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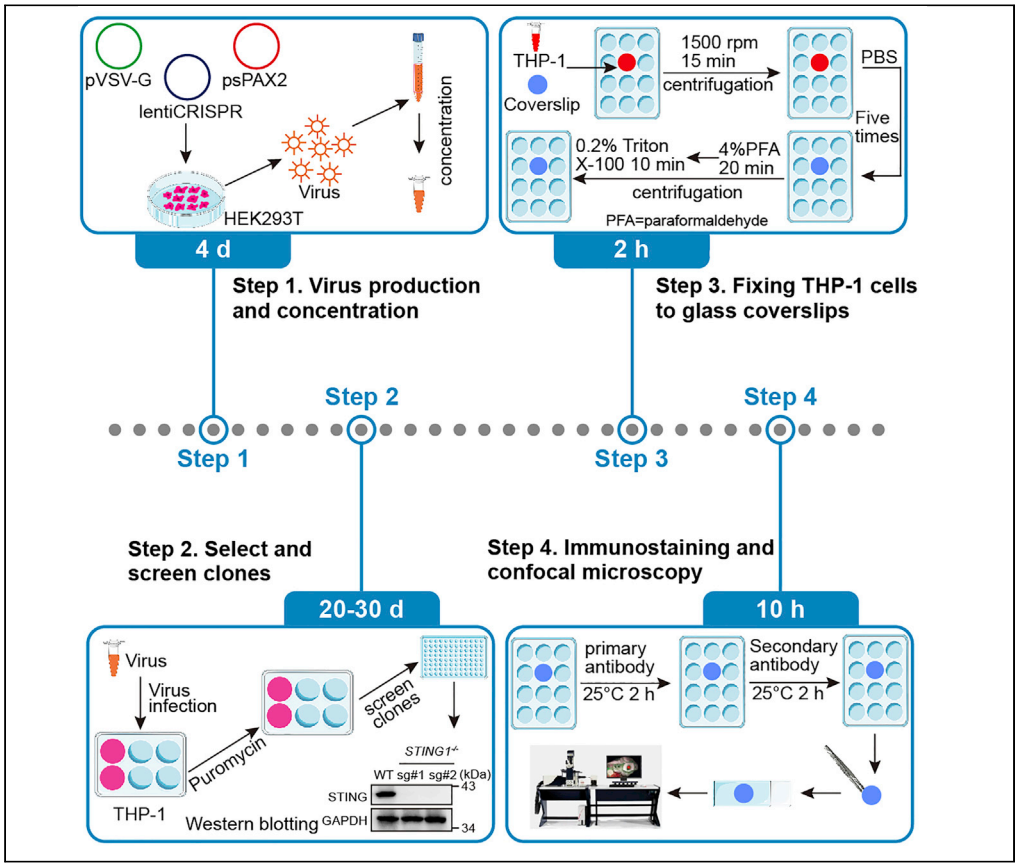
Protocol using lentivirus to establish THP-1 suspension cell lines for immunostaining and confocal microscopy



THP-1, a monocyte cell line growing in suspension, is widely used in immunology research. However, establishing suspension cell lines and performing confocal microscopy can be challenging. Here, we present a protocol to efficiently generate THP-1 cell lines using lentivirus and perform immunostaining and confocal microscopy. We detail steps for virus production, THP-1 cell infection and clone selection, fixing the suspension cells to the glass slide for immunostaining, and subsequent confocal microscopy. This protocol can be applied to other suspension cells.

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

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Highlights

Highly efficient screening of THP-1 suspension cell lines using lentivirus system

Protocol for immunostaining and confocal imaging of suspension cells

Applicable for multiple suspension cells including Jurkat cells

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Protocol using lentivirus to establish THP-1 suspension cell lines for immunostaining and confocal microscopy

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SUMMARY

THP-1, a monocyte cell line growing in suspension, is widely used in immunology research. However, establishing suspension cell lines and performing confocal microscopy can be challenging. Here, we present a protocol to efficiently generate THP-1 cell lines using lentivirus and perform immunostaining and confocal microscopy. We detail steps for virus production, THP-1 cell infection and clone selection, fixing the suspension cells to the glass slide for immunostaining, and subsequent confocal microscopy. This protocol can be applied to other suspension cells.

