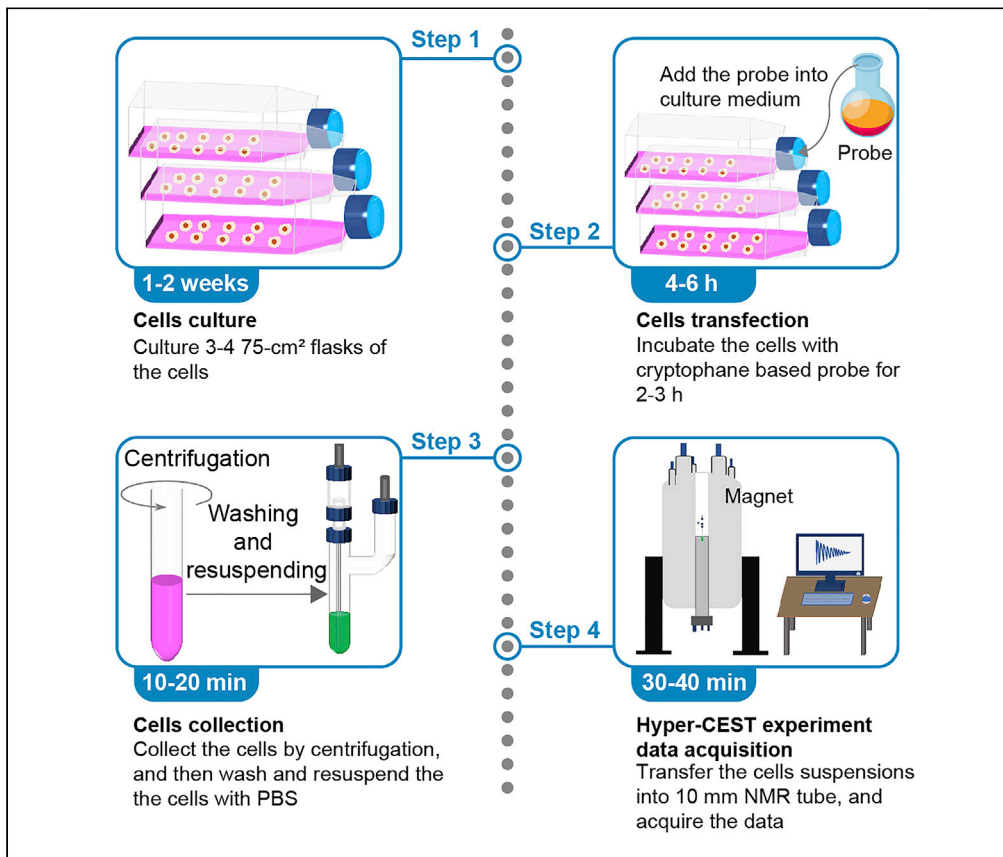


## Protocol

# Protocol for detecting substrates in living cells by targeted molecular probes through hyperpolarized $^{129}\text{Xe}$ MRI



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### Highlights

Protocol for detecting substrates in living cells with hyperpolarized  $^{129}\text{Xe}$  MRI

Procedure for culturing and collecting cells for hyperpolarized  $^{129}\text{Xe}$  MRI

Optimized for a cryptophane-based probe sensitive to biothiols

Due to limited detection sensitivity and contrast limitation, imaging substrates with  $^{129}\text{Xe}$  MRI in living cells is still a challenge. Here, we present an effective protocol to detect and image substrates in human lung cancer cells A549 with hyperpolarized  $^{129}\text{Xe}$  MRI. This protocol was optimized for a cryptophane-based probe sensitive to biothiols and can be expanded to other Xe-based probes to detect potential biomarkers in other mammalian cells.

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

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## Protocol

Protocol for detecting substrates in living cells by targeted molecular probes through hyperpolarized  $^{129}\text{Xe}$  MRI

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## SUMMARY

Due to limited detection sensitivity and contrast limitation, imaging substrates with  $^{129}\text{Xe}$  MRI in living cells is still a challenge. Here, we present an effective protocol to detect and image substrates in human lung cancer cells A549 with hyperpolarized  $^{129}\text{Xe}$  MRI. This protocol was optimized for a cryptophane-based probe sensitive to biothiols and can be expanded to other Xe-based probes to detect potential biomarkers in other mammalian cells.

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

## BEFORE YOU BEGIN

Hyperpolarized  $^{129}\text{Xe}$  nuclear magnetic resonance (NMR) shows great advantage for the noninvasive diagnosis of respiratory system diseases (Doganay et al., 2019; Li et al., 2021; Wang et al., 2018). Through hyperpolarized  $^{129}\text{Xe}$  magnetic resonance imaging (MRI), one can simultaneously visualize both the structure and function of lung in a single scan (Li et al., 2016a, 2016b, 2018, 2021; Qing et al., 2014; Xiao et al., 2018; Xie et al., 2019). Once the hyperpolarized  $^{129}\text{Xe}$  combined with molecular cages, such as cryptophane-A (Spence et al., 2001), cucurbit[6]uril (Kunth et al., 2015), protein (Wang et al., 2016), gas vesicles (Shapiro et al., 2014), metal-organic capsules (Du et al., 2020), and metal-organic frameworks (Yang et al., 2021; Zeng et al., 2020), it does show great promise for molecular imaging and trace molecules detection in living cells and *in vivo*.

