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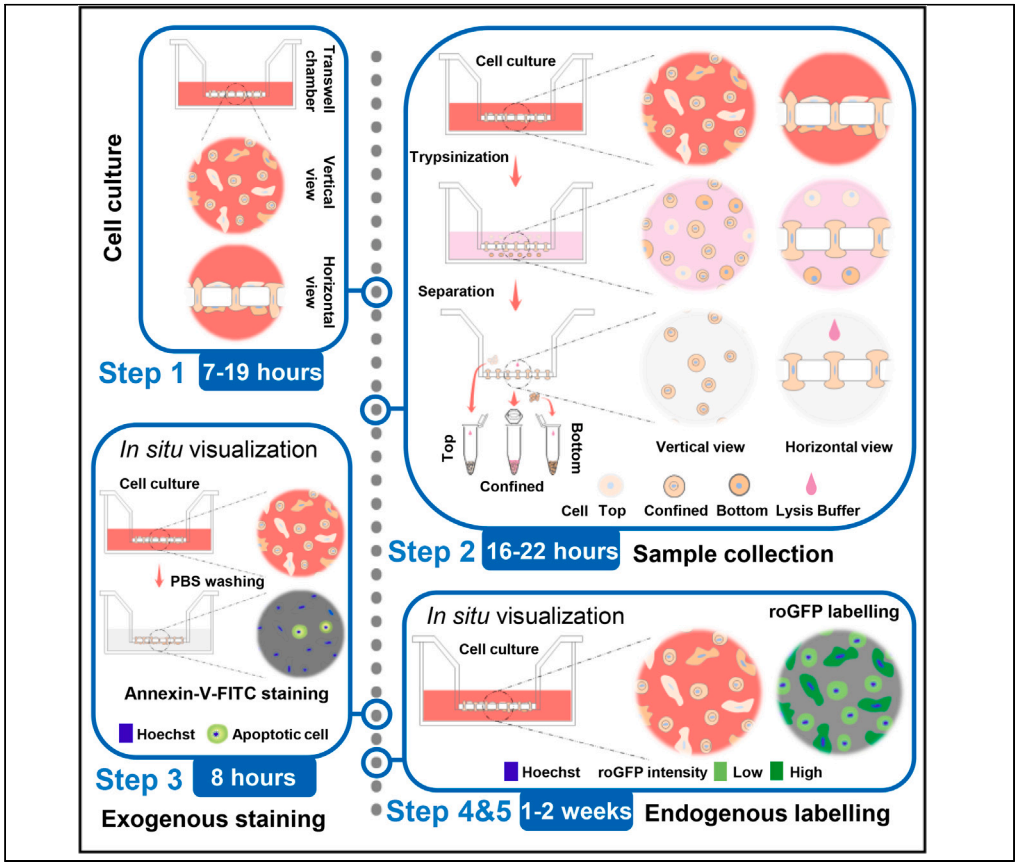
Protocol

Protocol for *in situ* visualization of mitochondrial ROS and apoptosis in spatially confined cells and sample preparation for biochemical analysis



Locomotion through spatially confining spaces is an important *in vivo* migration mode. Here, we present a protocol for *in situ* visualization of mitochondrial reactive oxygen species and apoptosis in cancer cells during confined migration. We then detail sample preparation of confined cells for transcriptome and immunoblotting analysis by using transwell chambers. This approach allows *in situ* evaluation of a variety of cellular functions during confined migration and preparation of the samples of confined cells for further biochemical analysis.

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

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Highlights

Steps described to study confined migration using transwell chamber

Sample preparation of confined cells for molecular and biochemical analyses

Procedures for evaluating a variety of cellular behaviors of confined cells

Qi et al., STAR Protocols 5, 102802
March 15, 2024
https://doi.org/10.1016/j.xpro.2023.102802

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Protocol for *in situ* visualization of mitochondrial ROS and apoptosis in spatially confined cells and sample preparation for biochemical analysis

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https://doi.org/10.1016/j.xpro.2023.102802

SUMMARY

Locomotion through spatially confining spaces is an important *in vivo* migration mode. Here, we present a protocol for *in situ* visualization of mitochondrial reactive oxygen species and apoptosis in cancer cells during confined migration. We then detail sample preparation of confined cells for transcriptome and immunoblotting analysis by using transwell chambers. This approach allows *in situ* evaluation of a variety of cellular functions during confined migration and preparation of the samples of confined cells for further biochemical analysis.

