for preparing 4% PFA is to dissolve the PFA in warm water at an alkaline condition, then neutralize by adding 10× PBS. Usually, the PFA dissolves in less than 10 min if placed in pre-warmed water with NaOH. Longer times (~15 min) are required if starting with cold water. Do not heat PFA for an extended period. There is no need to adjust pH when done as the amount of NaOH is very low.

PBS-T		
Reagent	Final concentration	Amount
100% Tween-20	0.05%	0.5 mL
10× PBS	1× PBS	100 mL
Milli-Q water	n/a	Up to 1 L

Note: Keep the PBS-T solution at 20°C–25°C for up to a week.

Blocking Buffer for IF Experiment		
Reagent	Final concentration	Amount
BSA	2%	1 g
Fish Gelatin (45% solution)	0.10%	111 µL
1% NaN3 (Sodium Azide) solution in water	0.01%	500 µL
100% Tween20	0.10%	50 µL
10× PBS	1×	5 mL
Milli-Q water	n/a	Up to 50 mL

Note: Weigh 1 g of BSA in a 50 mL Falcon tube, add 5 mL 10× PBS, 111 μL Fish gelatin solution, 50 μL 100% Tween20 and 500 μL 1% Sodium Azide. Adjust volume to 50 mL with water. Vortex briefly and put on a rocker to dissolve completely.

Note: Fish gelatin stock concentration may vary depending on suppliers. In any case, make sure to use 0.1% final concentration.

Note: Make fresh on the day of immunofluorescent staining.

10× Transfer Buffer		
Reagent	Final concentration	Amount
Tris Base	250 mM	90.75 g
Glycine	1900 mM	435 g
Milli-Q water	n/a	Up to 3 L

Note: Keep the 10× Transfer Buffer at 20°C–25°C for 2–3 months.

1× Transfer Buffer		
Reagent	Final concentration	Amount
10× Transfer Buffer	25 mM Tris, 190 mM Glycine	400 mL
Methanol	20%	800 mL
Milli-Q water	n/a	2800 mL

Note: Keep the 1× Transfer Buffer at 20°C–25°C for a week.

10× TBS		
Reagent	Final concentration	Amount
Tris Base	200 mM	24.2 g
NaCl	1370 mM	80 g
Milli-Q water	n/a	Up to 1 L

Note: pH of the final solution should be adjusted to ≈7.4.

Note: Keep the 10× TBS at 20°C–25°C for a month.

1× TBS-T		
Reagent	Final concentration	Amount
10× TBS	20 mM Tris, 137 mM NaCl	100 mL
100% Tween-20	0.1 %	1 mL
Milli-Q water	n/a	899 mL

Note: Keep the 1× TBST at 20°C–25°C for a day.

Automated imaging system

IN Cell Analyzer 6000 (Manufacturer, GE Healthcare (https://www.environmental-expert.com/products/cytiva-model-6000-in-cell-analyzer-581291)) was used for acquisition.

Note: Other automated or semi-automated imagers may be used as an alternative. Imaging system must be capable of acquisition of 4D datasets (multiple channels in 3D).

The imaging system specifications are listed below:

- Imaging of 3 spectral channels with minimal crosstalk.

Dye	Excitation maximum, nm	Emission maximum, nm
DAPI	358	461
Alexa488	490	525
Alexa546	556	573

Note: Here, we used an Alexa546 fluorophore, however alternative fluorescent dyes such as Alexa633 may be used as long as the imaging system has the capability to minimize spectral crosstalk among the selected fluorophores.

- Nikon 20× Plan Fluor LWD 0.75/NA

Note: Objectives with higher NA may be used as an alternative.

- Nikon 60× CFI S Plan Fluor ELWD 0.7/NA

Note: Objectives with higher NA may be used as an alternative.

- 4 megapixel sCMOS camera

Note: Cameras with smaller chip size may be used, but may significantly slow down acquisition

- Automated laser-based focusing for consistent selection of a focal plane

Note: Image based or manual auto-focus may be used as an alternative, but it is not recommended.

STEP-BY-STEP METHOD DETAILS

Reverse siRNA transfection of EpH4 cells

Timing: [~ 24 h] for step 1

Timing: [~ 25 h] for step 2

This step describes the transfection of EpH4 cells with siRNAs targeting Taz (Wwtr1) and Yap (siTaz/Yap) and controls (siCTL) for automated quantitative imaging in a 96-well dish and for parallel monitoring of knockdown efficiency by quantitative PCR (qPCR) and immunoblotting in 12-well dishes.