In the past ten years, a large numbers of hyperpolarized  $^{129}\text{Xe}$  probes have been developed for molecular detection (Rose et al., 2014; Witte et al., 2015; Yang et al., 2017; Zeng et al., 2017, 2021; Zhang et al., 2018), but the step-by-step procedure for the detection of molecules in living cells by hyperpolarized  $^{129}\text{Xe}$  technique has not been reported. Herein, we present a detailed protocol of how hyperpolarized  $^{129}\text{Xe}$  NMR is performed by using cryptophane-based probes to detect biothiols in living cells with as much details as possible. Although the protocol describes the specific steps for using cryptophane-based probes to image the biothiols in living cells by hyperpolarized



$^{129}\text{Xe}$  MRI, it can be adjusted to other molecular cages based Xe probes. Before one begins, the following preparations need to be carried out.

### Prepare phosphate buffer solution for cells washing

⌚ Timing: 2–3 h

1. Prepare phosphate buffer solution (PBS).
  - a. Dissolve  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (1.15 g),  $\text{KH}_2\text{PO}_4$  (0.20 g), KCl (0.20 g), and NaCl (8.00 g) in 1000 mL ultrapure water, and then adjust the pH value of the solution to 7.4 with dilute HCl and NaOH aqueous solution.
  - b. Transfer the PBS to a 2 L Erlenmeyer flask, and autoclave for 1 h. Let it cool to room temperature. After that, store the sterile PBS at 4°C.

⚠ **CRITICAL:** Store the buffer at 4°C, and the buffer should be filtered with a 0.22  $\mu\text{m}$  sterilized filter before being used to wash cells.

### Prepare trypsin solution for cells trypsinization

⌚ Timing: 1 h

2. Prepare trypsin solution.
  - a. Dissolve the trypsin (0.25 g) and ethylene diamine tetraacetic acid (EDTA) (0.1 g) in 500 mL sterile PBS (without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ).
  - b. Filter the solution with 0.22  $\mu\text{m}$  sterilized filter after the solid completely dissolved. Store the solution in 2 mL aliquots under  $-20^\circ\text{C}$ .

⚠ **CRITICAL:** The trypsin solution should be filtered with a 0.22  $\mu\text{m}$  sterilized filter before storing at  $-20^\circ\text{C}$ .

### Prepare culture medium for cells growth

⌚ Timing: 1 h

3. Prepare culture medium.
  - a. Add 1 mL of penicillin–streptomycin (penicillin, 10000 U/mL; streptomycin, 10 mg/mL) and 10 mL of fetal bovine serum (FBS) into 89 mL of Ham's Kaighn's Modification (F12K) culture medium. The volume fraction of the penicillin–streptomycin is 1%, and the FBS is 10%.
  - b. Filter the mix culture medium with 0.22  $\mu\text{m}$  sterilized filter and store at 4°C.

⚠ **CRITICAL:** The culture medium should be filtered with a 0.22  $\mu\text{m}$  sterilized filter before storing at 4°C.

### Prepare probe stock solution for cells experiment

⌚ Timing:  $\sim 0.5$  h

4. Prepare probe stock solution.
  - a. Dissolve 4.92 mg of the cryptophane-based probe (this cryptophane-based probe was synthesized by ourselves, refer to [Zeng et al., 2021](#)) in 1 mL of spectral grade dimethyl sulfoxide (DMSO) to obtain a 3.0 mM probe stock solution. Store the stock solution at room temperature.

### Prepare N-ethylmaleimide stock solution for biothiols block

⌚ Timing: ~0.5 h

5. Prepare NEM stock solution.
  - a. Dissolve the N-ethylmaleimide (NEM) in 1 mL of spectral grade DMSO to obtain a 0.3 M stock solution. Store the stock solution at room temperature.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ethylene diamine tetraacetic acid (EDTA)	Macklin	Cat#E917522
Na <sub>2</sub> HPO <sub>4</sub> •12H <sub>2</sub> O	Sinopharm	Cat#10020328
KH <sub>2</sub> PO <sub>4</sub>	Sinopharm	Cat#10017628
KCl	Sinopharm	Cat#10016328
NaCl	Sinopharm	Cat#10019328
NaOH	Sinopharm	Cat#10019764
HCl	Sinopharm	Cat#10011028
Dimethyl sulfoxide (DMSO), spectral grade	Tianjin Kemiou	N/A
Cremophor®EL	Aladdin Chemistry	Cat#C107105
N-ethylmaleimide (NEM)	Sigma-Aldrich	Cat#E1271
Trypsin	Sigma-Aldrich	Cat#T1426
Penicillin–streptomycin	Sigma-Aldrich	Cat#V900929
Fetal bovine serum	Macklin	Cat#M6546
Ham's Kaighn's Modification (F12K) culture medium	Boster	N/A
Cryptophane based probe	Synthesized by ourselves	N/A
Experimental models: Cell lines		
A549	Shanghai Cell Bank	N/A
Software and algorithms		
OriginPro 2016	OriginLab	<a href="https://www.originlab.com">https://www.originlab.com</a>
Matlab R2014a	MathWorks	<a href="https://ww2.mathworks.cn/en">https://ww2.mathworks.cn/en</a>
Other		
Cell counter	Nexcelom	<a href="https://www.nexcelom.com/">https://www.nexcelom.com/</a>
Hyperpolarizer	Home-built	N/A
AV-III400 (400 MHz) spectrometer	Bruker	<a href="https://bruker.com">https://bruker.com</a>
10 mm double resonant probe ( <sup>129</sup> Xe and <sup>1</sup> H, PABBO BB- <sup>1</sup> H-D Z-GRD)	Bruker	<a href="https://bruker.com">https://bruker.com</a>
10 mm microimaging probe (MICWB40 RES 400 <sup>1</sup> H/ <sup>129</sup> Xe 040/010 LLTR)	Bruker	<a href="https://bruker.com">https://bruker.com</a>