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

BEFORE YOU BEGIN

Tumor metastasis leads to the death of more than 90% of tumor patients.[2] The tumor microenvironment provides many paths for confined cell migration.[3] Understanding the mechanism underlying cancer cell migration in confining spaces will accelerate the development of therapeutic intervention to prevent metastasis. Although PDMS (polydimethylsiloxane) system has been adopted in many studies to observe the confined migration of cells,[4,5] the PDMS system is not suitable for the preparation of the cells for further molecular and biochemical analyses and the *in situ* cell staining with exogenous materials.

This protocol describes the steps of using Transwell assays, where we can collect samples of and perform the in *situ* visualization of confined and unconfined cells. After trypsin digestion, divide the cells in Transwell assay into three parts, named Confined (with confinement), Top side and Bottom side (without confinement) for further biochemical and molecular studies. By exogenously staining with Annexin-V-FITC, we can detect cell apoptosis. At the same time, we can detect ROS changes by detecting the intensity changes of fluorescence emitted by endogenously expressed roGFP.

Before beginning, we need to prepare the following essential elements.

Preparation of target plasmid

Timing: 2–3 weeks

Amplify EGFP by PCR, and generate combined mutation of C48S, Q80R, S147C and Q204C (C, cysteine, Q, glutamine, R, arginine, S, serine) in EGFP, transform it into roGFP fluorescent protein sensitive to ROS according to the literature.[6] Then, clone roGFP into PCDH-CMV-MCS-EF1-Hygro vector to form the PCDH-CMV-roGFP-EF1-Hygro plasmid expressing roGFP in the cytoplasm. Then clone the signal peptide sequence of mitochondrial protein COX4 into the 5 ′end of roGFP to form the PCDH-CMV-Mito-roGFP-EF1-Hygro plasmid expressing roGFP in mitochondria.

Preparation of cells

Timing: 1 week

Purchase A549, HEK293T and SK-Hep1 cells from the Type Culture Collection of the Chinese Academy of Sciences and cultured at 37°C, 5% CO2. Maintain A549 cells in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Maintain HEK293T and SK-Hep1 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Preparation of transwell

Timing: 1 week

There are many different sizes of transwell chambers, and different experimental purposes use different sizes of transwell chambers. Typically, cell migration experiments use the transwell chamber parameters with diameters of 6.5 mm (24-well plate) and 24 mm (6-well plate) and pore sizes of 8 μm. For the observation of cell states *in situ*, such as cell apoptosis and intracellular ROS levels, we choose to use transwell chambers with a diameter of 6.5 mm (24-well plate) and a pore size of 8 μm. For sample collection, such as RNA sample, we choose to use transwell chambers with a diameter of 24 mm (6-well plate) and a pore size of 8 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM	Sigma	D5796
RPMI-1640	Gibco	C11875500BT
Fetal bovine serum	ExCell Bio	FSD500
Penicillin-Streptomycin solution	Gibco	15140163
LipoFiter liposomal transfection reagent	Hanbio	HB-LF-1000
Polybrene	Sigma	TR-1003-G
PBS	Meilunbio	MA0008
Trypsin	Gibco	25200072
Annexin-V-FITC Apoptosis Detection Kit	BD Biosciences	556547
tBH	Sigma	458139
DTT	Sigma	D0632
Hoechst	Invitrogen	H3570
Experimental models: Cell lines		
Human: A549 cells	Cell library of the Chinese Academy of Sciences	SCSP-503
Human: HEK293T cells	Cell library of the Chinese Academy of Sciences	SCSP-502
Human: SK-Hep1 cells	Cell library of the Chinese Academy of Sciences	TCHu109
Experimental models		
Transwell, 8 μm, 24 well	Corning	3422

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Transwell, 8 μm, 6 well	Corning	3428
Recombinant DNA		
PCDH-CMV-roGFP-EF1-Hygro	This paper	N/A
PCDH-CMV-Mito-roGFP-EF1-Hygro	This paper	N/A
Software and algorithms		
Operetta high-content imager and Harmony software	PerkinElmer	N/A
GraphPad Prism	GraphPad	http://www.graphpad.com/support
Other		
1.5 mL centrifuge tube	NEST	615001
50 mL centrifuge tube	NEST	601002
Millex-HV	Millipore	SLHV033RB