1. Cell preparation for transfections
   a. Split sub-confluent EpH4 cells into DMEM supplemented with 10% FBS in 100 mm dishes such that cells reach 50–70% confluency after 24 h. Typically, this can be achieved by a 1:10 split from a confluent dish.

b. Trypsinize EpH4 cells for 5 min, neutralize with DMEM containing 10% FBS and count cells using a hemocytometer. Using 50 mL tubes, prepare two 15 mL cell dilution stocks in DMEM containing 10% FBS at 56,000 or 67,200 cells/mL for siCTL or siTaz/Yap, respectively.

⚠ CRITICAL: The Hippo pathway is highly responsive to the increases in cell density, thus, to avoid pathway activation, cells must be maintained at low density up to and including the assay endpoint. In addition, to ensure that siCTL and siTaz/Yap transfected cells, which grow at different rates, are at the same confluency, transfected cells should be seeded at a ratio of 1:1.2, respectively. These requirements are achieved when cells are plated at a final density of 20,000 and 24,000 cell/cm^2 for siCTL and siTaz/Yap transfectants, respectively.

Note: For imaging, 0.2 mL of the cell suspension/transfection solution will be used in each of 48 wells of a 96-well high optical quality μ-Plate, (area = 0.56 cm^2/well), which is 11200 or 13440 cells/well for siCTL or siTaz/Yap, respectively. For verification of knockdown, 1.25 mL of the cell suspension/transfection solution will be used in each of 2 wells (area = 3.5 cm^2 / well) of a 12 well plate. Thus, the total volume required for the experiment is 12.1 (48 x 0.2 + 2 x 1.25) mL per condition.

2. Preparation of siRNA transfection mix and cell transfection
   a. Label two 50 mL tubes as siCTL and siTaz/Yap for preparation of transfection solutions.
   b. Each mL of cell suspension requires 196 μL of Opti-MEM and 2 μL of siRNAs. For siTaz and siYap, use 1 μL of Taz and 1 μL of Yap siRNApools and for siCTL, use 2 μL of the siRNA pool from a 20 μM siRNA stock solution. Thus, to prepare sufficient mix for a final volume of 13 mL of cell suspension/transfection solution, use 2.55 mL of Opti-MEM and 26 μL of siRNAs (13 μL each of siTaz or siYap and 26 μL of siCTL). Gently mix by tapping or pipetting and then incubate at RT for 5 min.
   c. For each mL of transfection mix, add 2 μL of Lipofectamine RNAiMAX (i.e., 26 μL to each siRNA solution). Mix gently and incubate at RT (20°C–25°C) for 20 min.
   d. Add 10.4 mL of each cell suspension prepared in step 1 to the corresponding transfection mix and pipet up and down gently, avoiding bubbles to thoroughly mix cells and reagents.
   e. Distribute the cell suspension/transfection solution into individual wells. For the 96-well imaging plate, use a multichannel pipette to seed 0.2 mL of the siCTL or siTaz/Yap cell suspension/transfection mix into each of 48 wells in either the 6 left columns or 6 right columns, respectively of the 96 well plate. Gently shake the plate to distribute cells evenly in wells.
   f. For immunoblotting and/or qPCR to confirm knockdown efficiency, add 1.25 mL of the siCTL or siTaz/Yap cell suspension/transfection mix per well in duplicate into a 12-well dish. To monitor both protein and mRNA levels and to allow for independent sample processing, use a single well for each condition plated into 2 separate 12-well plates.
   g. Place plates in a tissue culture incubator for 20 h.

Note: Be sure to time this incubation accurately for optimal transfection efficiency.

   h. The next day (after 20 h) add fresh media (0.2 mL per well of DMEM 10% FBS to the 96 well plate and 1.25 mL to wells in the 12 well plates) and incubate for 4 h.

Cell starvation and TGFβ stimulation

Timing: [~ 6 h]

To determine temporal patterns of Smad localization, EpH4 cells transfected with siCTL or siTaz/Yap are treated with different doses of TGFβ from 1 to 50 pM at varying times from 0 to 3 h. Optimal activation of TGFβ signaling occurs in low serum conditions, possibly due to the presence of latent TGFβ in Fetal bovine serum (FBS) (Oida and Weiner 2010). For imaging, all cells are simultaneously fixed and stained. Thus, to vary the time of TGFβ treatment and ensure that all cells experience equivalent serum-deprivation conditions, a reverse time course approach is required.