**Optional:** The resources listed in the below table were based on our experience. Generally, the chemicals and resources can be purchased from any reliable commercial sources and do not need to be limited to those listed in our table.

### MATERIALS AND EQUIPMENT

Buffer and probe for cell experiment		
Name	Composition	pH
PBS	Na <sub>2</sub> HPO <sub>4</sub> •12H <sub>2</sub> O (1.15 g), KH <sub>2</sub> PO <sub>4</sub> (0.20 g), KCl (0.20 g), and NaCl (8.00 g) in 1 L ultrapure water	pH 7.4
Probe stock solution	3 mM in DMSO	N/A
NEM stock solution	0.3 M in DMSO	N/A

Equipment for cell count and NMR experiment		
Name	Function	Identifier
AV-III 400 spectrometer	MRS and MRI acquisition	Bruker
10 mm double resonant probe ( $^{129}\text{Xe}$ and $^1\text{H}$ , PA BBO BB- $^1\text{H}$ -D Z-GRD)	For MRS experiment	Bruker
10 mm microimaging probe (MICWB40 RES 400 $^1\text{H}/^{129}\text{Xe}$ 040/010 LLTR)	For MRI experiment	Bruker
Cell counter	Count the number of cells	Nexcelom

**Alternatives:** Other cell counters such as Beckman Coulter Vi-CELL XR, Thermo Countess 3 can also be used.

## STEP-BY-STEP METHOD DETAILS

### Cell resuscitation and culture

⌚ Timing: 1–2 weeks

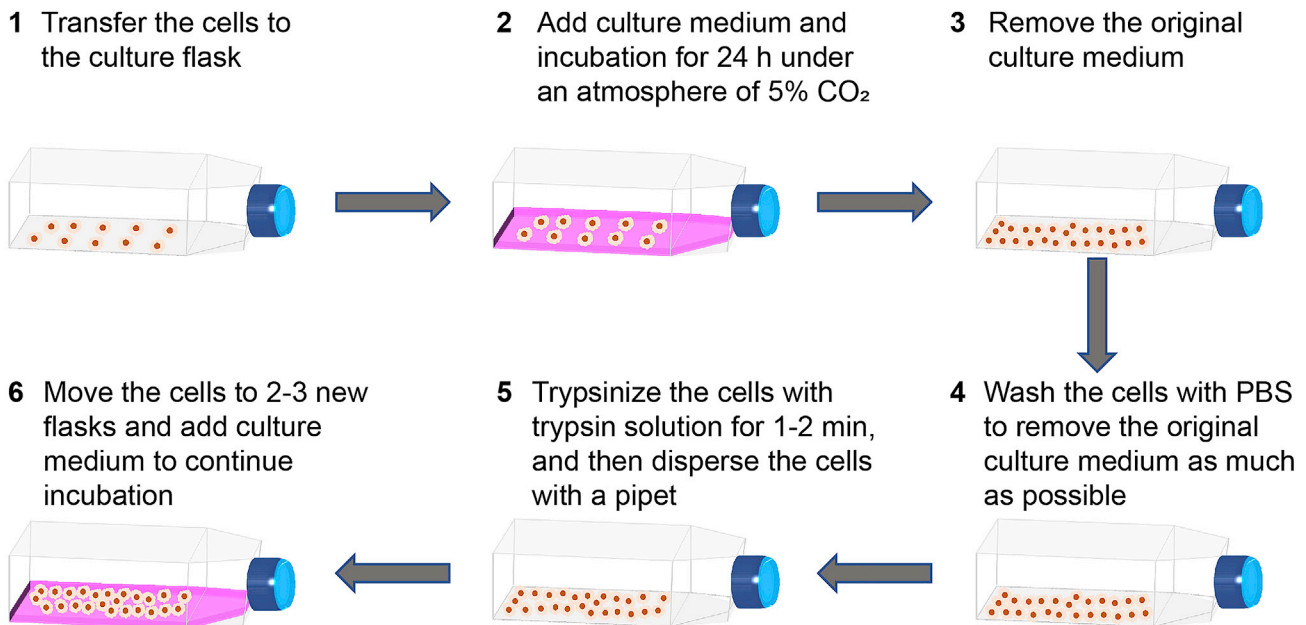
The main step is to obtain enough cells for hyperpolarized  $^{129}\text{Xe}$  NMR experiment. The following procedures are special to lung cancer cell A549. It may be suitable for other cells. The cell culture steps is shown in [Figure 1](#).