MATERIALS AND EQUIPMENT

Cell Culture medium	
Reagent	Final concentration
FBS	10%
Penicillin-Streptomycin Liquid	1%
RPMI-1640/DMEM	N/A
Total	N/A

Note: Store this medium at 4°C for 1 month. Maintain A549 cells in RPMI-1640 medium, maintain HEK293T and SK-Hep1 cells in Dulbecco's modified Eagle's medium (DMEM).

STEP-BY-STEP METHOD DETAILS

Cell culture in transwell chamber

Timing: 7–19 h

1. Perform Transwell assay follows the normal procedure.
   a. Digest A549 cells with 0.05% trypsin and wash twice with cold PBS.
   b. Resuspend cells with cold PBS and count them using an automatic cell counter (Invitrogen Countess).
   c. For 24-well plate Transwell: Resuspend 5×10^4 of the counted cells in 300 μL RPMI-1640 and load into the transwell chamber inserted in the well added with 500 μL of RPMI-1640-10% FBS. For 6-well plate Transwell: Resuspend 1×10^6 of the counted cells in 2 mL RPMI-1640 and load into the transwell chamber inserted in the well added with 2 mL of RPMI-1640-10% FBS.

Note: The membrane pore density of the transwell chamber we used is 1×10^5 per cm^2. For a transwell chamber with a diameter of 6.5 mm, the area of the membrane is 0.33 cm^2 and there are about 3.3×10^4 pores, for which a suitable cell density is extremely important (e.g. 5×10^4). Too many cells (e.g. 1×10^5) will crowd the cells on the membrane, making it difficult to clearly distinguish between confined and unconfined cells. Too few cells (e.g. 2×10^4) would make it difficult to find a field of view with a suitable number of cells for analysis.

⚠ CRITICAL: In general, the total volume of culture medium required for a transwell chamber with a diameter of 6.5 mm is 700 μL (inside: 100 μL + outside: 600 μL). In order to maximize the number of cells confined in the pores, it is necessary to change the distribution ratio of culture medium inside and outside. Add a little more suspension on the inside and a little less on the outside (e.g. inside: 300 μL + outside: 500 μL) to create a drop between the

inside and outside levels, so that the medium flow will draw most of the cells to the pores at the beginning and ensure that the majority of cells are located in the pores during subsequent migration induced by chemotaxis, *in situ* observation and cell sample collection.

d. Culture the Transwell samples in 37°C, 5% CO_2 incubator for 6–18 h.

Note: The culture time needs to be adjusted according to the purpose of the experiment.

Sample preparation of confined and unconfined cells

Timing: 16–22 h

2. Perform Transwell assay with A549 cells by using 6-well plate transwell chambers and obtain confined and unconfined samples.
   a. Sample preparation steps follow the cell culture in transwell chamber (Step 1: a, b, c) procedure.
   b. After culturing in 37°C, 5% CO_2 incubator for 12–18 h, gently remove the medium in the transwell chambers and culture wells with pipette and wash the transwell chambers with cold PBS twice.

Note: To avoid too few cells in either fraction of the cells (top side, confined and bottom side), the culture time should be optimized.

   c. Load 2 mL 0.05% trypsin into transwell chambers to digest cells for 10 min in 37°C, 5% CO_2 incubator. Gently pat the transwell chambers to help cells detach from the membrane after 5 min of digestion.

Note: The digestion of cells is a key step in the separation of cells on the top side, in the pores and on the bottom side of the Transwell membrane, for which the control of digestion time is extremely important. Too short digestion time prevents the detachment of cells on the membrane (on the top and bottom side), resulting in the confined cell samples being contaminated by unconfined cells. If the digestion time is too long, the part of the confined cells that is not very tightly stuck will fall off, which will lead to the loss of the confined cells and contaminate the samples of unconfined cells.