Table 1. Template of 96 well plate and starvation/treatment timing

Row	Starvation start time	TGFβ treatment start time	Total time of starvation & treatment
H	0	0	6 h
G	1 h	1 h	5 h
F	1 h 30 min	1 h 30 min	4 h 30 min
E	2 h	2 h	4 h
D	2 h 20 min	2 h 20 min	3 h 40 min
C	2 h 40 min	2 h 40 min	3 h 20 min
B	2 h 50 min	2 h 50 min	3 h 10 min
A	3 h	(no treatment)	3 h

3. Cell starvation
   a. Change media with starvation media (media with low serum, 0.1% FBS). Aspirate old media and add 0.2 mL of starvation media for varying times, as listed in Table 1 (Column 2) such that all cells are starved for 3 h at the assay endpoint. Cells in 12 well plates for immunoblotting and qPCR experiments are starved for 3 h.

4. TGFβ stimulation
   a. Prepare TGFβ-containing media in PCR tubes by diluting the TGFβ stock solution into starvation media to achieve a final concentration of 21, 52.5, 105, 210, 420 and 1050 pM.

   ⚠ CRITICAL: Dilute TGFβ into final concentration immediately after starvation and before treatment as TGFβ can be easily absorbed on the wall of the tubes.

   b. Using a micro multichannel pipette, add TGFβ at the indicated times (Table 1, Columns 3-4) to obtain the desired treatment dose and time by adding 10 μL of each of the different concentrations to the corresponding wells to achieve a final concentration of 1, 2.5, 5, 10, 20, and 50 pM. Table 2 shows the timing and concentration of TGFβ treatment.

   Note: A similar protocol is followed for transfection, seeding, starvation and TGFβ treatment of cells for tracking localization of Smad4.

Cell fixing and antibody staining

Timing: [~ 0.5 h] for step 5

Timing: [~ 18 h] for step 6

This step describes fixation and immunofluorescent staining of cells in 96-well dishes to monitor localization of Smads and Taz/Yap proteins in cells transfected with siRNAs. The following steps do not require sterile conditions.

5. Cell Fixing.
   a. Remove media using a multichannel aspirator and wash cells 3 times with PBS at RT (20°C–25°C) using a multichannel pipette.

   ⚠ CRITICAL: Use PBS at RT (20°C–25°C) to wash treated cells before fixation to avoid cold stress to cells.

   b. Fix cells using 100 μL per well of 4% PFA in PBS at RT (20°C–25°C) for 20 min and then wash 3 times with PBS-T.

6. Antibody staining.

Table 2. Template of 96 well plate and dose/time of TGFβ treatment

Condition	siCTL						siTaz/Yap					
TGFβ Dose (pM)	1	2.5	5	10	20	50	1	2.5	5	10	20	50
Column	1	2	3	4	5	6	7	8	9	10	11	12
Row A	Starved for 3 h, no treatment											
Row B	Starved for 3 h, TGFβ treated for 10 min.											
Row C	Starved for 3 h, TGFβ treated for 20 min											
Row D	Starved for 3 h, TGFβ treated for 40 min											
Row E	Starved for 3 h, TGFβ treated for 1 h											
Row F	Starved for 3 h, TGFβ treated for 1 h 30 min											
Row G	Starved for 3 h, TGFβ treated for 2 h											
Row H	Starved for 3 h, TGFβ treated for 3 h											

a. Permeabilize cells using 100 μL per well of 0.5% Triton X-100 in PBS for 10 min, and then wash 3 times with PBS-T.
b. Block using 100 μL per well of blocking buffer for 1 h at RT (20°C–25°C).
c. Incubate with 70–100 μL per well of primary antibodies diluted in blocking buffer at 2°C–8°C overnight (16–18 h), with rocking. The plate is stained simultaneously for both Smad2/3 and Taz/Yap, using dilutions of 1:500 for rabbit anti-Smad2/3 and 1:300 for mouse anti-Yap (see key resources table).
d. Wash cells 3 times with PBS-T for 5 min each.
e. Incubate with 100 μL per well of secondary antibodies (Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) and Alexa Fluor® 546 Goat Anti-Mouse IgG (H+L), for Smad2/3 and Taz/Yap, respectively, each at a dilution of 1:1000 and DAPI at 1 μg/mL, diluted in blocking buffer at RT (20°C–25°C) for 2 h. Cover the plate with foil to prevent light exposure as these antibodies are light sensitive.
f. Wash 3 times with PBS-T for 5 min each.
g. Add 400 μL of cold PBS to each well and seal the plate with aluminum foil sealing film.

⚠ CRITICAL: The sealed plate can be stored at 2°C–8°C prior to image acquisition (for up to 1 day).

Note: Antibody specificity and low background signals (roughly 5% of total intensity) were confirmed by testing all individual primary and secondary antibodies (see key resources table).