⚠ **CRITICAL:** For other cells, the procedures should adjust.

1. Resuscitate the cells.
  - a. Transfer the frozen tube containing A549 cells from the liquid nitrogen tank to 37°C warm water, hold the tube with tweezers and gently shake it to make it melt quickly.
  - b. Centrifuge the cells for 3–5 min at 100–400 XG.
  - c. Wipe the surface of the freezing tube with 75% ethanol in a laminar hood and then open the tube gently.
  - d. Remove the supernatant with a 1-mL spear tip, add 1 mL of warm (37°C) F12 K culture medium to homogeneously resuspend the cell pellet, and then transfer cells to a 25 cm<sup>2</sup> culture flask.
  - e. Add 4 mL of F12K culture medium (containing 10% FBS, 1% penicillin–streptomycin) into the culture flask.
  - f. Incubate the cells at 37°C under an atmosphere containing 5% CO<sub>2</sub>.
  - g. Replace the culture medium with fresh F12K medium (containing 10% FBS, 1% penicillin–streptomycin) after 24 h incubation.
2. Culture the cells.

**Optional:** Observe the morphology of the cells under an inverted microscope, while the cells bespread the culture flask, trypsinize, and continuous culture cells.

- a. After the cells bespread the culture flask, open the cap of the flask in a laminar hood, and remove the culture medium.
- b. Wash the cells with sterile PBS for 2–3 times to remove the culture medium as much as possible.
- c. Add 1 mL of trypsin solution to the culture flask cells, shake the flask gently to make the trypsin solution flow all over the cell surface.
- d. Place the flask under an inverted microscope, and observe the morphology of cells. When the cytoplasm retraction and intercellular stroma show obvious enlargement, the trypsinization was terminated immediately.
- e. Remove the trypsin solution and add 5 mL of fresh F12K culture medium (containing 10% FBS, 1% penicillin–streptomycin) into the flask.



**Figure 1.** Illustration of the cell culture steps

- f. Disperse the cells through a pipet smoothly and repeatedly, and avoid to produce gas bubbles when dispersing.
- g. Transfer the cells suspension into a new 75 cm<sup>2</sup> culture flask, and add 5 mL of F12K culture medium (containing 10% FBS, 1% penicillin–streptomycin).
- h. Incubate the cells at 37°C under an atmosphere of 5% CO<sub>2</sub>.
- i. Replace the culture medium with 10 mL of fresh F12K medium (containing 10% FBS, 1% penicillin–streptomycin) after 24 h.
- j. When the cells bespread the culture flask, repeat the procedures as previously described “a–i”, and make sure to culture enough cells (in our experiments, one sample needs 3–4 flasks of cells) for hyperpolarized <sup>129</sup>Xe NMR experiments.

**△ CRITICAL:** All solutions should be preheated to 37°C before being added into culture flask; do not produce bubbles when resuspend the cells, mix the cells softly and smoothly by a pipet to avoid damaging the cells; all operations should keep strictly aseptic; minimize the residual amount of trypsin solution to avoid cell damage, and neutralize the residual trypsin by culture medium.

The amount (mL) of the buffer, trypsin solution and culture medium for cells culture and resuscitation.

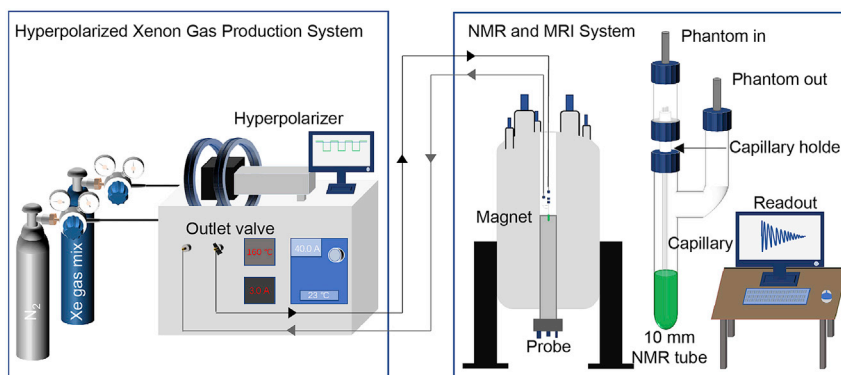
Solution	25 cm <sup>2</sup> culture flask	75 cm <sup>2</sup> culture flask
PBS buffer	3 mL	5 mL
trypsin solution	1–2 mL	2–3 mL
culture medium	4–5 mL	8–10 mL

### Incubate cells with cryptophane-based probes

⌚ Timing: 4–6 h

This main step is to transfect the probe into cells. Although the following procedures are special for cryptophane based probes, it is suitable for others probes too.