⚠ CRITICAL: A reasonable digestion time can be determined by observing the cell digestion status under a microscope.

   d. Stop digestion by adding 2 mL of complete medium.
      i. Move the cells on the top side and bottom side (*without confinement*) of the transwell chambers into 1.5 mL centrifuge tubes respectively and wash with cold PBS.
      ii. Lyse the cell samples by different lysis buffer, such as TRIzol reagent for RNA extraction, RIPA buffer for protein extraction.
   e. Wash the transwell chambers with cold PBS.
      i. Lyse the cells confined (*with confinement*) in the pores of the Transwell membrane by adding different lysis buffer, such as TRIzol reagent (RNA extraction), RIPA buffer (protein extraction) into the transwell chambers directly.
      ii. Then insert the transwell chambers into 50 mL centrifuge tubes and centrifuge for 2 min with 4000 g.
   f. Perform RNA and protein quantification on three parts of the cells (Table 1), top side and bottom side (without confinement), confined (with confinement). Alternatively, choose to count three parts of the cells for subsequent experiments (Table 2).

Table 1. Quantification of the RNA and protein samples extracted from three parts of the cells in transwell chamber

			RNA			Protein		
Cell line			Concentration (ng/μL)	Volume (μL)	Mass (μg)	Concentration (ng/μL)	Volume (μL)	Mass (μg)
A549	Sample 1	Top	122.80	25.72	3.16	704.00	50.00	35.20
		Confined	53.55	22.50	1.20	137.00	50.00	6.85
		Bottom	48.76	25.26	1.23	313.00	50.00	15.65
	Sample 2	Top	49.92	25.74	1.28	643.00	50.00	32.15
		Confined	41.19	24.59	1.01	216.00	50.00	10.80
		Bottom	61.32	22.03	1.35	423.00	50.00	21.15
	Sample 3	Top	62.21	22.67	1.41	669.00	50.00	33.45
		Confined	56.94	21.67	1.23	194.00	50.00	9.70
		Bottom	23.69	18.44	0.44	527.00	50.00	26.35
SK-Hep1	Sample 1	Top	107.70	20.00	2.15	750.00	50.00	37.50
		Confined	54.50	20.00	1.09	184.00	50.00	9.20
		Bottom	175.20	20.00	3.50	403.00	50.00	20.15
	Sample 2	Top	161.60	20.00	3.23	612.00	50.00	30.60
		Confined	80.80	20.00	1.62	336.00	50.00	16.80
		Bottom	85.30	20.00	1.71	454.00	50.00	22.70
	Sample 3	Top	259.10	20.00	5.18	1285.00	50.00	64.25
		Confined	111.80	20.00	2.24	141.00	50.00	7.05
		Bottom	79.10	20.00	1.58	207.00	50.00	10.35

6-well plate Transwell assays are performed with A549 and SK-Hep1 cells (1×10^6 per transwell chamber). After 18 h of culture, cells are trypsinized and separated into 3 groups, named confined (with confinement), top side and bottom side (without confinement). Sample 1, Sample 2, or Sample 3 represents a single well of 6-well plate, respectively. RNA and protein were extracted from three groups of the cells in the single well (transwell chamber). RNAs are extracted by TRIzol for subsequent RNA sequencing, and protein samples extracted by RIPA buffer for immunoblotting analyses. The concentration, volume and mass of the RNAs and proteins were shown in the table.

Note: In order to better collect the samples of confined cells, the lysis buffer treatment time can be prolonged appropriately, and the membrane of the transwell chamber can be scraped with a cell scraper before inserted in 50 mL centrifuge tubes for centrifugation.

In situ observation of confined and unconfined cells apoptosis

Exogenous staining - Cell apoptosis detection by Annexin-V-FITC staining

Timing: 8 h

3. Perform Transwell assay with A549 cells in 24-well plate transwell chambers and detect confined and unconfined cells apoptosis with Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences).
   a. Sample preparation steps follow the cell culture in transwell chamber (Step 1: a, b, c) procedure.
   b. After culturing in 37°C, 5% CO_2 incubator for 6 h, gently remove the medium in the transwell chambers and plates with pipette.
      i. Wash the transwell chambers with cold PBS twice.
   c. Stain cells in the transwell chambers with Annexin-V-FITC (An-V-FITC).