For complete details on the use and execution of this protocol, please refer to Ji et al. (2021).[1]

BEFORE YOU BEGIN

The following protocol explains how to detect endogenous protein, using confocal microscopy, and generate knockout in THP-1, suspension cells, using STING[2–5] (stimulator of IFN genes) as an example.

Compared with solid tumor-derived cells, most of leukemic cell lines (for example, THP-1 cells) could not be efficiently transfected with plasmid, and are often faced with some obstacles for constructing cell lines. To solve this problem, this protocol provides an option using lentivirus vector system.

At the same time, researchers may have some tricky issues when using cell lines growing in suspension for confocal microscopy. In this experiment, the biggest trouble for researchers is how to fix the suspension cells to the glass slide. This protocol describes detailed steps for solving this problem.

To ensure the cell viability, it is recommended to use cells that have been in culture between 1 week and 1.5 months. Furthermore, the recommended range of passage numbers of THP-1 and HEK293T cells was no more than 20 passages. At the same time, make sure that cells are growth at an appropriate density (For THP-1 cells, the appropriate density is $2–8\times10^5$ cells/mL. For HEK293T cells, ensure that cells can achieve 100% confluence after seeding for 2–3 d). This will greatly facilitate the progress of the experiment.

Institutional permissions

The virus studies in our research were approved by the academic committee of Nankai University.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GAPDH (6C5) (dilution 1:1000)	Santa Cruz	Cat#sc-32233
Rabbit polyclonal anti-TMEM173/ STING (dilution 1:1000)	ProteinTech	Cat#19851-1-AP; RRID: AB_10665370
Goat anti-mouse IgG-HRP (dilution 1:10000)	Sungene Biotech	Cat#LK2003
Goat anti-rabbit IgG-HRP (dilution 1:10000)	Sungene Biotech	Cat#LK2001
(TRITC)-conjugated affinipure goat anti-rabbit IgG (dilution 1:100)	ProteinTech	Cat#SA00003-2; RRID: AB_2890897
Chemicals, peptides, and recombinant proteins		
Bovine serum albumin (BSA)	Genview	Cat#FA016
Paraformaldehyde	Sangon Biotech	Cat#A500684
RIPA lysis buffer	Solarbio	Cat#R0020
PBS	Solarbio	Cat#P1020
Puromycin	Sangon Biotech	Cat#A610593
Polyethylenimine, linear, MW 25000, transfection grade	Polysciences	Cat#23966-100
ECL Western Blotting Substrate	Millipore	Cat#WBKLS0500
DAPI Fluoromount-G	Southern Biotech	Cat#0100-20
Fetal bovine serum	ExCell Bio	Cat#FND500
Dulbecco's modified Eagle's medium (DMEM)	Corning	10-013-CV
Roswell Park Memorial Institute (RPMI)-1640 medium	Corning	10-040-CV
0.05% (w/v) trypsin	HyClone	SH30236.02
SDS	Sangon Biotech	Cat#A600485
KCl	Sangon Biotech	Cat#A610440
KH_2PO_4	Sangon Biotech	Cat#A600445
NaCl	Sangon Biotech	Cat#A501218
EDTA	Sangon Biotech	Cat#B548402
TWEEN 20	Sigma-Aldrich	Cat#P2287
Triton X-100	Sigma-Aldrich	Cat#T8787
Agarose	Biosharp Life Sciences	Cat#BS081
$Na_2HPO_4 \cdot 12H_2O$	Sangon Biotech	Cat#A607793
Critical commercial assays		
ViraTrap™ Lentivirus Concentration Reagent	Biomiga	Cat#BW-V2001-03
Experimental models: Cell lines		
THP-1	ATCC	TIB-202
HEK293T	ATCC	CRL-3216
Oligonucleotides		
Sg-STING-A	This paper	GCGGGCCGACCGCATTTGGG
Sg-STING-B	This paper	ATCCATCCATCCCGTGTCCC
Recombinant DNA		
pBS-CMV-gagpol	Addgene	Cat#35614
psPAX2	Addgene	Cat#12260
pCMV-VSV-G	Addgene	Cat#8454
lentiCRISPR V2	Addgene	Cat#52961
Software and algorithms		
FiJi® (v2.0.0-rc-69/1.52i) ImageJ	Schindelin et al.[6]	https://imagej.net/software/fiji/
Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
Photoshop	Adobe	https://www.adobe.com/cn/products/photoshop.html
Adobe Illustrator	Adobe	https://www.adobe.com/cn/products/illustrator.html