Note: A similar protocol can be followed for staining of Smad4 alone using a dilution of 1:250 for mouse anti-Smad4 and 1:1000 of Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) secondary antibodies (see key resources table) with Yap staining done in parallel wells.

Note: Several Yap antibodies have been validated for immunocytochemistry in EpH4 cells (Narimatsu et al., 2016). The mouse anti-Yap antibody used in this protocol (see key resources table) recognizes both Yap and Taz. Validated alternatives that recognize only YAP are also available (Narimatsu et al., 2016).

Setting up image acquisition and analysis

Timing: [~1 h] for step 7

Timing: [~1–2 h] for step 8

Timing: [~ 1 h] for step 9

This step describes the setup for collecting images using IN Cell Analyzer 6000 (Manufacturer, Cytiva (https://www.environmental-expert.com/products/cytiva-model-6000-in-cell-analyzer-581291)), equipped with 20×/0.75 NA objective and sCMOS 2048×2048 camera. Image analysis using a custom image analysis routine in Columbus Image Data Storage and Analysis System v2.3 (PerkinElmer) is also described.

7. Acquisition and image pre-processing for high-content analysis.
   a. Select the 20× objective.

      Note: In alternative imaging platforms where the objective requires immersion media other than air, ensure that there is sufficient immersion medium on the objective throughout image acquisition.
   b. Place the 96-well plate into the sample holder.
   c. Position acquisition points in an unbiased way across all the wells in a plate.
   d. Set up 3 acquisition channels to acquire:
      Channel I: DAPI (*DAPI channel in IN Cell 6000*)
      Channel II: Alexa Fluor® 488 (*FITC channel in IN Cell 6000*)
      Channel III: Alexa Fluor® 546 (*dsRed channel in IN Cell 6000*)
   e. The number of acquisition points should be sufficient to capture 1000 cells per well. A recommendation is to start with 9 fields of view and adjust accordingly.
   f. Focusing must occur prior to each acquisition.
   g. Set up the 3D acquisition to acquire 4–6 μm along z-axis to capture the entire cell volume. Spacing between adjacent z-slices must be adjusted depending on objective's NA.
   h. Acquire images for wells of interest.
   i. Collapse the 3D datasets into 2D images using the maximum intensity projection method. Alternatively, individual z-slices or 3D rendering may be used to qualitatively examine images. Figure 1 shows representative images taken using this strategy.

      Note: Although not amenable to assessment of a large number of samples as in this method, a more accurate estimate of nuclear to cytoplasmic volumes can be obtained using 3D reconstruction from serial light sheet-based fluorescence microscopy optical sections.

8. Image analysis
   a. Segment nuclear masks in the DAPI (or other nuclei counterstained) channel (Figure 2A).
   b. Segment cellular masks in the Smad (Smad2/3 or Smad4) FITC channel (Figure 2B).

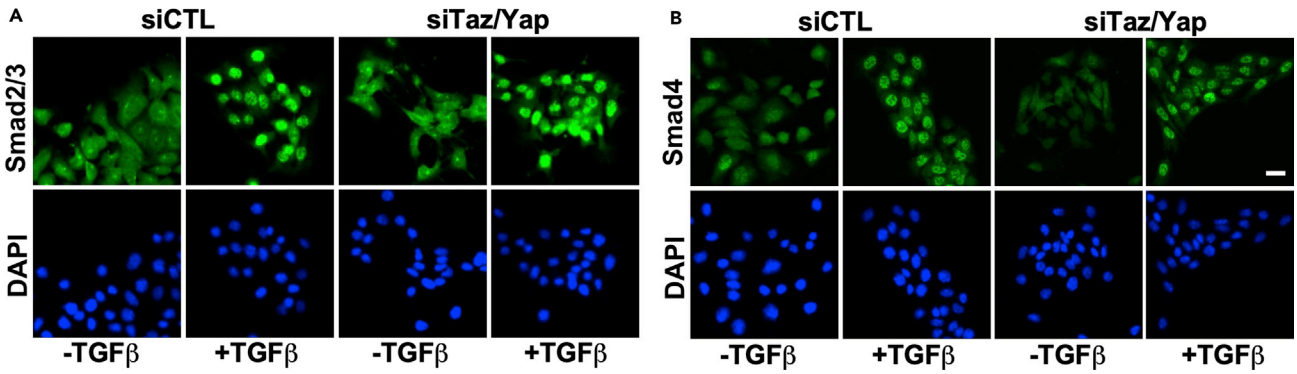

Figure 1. Representative images of cells used for quantification

EpH4 cells were transfected with siCTL or siTaz/Yap and then treated with 5 pM TGFβ for 1 h. Cells were fixed, nuclei visualized with DAPI and Smad proteins stained with antibodies against (A) Smad2/3 or (B) Smad4 and representative images, visualized using an IN Cell Analyzer 6000, are shown. Scale bar, 30 μm (Reprinted from (Labibi et al., 2020), Figure S1E and S1F).