Note: The details of Annexin-V-FITC staining procedures can be found from the protocols provided by the manufacturer.

   d. After staining, image the Transwell samples with Operetta high-content imager.
   e. Calculate the fluorescence intensities of Annexin-V-FITC and analyze with Harmony software. Count the percentage of cell apoptosis according to the fluorescence intensities of Annexin-V-FITC (Figure 1).

Table 2. The number of three parts of the cells in transwell chamber

Cell line			Cell number
A549	Sample 1	Top	3.87×10^5
		Confined	1.42×10^5
		Bottom	1.76×10^5
	Sample 2	Top	2.83×10^5
		Confined	7.75×10^4
		Bottom	2.67×10^5
	Sample 3	Top	3.57×10^5
		Confined	1.06×10^5
		Bottom	1.44×10^5
SK-Hep1	Sample 1	Top	4.10×10^5
		Confined	1.01×10^5
		Bottom	3.01×10^5
	Sample 2	Top	4.47×10^5
		Confined	7.46×10^4
		Bottom	2.72×10^5
	Sample 3	Top	3.53×10^5
		Confined	1.18×10^5
		Bottom	1.78×10^5

6-well plate Transwell assays are performed with A549 and SK-Hep1 cells (1×10^6 per transwell chamber). After 18 h of culture, cells are trypsinized and separated into 3 groups, named confined (with confinement), top and bottom (without confinement). Sample 1, Sample 2, or Sample 3 represents a single well of 6-well plate, respectively. The number of cells in each group was shown in the table.

In situ observation of confined and unconfined cells ROS

Endogenous labeling – Mito-roGFP intensities reflects mitochondrial ROS levels

Timing: 1–2 weeks

Construction of roGFP-expressing cell lines.

4. Produce lentivirus particles and infect A549 cells for stable expression of roGFP in mitochondria.
   a. Lentivirus packaging and production.
      i. Seed HEK293T cells in 6 cm dishes (3 mL, DMEM, 10% FBS) with a confluence of 50%–70% and culture in 37°C, 5% CO_2 incubator.
      ii. Change the medium of HEK293T cells with 2.5 mL fresh complete medium before transfection.
      iii. Co-transfect lentiviral vector (PCDH-CMV-Mito-roGFP-EF1-Hygro) with packing vector (psPAX2) and envelope vector (pMD2.G) into HEK293T cells using LipoFiter Liposomal Transfection Reagent (The mass ratio of 3 vectors is 4:3:3).

Note: The details of transfection procedures can be found from the protocols provided by commercial Liposomal Transfection Reagent.

      iv. After 6 h of culture, replace the medium with 3 mL fresh complete medium to remove the liposomes and residual plasmids.
      v. 48 h after medium replacement, collect the culture medium (lentivirus supernatant) and supplement with the same volume of fresh complete medium, which will be collected in another 24 h.
      vi. Filter the lentivirus supernatant with 0.45 μm Millipore filter membrane, and collect the lentivirus in a 15 mL centrifugal tube.

Note: Lentivirus supernatant can be temporarily stored at 4°C, but for long term storage it should be stored at −80°C.

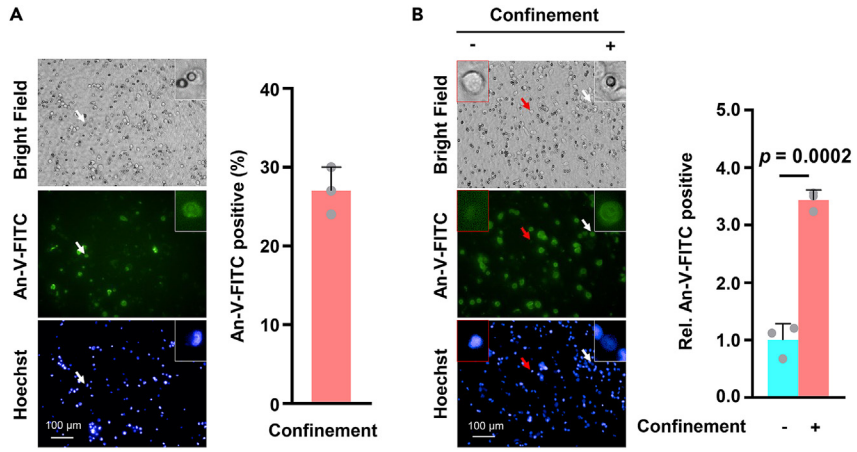

Figure 1. Cell apoptosis detection by Annexin-V-FITC staining

(A) 24-well plate Transwell assays are performed with A549 cells (5×10^4 per chamber). After 6 h of culture, the cells are stained with Annexin-V-FITC and imaged *in situ*. Representative images of the apoptotic confined cells are presented (Left panel). The percentages of apoptosis of confined cells are shown (Right panel).