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
6 well cell culture plate	Nest	Cat#703001
12 well cell culture plate	Nest	Cat#712001
15 mL centrifuge tube	Nest	Cat#601052
50 mL centrifuge tube	Nest	Cat#602052
Confocal laser scanning microscope	Leica	TCS SP5
Microscope slide	Sail Brand	Cat#7107
Circle microscope cover glass	Nest	Cat#801007
Centrifuge	Eppendorf	5810/5810R
Tannon-5500 gel imager	Tannon	Cat#5500

MATERIALS AND EQUIPMENT

[Complete RPMI-1640 or DMEM medium]		
Reagent	Final concentration	Amount
RPMI-1640 or DMEM	N/A	45 mL
Fetal bovine serum (FBS)	10%	5 mL
Total	**N/A**	**50 mL**

Note: Store at 4°C and prewarm at 37°C before use.

[PBS (phosphate buffered solution)]		
Reagent	Final concentration	Amount
Na_2HPO_4	10 mM	1.44 *g*
NaCl	136 mM	8 *g*
KH_2PO_4	2 mM	0.24 *g*
KCL	2.6 mM	0.2 *g*
Add ddH_2O to	**N/A**	**1 L**

Note: Store at 4°C for up to 2 years and prewarm at 37°C before use. Adjust the pH value to 7.2–7.4.

[4% paraformaldehyde]		
Reagent	Final concentration	Amount
Paraformaldehyde	4%	4 *g*
PBS	N/A	Adjust to 100 mL
Total	**N/A**	**100 mL**

Note: Store at 4°C for 1 month. To dissolve paraformaldehyde, we could adjust pH value to 11 and heat the solution to 60°C. In the end, when the solution is cooled to room temperature (20°C–25°C), adjust pH value to 7.0.

STEP-BY-STEP METHOD DETAILS

HEK293T cells seeding and transfection

Timing: 2 d

This step is to seed and transfect HEK293T cells for the production of high titer lentivirus, which is critical for subsequent cell line screening. The volumes used here are for 10 cm cell culture dish. We choose STING as an example, and you may set your own gene according to your study design.

1. HEK293T cells seeding.
   a. After HEK293T cells reached 100% confluence (incubation at 37°C, 5% CO_2), remove and discard culture medium. Then, briefly rinse the HEK293T cells with 5 mL PBS (phosphate-buffered saline) to remove all traces of serum which will inhibit the function of trypsin.
   b. Add 2–3 mL of 0.05% (w/v) trypsin - 0.53 mM EDTA solution to dish, then waiting for 2–5 min at room temperature (20°C–25°C) until cell layer is dispersed.

   Note: In this step, 0.25% trypsin is not recommended.

   c. Add 5 mL complete growth medium, then aspirate cells by gently pipetting.
   d. Add appropriate cell (3.5–3.8×10^6 cells) suspension to new 10 cm cell culture dish, and ensure that cells can achieve 80% confluence after 24 h (Figure 1). Incubate cells at 37°C, 5% CO_2.
2. HEK293T cells transfection.
   a. Add 6 μg retroviral vector (control or gene of interest for KO (here STING)), 6 μg psPAX2 and 3 μg pCMV-VSV-G to 1.5 mL centrifugation tube, then mix them with 400 μL serum-free DMEM (Dulbecco's modified Eagle medium). This tube is named as tube A.

   Note: For the details of the retroviral vector construction, you could refer to these publications.[7,8]

   b. Add 60 μL PEI (polyethylenimine, 1 mg/mL) to 1.5 mL centrifugation tube, then mix it with 400 μL serum-free DMEM. This tube is named as tube B.
   c. Add the mixture from tube B to tube A, then mix thoroughly by pipetting with 1 mL pipette.