3. Incubate the cells with probe (Experimental group).
  - a. Culture the cells in 75 cm<sup>2</sup> culture flask as described in step 2.
  - b. Observe the bespreading cells (percent confluence>80%) under an inverted microscope, wipe the surface of the freezing tube with 75% ethanol in a laminar hood.
  - c. Open the cap of the culture flask in a laminar hood, and remove the regular culture medium gently.
  - d. Wash the cells with sterile PBS for 2–3 times to remove the regular culture medium.
  - e. Add 100 μL of cryptophane-based probe (3 mM) and 100 μL of Cremophor® EL into 9.8 mL of fresh F12K culture medium (without penicillin–streptomycin and FBS), and mixing homogeneously. The final probe concentration is 30 μM in the culture medium.
  - f. Add the F12K culture medium mixture (contain probe and Cremophor® EL) into the culture flask.
  - g. Incubate the cells at 37°C under an atmosphere containing 5% CO<sub>2</sub> for 2–3 h.
4. Incubate the cells with NEM and probe (Control group).
  - a. Culture the cells in 75 cm<sup>2</sup> culture flask as step 2 described.
  - b. Observe the bespeading cells under an inverted microscope, wipe the surface of the freezing tube with 75% ethanol in a laminar hood.
  - c. Open the cap of the culture flask in a laminar hood, and remove the regular culture medium gently.
  - d. Wash the cells with sterile PBS for 2–3 times to remove the regular culture medium as much as possible.
  - e. Add 100 μL of NEM (a popular thiol blocking agent) (0.3 M) and 100 μL of Cremophor® EL into 9.8 mL of fresh F12K culture medium (without penicillin–streptomycin and FBS), and mix homogeneously. The final NEM concentration is 3 mM in the culture medium. And then add the culture medium into the culture flask.
  - f. Incubate the cells at 37°C under an atmosphere of 5% CO<sub>2</sub> for 2 h.
  - g. Open the cap of the culture flask in a laminar hood, and remove the regular culture medium gently.
  - h. Wash the cells with sterile PBS for 2–3 times to remove the residual quantities of NEM as much as possible.
  - i. Add 100 μL of cryptophane-based probe (3 mM) and 100 μL of Cremophor® EL into 9.8 mL of fresh F12K culture medium (without penicillin–streptomycin and FBS), and mix homogeneously. The concentration of the probe is 30 μM in the culture medium.
  - j. Add the F12K culture medium mixture (containing probe and Cremophor® EL) into the culture flask.
  - k. Incubate the cells at 37°C under an atmosphere of 5% CO<sub>2</sub> for 2–3 h.
5. Collect the cells (For experimental group and control group).
  - a. After incubating the cells with the probe for 2–3 h, open the cap of the culture flask in a laminar hood, and remove the regular culture medium.
  - b. Wash the cells with sterile PBS for 2–3 times to remove the regular culture medium and residual probes have not been uptake by cells.
  - c. Add 3 mL of trypsin solution to the culture flask cells, gently shake the flask to make the trypsin solution flow all over the cell surface.
  - d. Place the flask under an inverted microscope, and observe the morphology of cells. When the cytoplasm retraction and intercellular stroma show obvious enlargement, the trypsinization was terminated immediately.
  - e. Remove the trypsin solution and add 4 mL of F12K culture medium (containing 10% FBS, 1% penicillin–streptomycin).
  - f. Disperse the cells through smoothly and repeatedly blow, and avoid to produce gas bubbles when blowing.



**Figure 2. Illustration of hyperpolarized  $^{129}\text{Xe}$  NMR experiment**

The  $^{129}\text{Xe}$  nuclear spin polarization was enhanced 100,000-fold compared to its thermal equilibrium polarization after the Xe gas mixture was delivered into the hyperpolarizer. After that, the hyperpolarized  $^{129}\text{Xe}$  was directly bubbled into the NMR tube, followed by NMR and MRI data acquisition.

- g. Transfer the cells suspension into 2-mL centrifuge tubes, and spin at 100–400 XG for 3–5 min.
- h. Remove the supernatant with a 1-mL spear tip smoothly, and add 1 mL of PBS to blow the cells gently. And then centrifuge the cells for 3–5 min at 100–400 XG. Repeat this procedure 2 times to remove the residual probes.
- i. All cells are concentrated and transferred into a 5-mL centrifuge tube gently.
- j. Add 2 mL of PBS buffer into the tube, and blow the cells smoothly to make a cells suspension.
- k. Count the cells concentration by cell counter.
- l. Store the cells suspension in an ice-water mixture for a few minutes.

△ **CRITICAL:** To keep the viability of the cells, all procedures should be performed gently.

**Note:** The cell concentration was kept at  $\sim 6 \times 10^6$  cells/mL in this experiment. To obtain such concentration of cells, after the cells percent confluence > 80%, 3–4 75-cm<sup>2</sup> flasks of the cells should be combined.

### Production of hyperpolarized Xe and initial Xe NMR test

⌚ **Timing:** 1–1.5 h

This main step is to produce the hyperpolarized  $^{129}\text{Xe}$  for the initial  $^{129}\text{Xe}$  NMR test. Before opening the hyperpolarized  $^{129}\text{Xe}$  system, the experimenters should read and understand the standard operating procedures (SOP). The hyperpolarized system is shown in [Figure 2](#).