(B) 24-well plate Transwell assays are performed with A549 cells (5×10^4 per chamber). After 6 h of culture, the cells are stained with Annexin-V-FITC and imaged *in situ*. Representative images of the apoptotic confined cells (white arrows) or unconfined cells (red arrows) were presented (Left panel). The percentages of apoptosis of confined or unconfined cells were normalized to the percentage of apoptosis in unconfined cells and were shown (Right panel). Data represent the mean ± S.D. of three independent experiments. An-V-FITC: Annexin-V-FITC. Figure reprinted with permission from (Cai et al., 2023).[1]

b. Lentivirus infection of A549 cells.
   i. Dilute the lentivirus supernatant with fresh complete medium (vol/vol = 1:1) and add 3 mL of the dilution into 6 cm dishes seeded with A549 cells with a confluence of 30%–50%.

Optional: The efficiency of infection can be promoted by adding 8 μg/mL polybrene.

   ii. After 12 h of infection, replace the medium with 3 mL of fresh complete medium and culture the cells for another 24 h.
   iii. Add 100 μg/mL of hygromycin into the medium and culture the cells for another 48–72 h to screen the resistant cells.

Note: Remove the medium with hygromycin when the wild-type cells were all killed. The virus concentration and infection time are adjustable.

c. Detection of Mito-roGFP expression in A549 cells.
   i. After hygromycin screening, reseed A549 cells (5×10^5) expressing Mito-roGFP in 6 cm dishes respectively and culture for 24 h for good adhesion.
   ii. Take the images with an inverted fluorescence microscope (Figure 2).

d. Detection of the sensitivity of Mito-roGFP to ROS.
   i. Seed A549 cells expressing Mito-roGFP (5×10^3 per well) in 96-well plates respectively and culture for 24 h for good adhesion.
   ii. Treat the adherent cells with or without 1 mM tBH (a lipid-soluble organic peroxide) or 1 mM DTT (a reducing agent). Image the cells before and 15 min and 45 min after treatment.
   iii. Take the images with Operetta high-content imager and analyze by Harmony software (Figure 3).

In situ observation Mito-roGFP intensities with Operetta high-content imager

Timing: 8 h

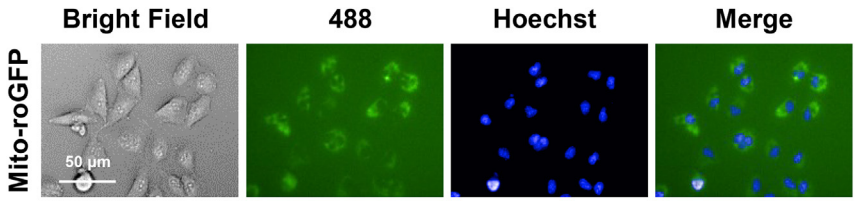

Figure 2. Mito-roGFP expression in A549 cells

A549 cells (5×10^5) expressing Mito-roGFP were seeded in 6 cm dishes. The images show the expression of Mito-roGFP in A549 cells.

5. Perform Transwell assay with A549 cells expressing Mito-roGFP in 24-well plate transwell chambers and reflect mitochondrial ROS levels in confined and unconfined cells with fluorescence intensity.
   a. Sample preparation steps follow the cell culture in transwell chamber (Step 1: a, b, c) procedure.
   b. After culturing in 37°C, 5% CO_2 incubator for 6 h, directly move into and image the Transwell samples with Operetta high-content imager.

   Note: In transwell chambers, the cells will adhere to the membrane and move into the pores (***confined cells***, forming a concentric circle structure, the diameter of the outer ring is smaller than the diameter of the cell when it is spherical) in 6 hours. If the culture time is too long (e.g. 12 hours), many cells will migrate through the pores to the bottom side of the membrane, resulting in fewer confined cells. On the other hand, the cells near the pores will form connections with the cells in the pores, which will make it difficult to accurately identify confined cells.

   c. Calculate the fluorescence intensities of Mito-roGFP and analyze with Harmony software (Figure 4).