   Note: To ensure high transfection efficiency, we should add the mixture of tube B to A, and the reverse is not advisable.

   d. Incubate for 20 min at room temperature (20°C–25°C), then add the mixture to 10 cm cell culture dish, which contains HEK293T cells (80% confluence) to be transfected.

   Note: To ensure even distribution of the mixture, gently swirl the dish for several times.

   e. Then incubate cells at 37°C, 5% CO_2.
   f. After 6 h, replace the medium with 10 mL prewarmed DMEM with 10% FBS (fetal bovine serum).
   g. Let the cells growth for 2 d.

Lentivirus collection and concentration

Timing: 2 d

In this part, we describe the protocol for the collection of the virus released into the supernatant. Then, we concentrate the virus by ViraTrap™ Lentivirus Concentration Reagent (Biomiga). With this procedure, the titer of the virus can be increased about ten-fold, which can greatly improve the efficiency of infection.

3. After virus production for 2 d, transfer the supernatant (10 mL) into a 15 mL centrifugation tube, then centrifuge (160×*g*) at 4°C for 15 min.
4. Carefully transfer the supernatant to a new 15 mL centrifugation tube, and filter it with 0.22 μm filters.

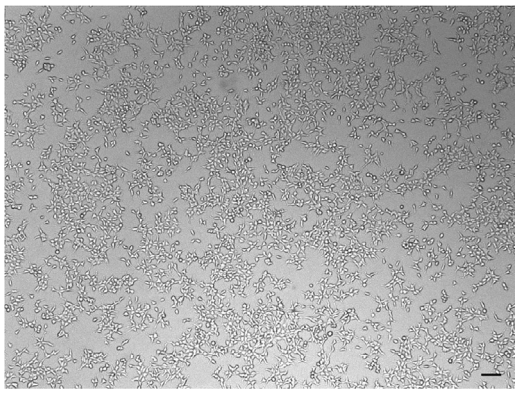

Figure 1. The appropriate cell confluence of HEK293T cells before transfection

Phrase contrast microscopy (objective magnification) images was shown. Scale bar: 100 μm.

Note: All virus-related experiments need to be done in the biological safety cabinet.

5. Add the ViraTrap™ Lentivirus Concentration Reagent (2.5 mL) to the tube, then mix thoroughly by pipetting. Subsequently, incubate the mixture at 4°C for 8–16 h.
6. Centrifuge the mixture (650×*g*) at 4°C for 20 min.

Note: In this step, you can observe the significant white pellet at the bottom of the 15 mL centrifugation tube.

7. Remove supernatant, leaving the white pellet at the bottom of the 15 mL centrifugation tube (Figure 2).
8. Repeat step 6 and 7.
9. Resuspend the white pellet using 0.5–1.0 mL Roswell Park Memorial Institute (RPMI)-1640 medium with 10% FBS.
10. The concentrated virus can be directly used to infect cells (THP-1) or can be stored at −80°C.

⚠ CRITICAL: Avoid multiple freeze/thaw cycles.

THP-1 cells infection and selection

Timing: 20–30 d

This is the final step of THP-1 cell line generation. THP-1 cells are infected with lentivirus, then selected with puromycin.

11. Resuspend THP-1 cells (3×10^5 cells) with 1 mL RPMI-1640 containing concentrated virus, then transfer them (1 mL) to 12 well plate. Incubate the mixture at 37°C, 5% CO_2 for 6 h.
12. Replace the medium with the prewarmed complete RPMI-1640 medium.

Note: The replaced medium containing the virus needs to be deactivated by soaking in a solution of sodium hypochlorite (0.02–0.05%).

13. After 48 h, replace the medium with the prewarmed complete RPMI-1640 medium, which contains 2 μg/mL puromycin. Incubate the mixture at 37°C, 5% CO_2.

Note: Here, it is important to choose the appropriate concentration of the puromycin. For THP-1 cells, we normally choose 1–2μg/mL puromycin.