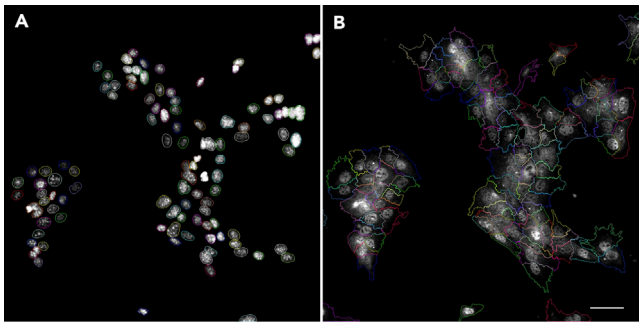

Figure 2. Steps of the image analysis routine

Nuclear (A) and Cellular (B) masks are indicated in the DAPI and FITC channels, respectively. Scale = 50 μm.

c. Identify the cytoplasmic mask by subtracting the nuclear segmentation mask from the cell segmentation mask.
d. Calculate median intensity for the Smad channel (Channel II, see 7d) within the nuclear segmentation mask ($Smad_N$) and the cytoplasmic segmentation mask ($Smad_C$)
e. Calculate the ratio of $\frac{Smad_N}{Smad_C}$ for each cell within a well.
f. For each well, calculate the median value of all the $\frac{Smad_N}{Smad_C}$ values obtained for each individual cell in a given well.
g. Calculate median intensity for the Taz/Yap channel (Channel III, see 7d) within the nuclear segmentation mask (Taz/Yap_N) and the cytoplasmic segmentation mask (Taz/Yap_C).
h. Calculate the ratio of $\frac{Taz/Yap_N}{Taz/Yap_C}$ for each cell within a well.
i. For each well, calculate the median value of all the $\frac{Taz/Yap_N}{Taz/Yap_C}$ values obtained for each individual cell in a given well.

Note: Steps 8g-i are used to verify that Taz/Yap localization remains constant throughout the experimental conditions.

j. Calculate median intensity with a cell mask for the Taz/Yap channel (Channel III, see 7d)
k. Identify cells with high intensity signals (which correspond to wild type cells) and those with low intensity signals, which correspond to Yap/Taz depleted cells.

Note: Steps 8j and k are used to verify Taz/Yap knockdown efficiency.

9. Estimating normalized masses of proteins in the nucleus and cytoplasm
   a. Estimate the pixels covered by nucleus and cytoplasm (determined by subtracting nucleus from entire cell) and calculate the areas of each region assuming each pixel is 0.325 micrometers.
   b. Calculate the ratio of the nuclear to cytoplasmic volume. Volume is the base area times height and as a rough approximation, the ratio of the volumes is the same as the ratio of the areas. The ratio of cytoplasmic to nuclear volumes was determined to be 2.52 ± 0.023 for EpH4 cells transfected with siRNAs.
   c. Estimate the normalized cytoplasmic and nuclear masses. Fluorescence data are proportional to concentrations (Schmierer et al., 2008), thus, to estimate normalized cytoplasmic and nuclear masses, R and R_v, the ratios of cytoplasmic to nuclear concentration and volumes, respectively, as defined in Table 3 are measured by IF experiments. Then, accordingly the ratio of nuclear to cytoplasmic masses of the proteins, R_m (Table 3) is calculated.
   d. Considering $1 + R_m = 1 + \frac{M_{Nuc}}{M_{Cyt}} = \frac{M_{Nuc} + M_{Cyt}}{M_{Cyt}}$, and since during the signaling period (3 h) there is negligible degradation of the Smad/Taz/Yap proteins as shown by immunoblotting (Labibi et al., 2020), by assuming $M_{Nuc} + M_{Cyt} = 100$, the normalized cytoplasmic and nuclear masses

Table 3. Different ratios estimated in the process of normalized mass quantification

Ratio	Factors
$R = \frac{C_{Nuc}}{C_{Cyt}}$	C_{Nuc} , nuclear concentration, C_{Cyt}, cytoplasmic concentration
$R_v = \frac{V_{Nuc}}{V_{Cyt}}$	V_{Nuc} , nuclear volume, V_{Cyt}, cytoplasmic volume
$R_m = \frac{M_{Nuc}}{M_{Cyt}}$	M_{Nuc} , nuclear mass, M_{Cyt}, cytoplasmic mass

of the proteins can be calculated as: $M_{Cyt} = \frac{100}{1+R_m}$ *and* $M_{Nuc} = \frac{100R_m}{1+R_m}$, respectively. Example plots of results are shown in Figure 3.