EXPECTED OUTCOMES

This method can collect samples of confined and unconfined cells. By using 6-well plate transwell chambers, we successfully separated the confined and unconfined cells (top and bottom side) and collected large amount of RNA samples, protein samples (Table 1), and also counted the confined and unconfined cells (Table 2) for further biochemical analysis. This method can also be used to observe the confined cells *in situ* clearly. By Annexin-V-FITC staining, cells could be imaged and quantified by fluorescence intensity to determine cell apoptosis (Figure 1). Mito-roGFP is successfully expressed in A549 cells (Figure 2) and is sensitive to ROS changes (Figure 3). By the expression of roGFP, cells could be imaged and quantified by fluorescence intensity to determine the level of intracellular ROS (Figure 4).

LIMITATIONS

From the perspective of *in situ* observation, compared with the PDMS models, in which cells can be observed in the direction perpendicular to the direction of cell movement, used in previous studies, in the Transwell model, cells can only be observed in the direction parallel to the direction of cell movement, so the dynamic shape changes of cells, nuclei, and more microscopic structures cannot be clearly observed.

In the 6-well plate Transwell experiment, we could control the digestion time of trypsin and separate the cells on the top side, in the pores and on the bottom side of the transwell chambers. But it is still difficult to ensure that there is no cross contamination among the three groups of cells. Because trypsin digestion, on the one hand, part of the confined cells will inevitably fall off, on the other hand, the top side and bottom side part of the cells may not be able to completely detached from the membrane, so there will be a little cross-contamination between each part. As a result,

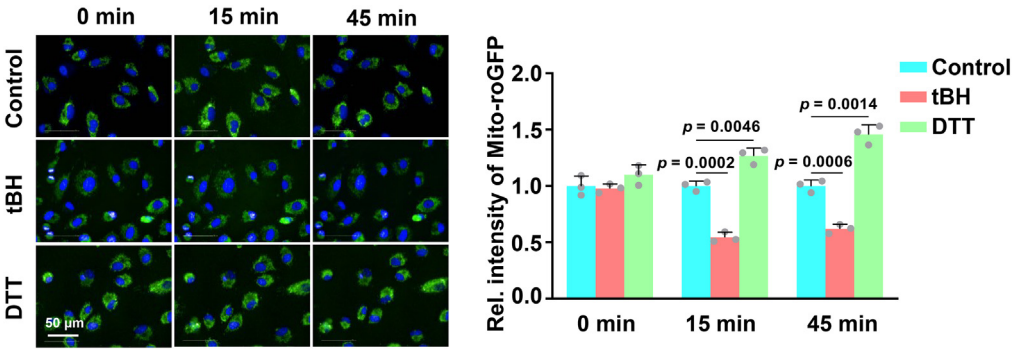

Figure 3. The sensitivity of Mito-roGFP to ROS in A549 cells

Mito-roGFP-expressing A549 cells were seeded in 96-well plates and treated with or without 1 mM tBH or 1 mM DTT. The cells were imaged before treatment 0 min, 15 min and 45 min after treatment respectively. Representative images of Mito-roGFP were shown (Left panel). Relative Mito-roGFP fluorescence intensities of each time point were normalized to those of the untreated Mito-roGFP-expressing A549 cells (Right panel). Data represent the mean ± s.d. of three independent experiments. Figure reprinted with permission from (Cai et al., 2023).[1]

the experimental data we collected is difficult to perfectly reflect the actual situation, such as the error between the results of RNA sequencing and the actual level of RNA changes.

In addition to the purity of samples, there is also a problem in the quantity of samples obtained. Since nucleic acid samples can be amplified by PCR, there is no need to worry about its quantity. However, in terms of protein, although we can collect enough protein samples for immunoblotting analyses, it is difficult to do mass spectrometry and other experiments that require a large amount of protein. But theoretically, it can be solved by using enough or larger transwell chambers for sample collection.