14. Waiting for the completion of cell screening, which takes approximately 7–10 d. troubleshooting 1.

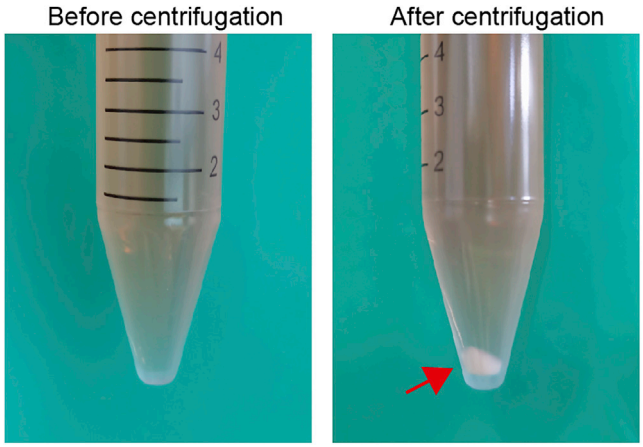

Figure 2. Virus concentration using ViraTrap™ Lentivirus Concentration Reagent
The red arrow indicated the white pellet containing the virus.

Note: If necessary, we can change fresh medium 2–3 times in this process.

15. When the cells proliferate normally, monoclonal cell line generation is performed by limited dilution method. troubleshooting 2.

Note: If the knockout effect is excellent (the target protein could not be detected) prior to monoclonal cell line generation, then the monoclonal cell line generation step can be omitted.

16. After 15–20 d, the monoclonal cells are validated by Western Blot (Figure 3). troubleshooting 3.

⚠ CRITICAL: In this part, the viability of THP-1 cells is critical for the successful completion of the experiment. To ensure the THP-1 cells viability, make sure that the cells are growth in an appropriate density (3×10^5–8×10^5/mL). Furthermore, high quality FBS is also essential.

Fixing THP-1 cells to the glass coverslip

Timing: 2 h

In this section, we describe the protocol for fixing THP-1 cells (growing in suspension) to the glass coverslip. This is the difficult part for performing confocal microscopy in suspension cells. This protocol is generally useful for other suspension cells.

17. Place the glass coverslip in a 12 well plate, then add 1 mL THP-1 cells (2×10^6 cells).

Note: Before centrifugation, ensure the glass coverslip is fully attached to the bottom of the 12 well plate.

18. Centrifuge the 12 well plate at $120 \times g$ for 20min.
19. Gently remove the 12 well plate from centrifuge and wash 3–5 times using PBS.

⚠ CRITICAL: Shaking is not recommended during washing.

20. Add 1 mL 4% paraformaldehyde, then fix cells at $120 \times g$ for 20min.

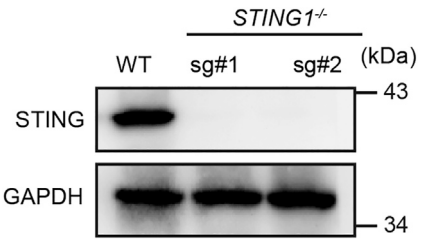

Figure 3. The expression of STING was detected in STING knockout THP-1 cell lines

The levels of STING in WT (wild type) and STING knockout THP-1 cells were detected by western blotting. Representative blots from three biologically independent replicates with similar results are shown.

21. Aspirate 4% paraformaldehyde carefully.

Note: After this step, cells fixed to the coverslips are relatively stable.

22. Add 1 mL 0.1% Triton X-100, and permeabilize cells at 120×*g* for 10 min.
23. Gently remove the 12 well plate from centrifuge and wash 3–5 times using PBS (Figure 4). trou-bleshooting 4.

⚠ CRITICAL: In this fixing progress, it is inevitable that a part of cells will fall off from the coverslip. Thus, we need to provide enough cells in the beginning (approximately 2–3× 10^6 cells/well). Certainly, too many cells are also undesirable, which will influence the following experiments including immunostaining and confocal microscopy.