Confirmation of knockdown efficiency of Taz and Yap by qPCR

Timing: [~ 6 h]

This step describes parallel monitoring of knockdown efficiency of Taz/Yap and the effect on target gene expression by qPCR using cells plated in a 12-well dish as described above (step 2f). The expression levels of Taz, Yap and the Hippo target genes Ankrd1 and Cyr61 are compared in cells transfected with siCTL or siTaz/Yap. The expression levels of the Hippo target genes are expected to be reduced in siTaz/Yap transfected cells.

10. RNA isolation, cDNA preparation and qPCR
    a. Isolate total RNA from siCTL and siTaz/Yap transfected and serum starved (3 h) cells using PureLink RNA Mini Kit according to the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fpurelink_rna_mini_kit_man.pdf&title=UHVyZUxpbmsgUk5BIE1pbmkgS2l0). Store purified RNA on ice. For long-term storage, store at −80°C.
    b. Determine RNA concentration by a NanoDrop One-C.
    c. Using 1 μg of total RNA, generate cDNA using oligo (dT) primers and M-MLV reverse transcriptase using standard protocols that include a DNaseI pre-digestion step.
    d. Perform Real-time PCR using a 2X SYBR Green PCR master mix and validated primers (see key resources table) using an appropriate Real-Time PCR instrument such as the QuantStudio™ 6 flex real-time PCR. For each well of a 384-well dish use 6.25 ng of cDNA template and 250 nM of each primer in a 10 μL reaction volume.
    e. Perform PCR using the following cycle conditions: 1 denaturing cycle of 95°C for 10 min, 40 qPCR cycles of 95°C for 15 s, 60°C for 1 min, and 1 melting curve cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.
    f. Normalize gene expression to Hprt and calculate relative expression by the ΔΔCt method.

Immunoblotting to confirm knockdown efficiency of Taz and Yap

Timing: [~ 24 h]

This step describes parallel monitoring of knockdown efficiency of Taz/Yap at the protein level by immunoblotting using extracts from cells plated in 12-well dish (step 2f). The expression levels of Taz/Yap proteins are compared in cells transfected with siCTL or siTaz/Yap.

11. Preparation of Cell Lysates, Electrophoresis, and Immunoblotting.
    a. Place the 12-well dish of siTaz/Yap or siCTL transfected and serum starved (3 h) cells on ice and wash cells 3 times with 1 mL of ice-cold PBS.