6. Produce the hyperpolarized  $^{129}\text{Xe}$  and acquire the  $^{129}\text{Xe}$  NMR signal in PBS.
  - a. Startup the hyperpolarizer. Turn on the hyperpolarizer according to the standard operating procedures.
  - b. Turn on the  $\text{N}_2$  tank (control the pressure valve to bubble or stand by), set the relative gas pressure at 0.5 MPa (5 bar) (i.e., 5 bar above atmospheric pressure). This pressure controls the pressure controller behind the sample, which controls the valve to open or close.
  - c. Turn on the Xe mixture tank, set the relative gas pressure at 51 psig (3.5 bar) (i.e., 3.5 bar above atmospheric pressure). The gas mixture is delivered to the hyperpolarizer to produce the hyperpolarized  $^{129}\text{Xe}$  gas.
  - d. Add 2 mL of PBS into the 10 mm NMR tube, and screw the capillary holder onto the inlet thread.



- e. Connect the capillary holder of the NMR tube with the gas-delivery tube (coming from the hyperpolarizer) and the other chem thread of the NMR tube with the gas outlet tube (going into the pressure controller).
- f. Slowly open the outlet valve of the hyperpolarizer.
- g. Set the gas flow rate into the hyperpolarizer to 0.1 SLPM, to bubble the gas mixture into the NMR tube, and check the status of the gas flow.
- h. Close the outlet valve of hyperpolarizer to stop the gas flow, while the output pressure decreases to 0 psig, set the gas flow rate at 0 SLPM.
- i. Put the NMR tube carefully into NMR magnet.
- j. Adjust the experiment temperature with the VTU of the spectrometer. The NMR data are acquired at room temperature ( $T = 298$  K).
- k. Create a new  $^1\text{H}$  NMR experiment.
- l. Tune and match the  $^1\text{H}$  resonators, and manually shim the magnetic field.
- m. Adjust the magnetic field to make the proton chemical shift of the  $\text{H}_2\text{O}$  to 4.8 ppm.
- n. Acquire a  $^1\text{H}$  NMR spectrum, to evaluate the homogeneity of the magnetic field. If the homogeneity is good, proceed to the next step.
- o. Create a new  $^{129}\text{Xe}$  NMR experiment.
- p. Tune and match the  $^{129}\text{Xe}$  resonators.
- q. Set the number of repetitions to one.
- r. Choose a bubble time of 60 s, and a waiting time after bubbling of 3 s to allow the bubbles to collapse.
- s. Slowly open the outlet valve of the hyperpolarizer.
- t. Set the gas flow rate into the hyperpolarizer to 0.1 SLPM, to bubble the gas mixture into the NMR tube
- u. Acquire a  $^{129}\text{Xe}$  NMR spectrum, to evaluate the status of the hyperpolarizer through the signal to noise ratio (SNR) of  $^{129}\text{Xe}$  NMR spectrum. If the status of the hyperpolarizer is good (for our hyperpolarizer,  $\text{SNR} > 400$ ), continue to experiment.
- v. Close the outlet valve of hyperpolarizer to stop the gas flow, while the output pressure decreases to 0 psig, set the gas flow rate to 0 SLPM.
- w. Take out the NMR tube from the magnet, and unscrew the NMR tube connector carefully while keeping the hyperpolarizer on standby.
- x. Store the NMR tube for later experiments.

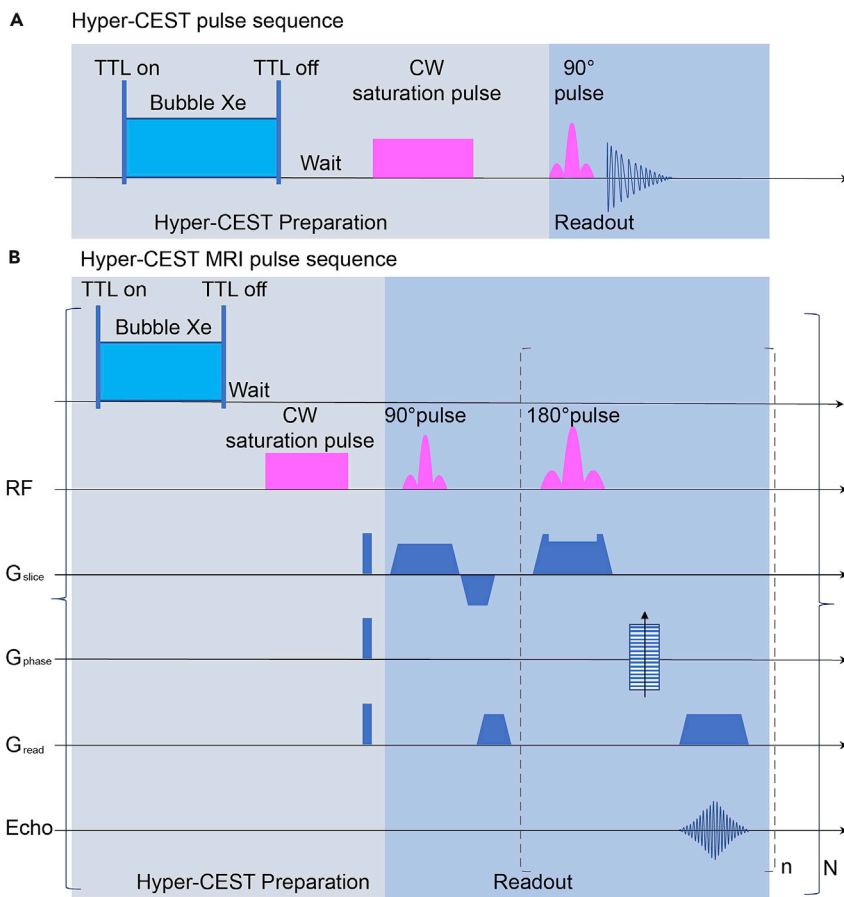
**⚠ CRITICAL:** The laser system of the hyperpolarizer must be used in strict accordance with the operating procedures to prevent damage. While turning on the hyperpolarizer, all procedures should perform accurately as the description in the SOP. Due to the different hyperpolarizer system having different SOP, the experimenters should read and understand the SOP before turning on the system. Before unscrew the NMR tube connector, the output pressure should decrease to 0 psig, which can avoid the sample suck in gas-delivery tube to pollute the gas-delivery tube.