Immunostaining and confocal microscopy

Timing: 10 h

This step describes the approach to probe STING with immunostaining. Subsequently, capture the images with confocal microscopy. You can analyze different proteins according to your study design, which can be completed by choosing different primary antibody and secondary antibody.

24. Add 1 mL 3% BSA (bovine serum albumin) to the 12 well plate, which contains the fixed THP-1 cells.
25. Incubate at room temperature (20°C–25°C) for 2 h.

Note: In this step, you can also choose to incubate overnight (12–16 h) at 4°C.

26. Wash 2 times using PBS.
27. Add 400μL primary antibody (anti-STING, Proteintech, 19851-1-AP, 1:100), and incubate at room temperature (20°C–25°C) for 2 h.
28. Wash 4–5 times using PBS.
29. Add 400 μL TRITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 1:100).
30. Incubate at room temperature (20°C–25°C) for 2 h, protected from light.

Note: In this part, it is indispensable to protect from light since this step.

31. Wash 4–5 times using PBS.
32. Mount the coverslips onto the glass slides using antifade mounting medium with DAPI.

Note: If necessary, antifade mounting medium without DAPI is also available, thus you can use the blue fluorescence to stain you own protein.

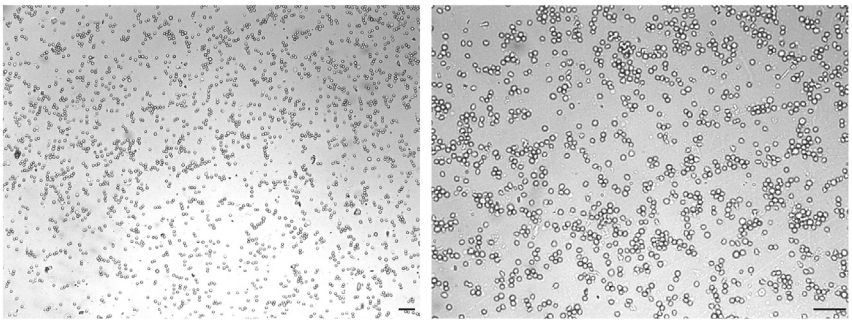

Figure 4. Example of fixed THP-1 cells to the glass coverslip

The phase-contrast images of THP-1 cells morphology, fixing to the glass coverslip, were shown. The magnification on the right is twice that on the left. Scale bar: 100 μm.

⚠ CRITICAL: The volume of the antifade mounting medium can severely affect the quality of your sample. Normally, 3–5 μL is the best choice.

33. Capture the images with the Leica TCS SP5 confocal laser scanning microscope (Figure 5). troubleshooting 5.

EXPECTED OUTCOMES

In the cell line screening experiments, we generated two STING knockout cell lines, in which STING could not be detected.

Thanks to this protocol, we could provide confocal image to study at subcellular resolution in the, THP-1, suspended cells. Endogenous STING in THP-1 cells, treated with or without cGAMP (cyclic GMP–AMP),[9,10] was immunostained by anti-STING antibody and TRITC-conjugated secondary antibody, followed by detection with confocal microscopy. The endogenous STING is uniformly distributed in the cytoplasm, and strongly translocated to the perinuclear region upon stimulation with cGAMP. This phenomenon is consistent with previous studies.[1,11]

LIMITATIONS

Sometimes, cell line screening may fail due to low cell viability. To solve this problem, it is truly important to keep cells at appropriate density (For THP-1 cells, the appropriate density is 2–8×10^5 cells/mL. For HEK293T cells, ensure that cells can achieve 100% confluence after seeding for 2–3 d.). At the same time, high quality serum would also be of great help.

In this protocol, a centrifuge capable of 12 well plate centrifugation is essential when using THP-1 cells for confocal microscopy. This type of centrifuge may not be available to some laboratories. Furthermore, perfect confocal microscopy experiments rely on high-quality primary antibodies, which are not always available.

TROUBLESHOOTING

Problem 1

In the process of THP-1 cell line screening, the cells appear adherent and are significantly altered in morphology (related to step 14).