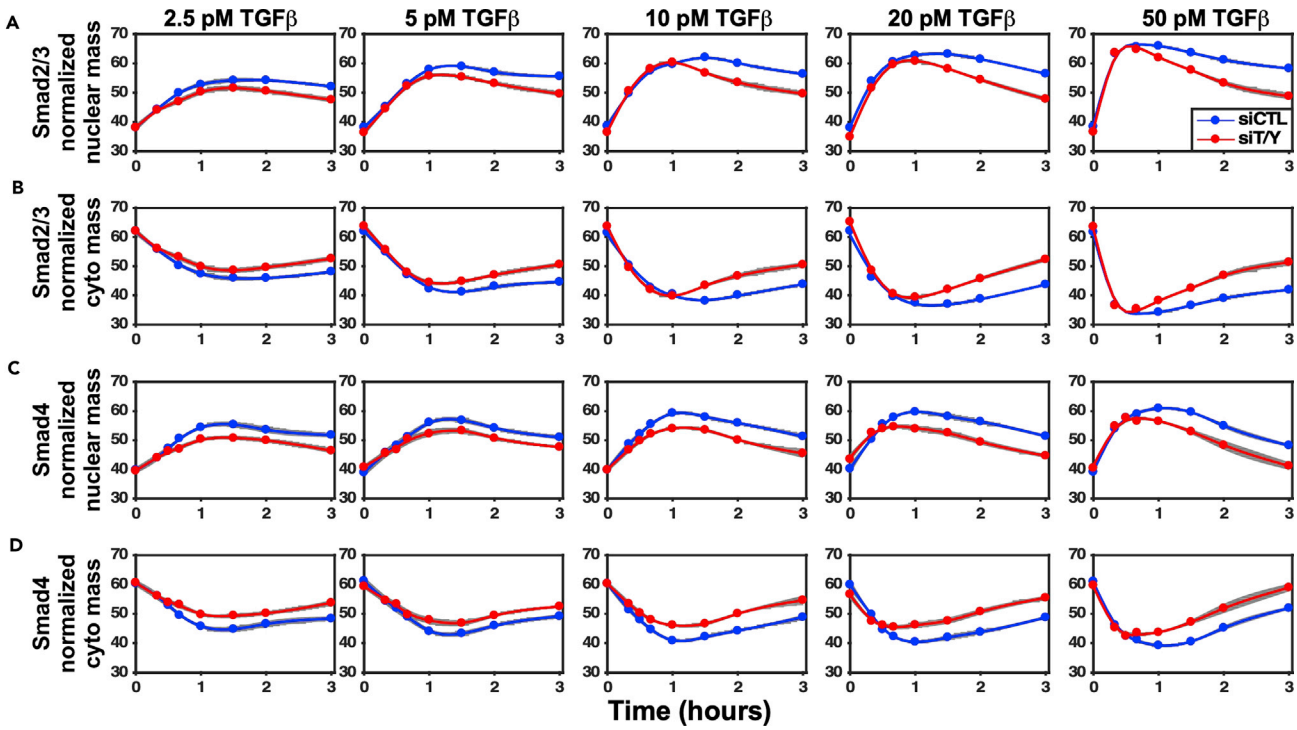

Figure 3. Normalized nuclear and cytoplasmic masses of Smad2/3 and Smad4 upon loss of Taz/YAP expression

The nuclear to cytoplasmic ratios obtained by IF imaging were converted into normalized mases by taking into account the volumes of the nucleus and cytoplasm. The gray area indicates the mean +/- SEM of at least 9 biological replicates and the full circles show the average values of the original data. (A) Normalized mass of nuclear Smad2/3, (B) Normalized mass of cytoplasmic Smad2/3, (C) Normalized mass of nuclear Smad4 and (D) Normalized mass of cytoplasmic Smad4. (Reprinted from (Labibi et al., 2020), Figure S4 panels B to E).

b. Add 150 μL of lysis buffer per well. Rock the plate at 2°C–8°C for 15 min.

⚠ CRITICAL: Prepare the lysis buffer immediately before lysing and keep on ice until use.

c. Scrape wells with a cell scraper and collect lysates into 1.5 mL microcentrifuge tubes. Centrifuge at 15,000 × *g* for 10 min.
d. Collect supernatants, transfer into new tubes and determine total protein concentration using the Bradford assay (He 2011).
e. Adjust to achieve equal protein concentrations in all samples, add sample buffer, and heat samples for 5 min at 95°C. Load 15–25 μg of protein per lane and separate by SDS-PAGE gel electrophoresis.
f. Transfer proteins to a nitrocellulose membrane using wet transfer.
g. Block membranes using 5% skim milk in TBS-T for 1 h.
h. Remove blocking solution and incubate membranes in primary antibodies diluted in TBS-T with 5% skim milk (for mouse anti-Yap, which also recognizes Taz, use 1:1000 and for rabbit anti-Actin use 1:8000) at 2°C–8°C overnight (16–18 h) on a rocker.
i. Wash membranes with TBS-T buffer 3 times for 10 min each time on a rocker.
j. Incubate membranes with HRP-conjugated secondary antibodies (anti-mouse for Yap and anti-rabbit for Actin, diluted 1:5000) in blocking solution at RT (20°C–25°C) for 1 h on a rocker.
k. Wash membranes with TBS-T buffer 3 times for 10 min each time on a rocker.
l. Detect chemiluminescent signals using SuperSignal™ substrate as described by the manufacturer (thermofisher.com)<https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%

2Fmanuals%2FMAN0011307_SupSig_West_Dura_Extend_Dur_Subs_UG.pdf&title=VXNIciBHdWlkZTogIFN1cGVyU2lnbmFsIFdlc3QgRHVyYSBFeHRlbmRlZCBEdXJhdGlvbiBTdWJzdHJhdGU) and image using an appropriate instrument such as the Versadoc (BioRad) or other Imager.