### Hyper-CEST NMR for cells

⌚ Timing: 35–40 min

This main step is to acquire the Hyper-CEST for the living cells after incubated with cryptophane-based  $^{129}\text{Xe}$  probes. Although the following procedures are specially for cryptophane-based probes, it is suitable for others probes too. The Hyper-CEST pulse sequence is shown in [Figure 3](#).

7. Acquire the Hyper-CEST spectra for living cells.
  - a. Load 2 mL of cells suspension (the concentration of cells was kept at  $\sim 6 \times 10^6$  cells/mL) into the 10 mm NMR tube, and screw the capillary holder onto the inlet thread.



**Figure 3. Illustration of the pulse sequence of Hyper-CEST**

(A) The hyperpolarized  $^{129}\text{Xe}$  was bubbled into the NMR tube for 20 s and waited for another 3 s to allow the bubbles being collapsed. Then a continuous-wave pulse was applied to saturate the Xe in the host cage. After that, a  $\pi/2$  pulse was applied to acquire the  $^{129}\text{Xe}$  NMR spectrum in a single scan.

(B) For each excitation, the hyperpolarized Xe was bubbled into the NMR tube for 20 s, a wait time (3 s) allowed the bubbles to collapse. After that, a saturation pulse was applied to saturate the on or off-resonance frequency. Then, the image was acquired by a RARE sequence. The n is the echo chain length (rare factor), and N is the excitation number.

- b. Connect the capillary holder of the NMR tube with the gas-delivery tube (coming from the hyperpolarizer) and the other chem thread of the NMR tube with the gas outlet tube (going into the pressure controller).
- c. Put the NMR tube carefully into NMR magnet.
- d. Adjust the experiment temperature with the VTU of the spectrometer. The NMR data are acquired at room temperature ( $T = 298 \text{ K}$ ).
- e. Create a new  $^1\text{H}$  NMR experiment.
- f. Tune and match the  $^1\text{H}$  resonators, and manually shim the magnetic field.
- g. Adjust the magnetic field to make the proton chemical shift of the  $\text{H}_2\text{O}$  to 4.8 ppm.
- h. Acquire a  $^1\text{H}$  NMR spectrum, to evaluate the homogeneity of magnetic field. If the homogeneity is good, proceed to the next step.
- i. Create a new  $^{129}\text{Xe}$  NMR experiment.
- j. Tune and match the  $^{129}\text{Xe}$  resonators.
- k. Set up a series of Hyper-CEST experiments, and set the number of repetitions to one.
- l. Set the saturation frequency offset range from 55 ppm to 85 ppm with 1 ppm steps (the encapsulated  $^{129}\text{Xe}$  NMR signal appeared at  $\sim 71$  ppm, referenced to the Xe gas, the chemical shift of Xe gas was set as 0 ppm).

- m. Choose a 6.5  $\mu\text{T}$  continuous-wave (cw) saturation pulse with 10 s for saturation.
- n. Choose a bubble time of 20 s, and a waiting time after bubbling of 3 s to allow the bubbles to collapse.
- o. Slowly open the outlet valve of the hyperpolarizer.
- p. Set the gas flow rate into the hyperpolarizer to 0.1 SLPM, to bubble the gas mixture into the NMR tube.
- q. Acquire a series of Hyper-CEST experiments.
- r. After all of the Hyper-CEST experiment is completed, close the outlet valve of hyperpolarizer to stop the gas flow, while the output pressure decrease to 0 psig, set the gas flow rate to 0 SLPM.
- s. Remove the NMR tube from the magnet, and unscrew the NMR tube connector carefully while keeping the hyperpolarizer on standby. Store the NMR tube for later experiments.
- t. Process the series of Hyper-CEST experiments data to display the Hyper-CEST spectrum (Z-spectrum).
- u. After the CEST experiment, the integrities of cells was evaluated by an inverted microscope.