Comparison of PDMS and Transwell

	PDMS	Transwell
Direction of observation *vs.* Direction of cell migration	Perpendicular	Parallel
Can confined and unconfined cell samples be collected?	No	Yes
Can cells be easily stained with exogenous materials?	No	Yes
Can dynamic structural changes of confined cells be clearly observed?	Yes	No

TROUBLESHOOTING

Problem 1

The amount of sample obtained by trypsin digestion of cells in 6-well transwell chamber does not meet the demand (step 2).

Potential solution

Samples from multiple 6-well plates transwell chambers can be collected together, or larger transwell chambers, such as 10 cm transwell chambers, can be used for sample collection.

Problem 2

The three parts of the sample, (Confined (with confinement), Top side and Bottom side (without confinement)) cannot be completely separated, and there may be a little mutual contamination (step 2).

Potential solution

Pay attention to the time of cell digestion, the digestion of cells is a key step in the separation of cells, and observe the cell state multiple times under the microscope, promptly and gently separate the cells. After observing that there are no cells in the top and bottom side through the microscope, collect the sample from the confined space (see notes of steps 2).

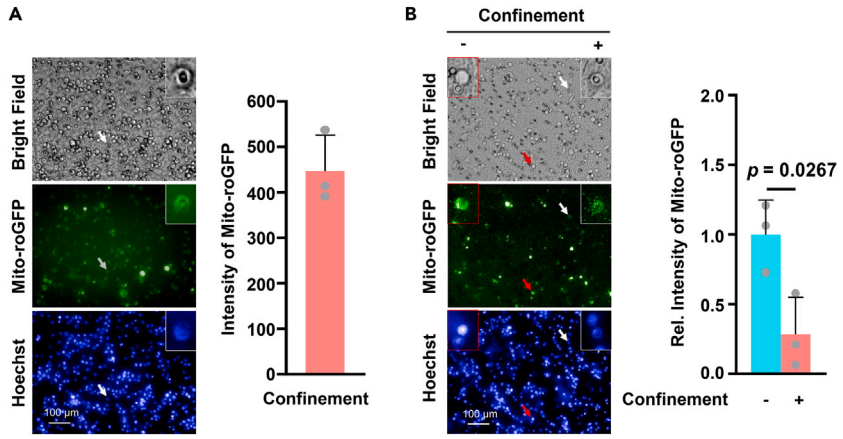

Figure 4. Mito-roGFP intensities reflect mitochondrial ROS levels of cells

(A) 24-well plate Transwell assays are performed with A549 cells (5×10^4 per chamber). After 6 h of culture, cells were imaged. Representative images show the fluorescence of Mito-roGFP of confined A549 cells and are presented (Left panel). roGFP fluorescence intensities are calculated and shown (Right panel).

(B) 24-well plate Transwell assays are performed with A549 cells (5×10^4 per chamber). After 6 h of culture, cells were photographed *in situ*. Representative images of Mito-roGFP in cells with confinement (white arrows) or without confinement (red arrows) were presented (Left panel). Mito-roGFP fluorescence intensities of cells with or without confinement were normalized to those of the Mito-roGFP-expressing cells without confinement and were shown (Right panel). Data represent the mean ± s.d. of three independent experiments. Figure reprinted with permission from (Cai et al., 2023).[1]

Problem 3

The fluorescence intensity of roGFP is insufficient, resulting in difficulties in detection (step 4).

Potential solution

This problem can be addressed in several ways, including increasing the virus titer, increasing the amount of virus and infection time during cell infection, and multiple rounds of superinfection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Weiwei Yang (wyang@sibcb.ac.cn).

Technical contact

If you have technical problems about this protocol, please contact Yijun Qi (qiyijun20@mails.ucas.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (92253305, 92053203, and 32025013) to W.Y., the National Key Research and Development Program of China (2022YFA0806200 and 2019YFA0802000) to W.Y., CAS Project for Young Scientists in Basic Research (YSBR-014) to W.Y., Shanghai Municipal Science and Technology Major Project, and the Innovative Research Team of High-level Local Universities in Shanghai (SHSMU-ZLCX20212302) to W.Y.

AUTHOR CONTRIBUTIONS

Y.Q. and G.C. performed the experiments, analyzed the data, and wrote the manuscript together. W.Y. conceived the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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