EXPECTED OUTCOMES

In order to undertake mathematical modeling of TGFβ/ Hippo pathway crosstalk, it is essential to collect accurate data for the concentrations of nuclear and cytoplasmic Smads and Taz/Yap at different time points and doses of ligand. Immunofluorescence (IF) microscopy is widely accepted and amenable to high-throughput approaches. By using a 96-well format, all time points and doses of TGFβ could be accommodated on one plate, providing increased experimental accuracy. Moreover, by double-staining for Smad2/3 and Taz/Yap, we are able to assess Smad2/3 localization while simultaneous confirming Taz/Yap knockdown in individual cells (Labibi et al., 2020). It is also worth noting, that in prior studies that modeled TGFβ/Smad signaling, the IF approach used was FRAP with EGFP-tagged Smads. While EGFP-tagged Smad2 was reported to behave in a manner that is experimentally indistinguishable from endogenous Smads (Schmierer et al., 2008), the presence of this additional tagged Smad2 may impact normal cell behavior by altering the physical limit and saturation phases. Thus, this approach, which tracks the localization of endogenous Smads, is preferred as it enables gathering more physiologically relevant data.

Automated IF imaging was used to estimate the nuclear to cytoplasmic ratios of Smads and Taz/Yap. The reason for selecting the ratio was to reduce intra assay variation and to normalize intensities in each individual IF plate. Fluorescence data are proportional to concentrations, whereas immunoblot data are proportional to total particle numbers (Schmierer et al., 2008). The nuclear to cytoplasmic ratio shows the ratio of intensities of the protein of interest in the nucleus to the cytoplasm. We converted the nuclear to cytoplasmic ratios obtained by IF imaging into normalized masses by taking into account ratio of the volumes of the nucleus and cytoplasm. The proposed strategy to acquire the required data can be used in mathematical modeling of cell signaling pathways at different conditions, doses, and time points.

LIMITATIONS

The two genes Taz and Yap are crucial for EpH4 cell survival, so cells with both genes simultaneously knocked down, do not appear healthy at long time points (>30 h). This limits the ability to analyze protein localization for extended periods of time. In this study, the 3 h period was sufficient to study the mechanism of Smad nuclear accumulation.

TROUBLESHOOTING

Problem 1

The Hippo pathway is highly responsive to increases in cell density (step 1b).

Potential solution

To avoid density-dependent Hippo pathway activation, cells must be maintained at low densities up to and including the assay endpoint. Cell counts should be determined accurately, and Taz/Yap localization should be monitored to ensure localization remains constant. In addition, it is important to ensure that siCTL and siTaz/Yap transfected cells, which grow at different rates, are at the same confluency at the start of the experiment. Therefore, cells should be seeded at a ratio of 1:1.2, respectively.

Problem 2

Optimal activation of TGFβ signaling occurs in low serum conditions, but prolonged serum depletion is well known to alter Taz/Yap localization (step 3a).

Potential solution

Taz/Yap localization should be carefully monitored under the experimental conditions to ensure localization remains constant. In our protocol, we observed that there was no change in Taz/Yap localization in cells treated with TGFβ (at 1–50 pM) for time points up to 6 h after the start of serum depletion, so experiments should be kept within these parameters (Labibi et al., 2020).

Problem 3

TGFβ can be easily absorbed on the wall of the tubes. This can affect the intended doses of TGFβ (step 4a).

Potential solution

To avoid absorption of TGFβ on the wall of the tubes, TGFβ should be diluted to the appropriate concentrations immediately after starvation and before treatment. Using immunoblotting and qPCR experiments, it was confirmed that dose of diluted TGFβ in tube is fairly constant during the 3 h treatment.

Problem 4

Images for Smad2/3 (Alexa Fluor® 488) and Taz/Yap (Alexa Fluor® 546) channels are very similar across the different treatments (step 7 h).

Potential solution

Similar images may indicate that there is spectral crosstalk between two channels. Ensure that the microscope used for acquisition has a set of filters capable of separating these fluorophores. Alternatively, replacing Alexa Fluor® 546 with a far-red label like Alexa Fluor® 647 can be used to improve spectral separation.

Problem 5

The calculated ratio $\frac{Smad_N}{Smad_C}$ does not significantly change when cells are treated with TGFβ despite a change being observed during qualitative assessment of images (step 8e).

Potential solution

The cellular mask might not have been detected correctly due to a strong decrease it the cytoplasmic signal of Smad2/3. Ensure that cell segmentation is accurate across the various treatments and perform routine quality control of the segmentation results on random samples and wells in each experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Liliana Attisano (liliana.attisano@utoronto.ca).

Materials availability

This study did not generate new unique materials.

Data and code availability

All data produced or analyzed for this study are included in (Labibi et al., 2020) and its Supplemental Materials.

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AUTHOR CONTRIBUTIONS

B.L. designed and performed experiments and wrote the manuscript. M.B. performed imaging and developed the image analysis protocol. T.C. established protocols, prepared figures, and reviewed the manuscript. L.A. supervised the study and wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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