Potential solution

This phenomenon suggest that THP-1 cells are differentiated., which is a common problem in THP-1 cell screening. To solve this problem, we should ensure the THP-1 cells density (the appropriate density is 2–8×10^5 cells/mL.) and supplement high quality serum during cell culture. Furthermore, we

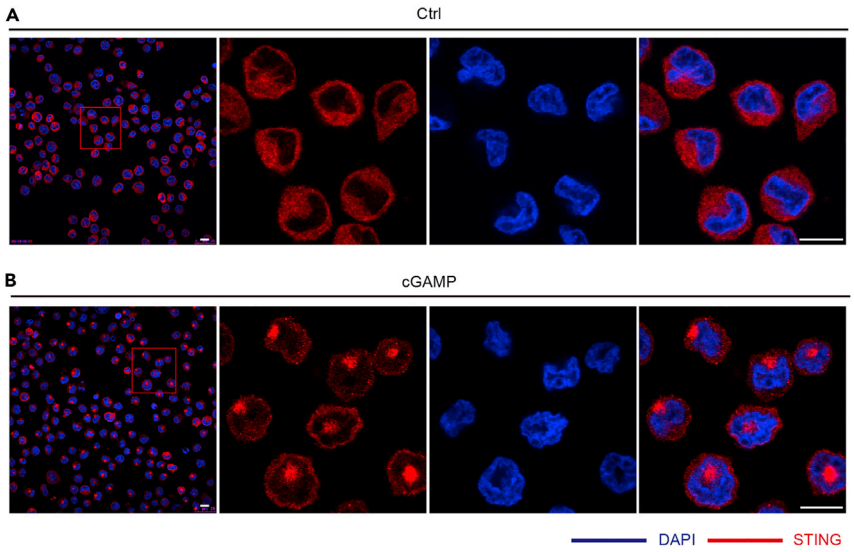

Figure 5. Confocal microscopy analysis of the endogenous STING in THP-1 cells

(A and B) THP-1 cells were treated without (A) or with (B) cGAMP, then endogenous STING was immunostained by anti-STING antibody and TRITC-conjugated secondary antibody, followed by detection with confocal microscopy. Scale bars: 10 μm. Representative data from one experiment are shown (n = 3 biologically independent experiments).

could transfer the suspension cells to a new well plate and discard the adherent cells, when the number of cells growing in suspension is sufficient.

Problem 2

At the end of cell line screening, all of the cells are dead (related to step 15).

Potential solution

First of all, we should ensure the THP-1 cells are successfully infected with virus. For this purpose, we could choose the retroviral vector with fluorescent proteins (GFP or RFP). Then, ensure that the concentration of puromycin is not too high. The appropriate concentrations of puromycin should completely kill wild-type THP-1 cells in 3–7 days, and normally is 1–2 μg/mL for THP-1 cells.

Problem 3

When the screening is completed, the target protein is not reduced at all (related to step 16).

Potential solution

The most likely of this problem is that inappropriate sgRNAs are selected.[7,8] You can try to use new sgRNAs that are frequently used by other researchers. Besides, you should make sure that the concentration of puromycin is not too low. Furthermore, you have to consider that antibodies sometimes recognize non- target proteins, thus giving us incorrect interpretation.

Problem 4

When the fixing of cells to the glass coverslip is complete, the density of cells is not appropriate (related to step 23).

Potential solution

To solve this problem, we should adjust the number of cells at the beginning. Furthermore, the number of cells on the glass coverslip in step 19 will vary considerably. Thus, we should make sure that this step is repeatable.

Problem 5

In the confocal microscopy experiment, no or weak fluorescent signal can be detected (related to step 33).

Potential solution

First of all, make sure your primary antibody is capable of being used for immunostaining and is highly sensitive. Then, antifade mounting medium should be of good quality, which can prevent the quenching of fluorescence.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xinqi Liu (liu2008@nankai.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Methodology, W.J., X.L.; Validation, W.J., L.Z.; Formal analysis, W.J., X.L.; Investigation, W.J., L.Z.; Data curation, W.J., X.L.; Writing - original draft, W.J., X.L., L.Z.; Visualization, W.J., L.Z.; Supervision, X.L.; Project administration, W.J., X.L.; Funding acquisition, X.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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