**△ CRITICAL:** This protocol's saturation frequency offset range is specific for the cryptophane-based  $^{129}\text{Xe}$  probes. While using other probes, the saturation frequency offset should be adjusted to a suitable range (the saturation frequency offset range was determined by the position of encapsulated  $^{129}\text{Xe}$  NMR signal peak, which should cover the  $^{129}\text{Xe}$  NMR signal completely). Before removing the NMR tube from the magnet, the output pressure should decrease to 0 psig. The control group and experimental group both perform the same procedures. To ensure the integrities of cells, the rate of the bubble should be well controlled and the Hyper-CEST experiments should be performed as soon as possible.

### Hyper-CEST MRI for cells

⌚ Timing: 30–40 min

This main step is to acquire the Hyper-CEST MRI for the living cells after incubated with cryptophane-based Xe probes. Although the following procedures are specific for cryptophane-based probes, it suitable for others probes too.

8. Acquire the Hyper-CEST MRI of the living cells.
  - a. Load 2 mL of cells suspension into the 10 mm NMR tube, and screw the capillary holder onto the inlet thread.
  - b. Connect the capillary holder of the NMR tube with the gas-delivery tube (coming from the hyperpolarizer) and the other chem thread of the NMR tube with the gas outlet tube (going into the pressure controller).
  - c. Put the NMR tube carefully into NMR magnet.
  - d. Adjust the experiment temperature with the VTU of the spectrometer. The MRI data are acquire at room temperature ( $T = 298\text{ K}$ ).
  - e. Create a new  $^1\text{H}$  MRI experiment. Tune and match the  $^1\text{H}$  resonators, perform a global automatic shim on the sample and acquire a proton reference image with automated adjustments for  $^1\text{H}$  resonance frequency, reference attenuation (flip angle) and receiver gain according to the spectrometer manufacturer's protocol.
  - f. Create a new  $^{129}\text{Xe}$  NMR experiment. Tune and match the  $^{129}\text{Xe}$  resonators.
  - g. Set the spectral width to 350 ppm, and set the signal average to four.
  - h. Choose a bubble time of 20 s, and a waiting time after bubbling of 3 s to allow the bubbles to collapse.
  - i. Slowly open the outlet valve of the hyperpolarizer.

- j. Set the gas flow rate into the hyperpolarizer to 0.1 SLPM, to bubble the gas mixture into the NMR tube.
- k. Acquire a  $^{129}\text{Xe}$  NMR spectrum.
- l. Process the  $^{129}\text{Xe}$  NMR spectrum with Fourier transform and automatic phase correction. And then, find the resonance frequency of the dissolved Xe peak and use it as the transmitter frequency for further  $^{129}\text{Xe}$  MRI acquisition.
- m. Create two  $^{129}\text{Xe}$  MRI experiments to acquire the on-resonant saturation and off-resonant saturation data.
- n. Choose RARE sequence to obtain the MRI data. Set the parameters as follow: set the transmitter frequency; the off-resonant saturation frequency offset set to 119 ppm (according to the z-spectrum interpretation, and the chemical shift of dissolved  $^{129}\text{Xe}$  was set as 0 ppm), and the on-resonant saturation frequency offset set to -119 ppm (according to the z-spectrum interpretation, and the chemical shift of dissolved  $^{129}\text{Xe}$  was set as 0 ppm); the number of acquisitions set to 16; the saturation power  $B_1$  set to 13  $\mu\text{T}$  and saturation time set to 5 s; the rest general parameters set as follow: number of dummy scan =4, slice thickness=25 mm, matrix size=32  $\times$  32, in-plane resolution= 0.9375  $\times$  0.9375  $\text{mm}^2$ , field of view =30  $\times$  30  $\text{mm}^2$ , centric k-space encoding, bandwidth=5400 Hz, echo time=4.65 ms, repetition time=28077 ms (including saturation time and xenon delivery), centric k-space encoding, no partial Fourier transform acceleration, rare factor=16.
- o. Set the gas mixture delivery flow into the hyperpolarizer to 0.1 SLPM.
- p. Open the outlet valve of the hyperpolarizer slowly to bubble the gas mixture into the NMR tube.
- q. Run the sequence to acquire the Xe MRI data.
- r. After all of the Xe MRI experiments were completed, close the outlet valve of hyperpolarizer to stop the gas flow, while the output pressure decrease to 0 psig, set the gas flow rate to 0 SLPM.
- s. Take out the NMR tube from the magnet, and unscrew the NMR tube connector carefully while keeping the hyperpolarizer on standby.
- t. Process the Xe MRI data on MATLAB to display the Hyper-CEST response. The image matrix 32  $\times$  32 was interpolated into a 64  $\times$  64 image matrix. Hyper-CEST effect for on-resonant saturation was analyzed compared to off-resonant saturation for each pixel by the formula (CEST effect =  $(\text{Intensity}_{\text{off}} - \text{Intensity}_{\text{on}}) / \text{Intensity}_{\text{off}}$ ) point by point. The mask was used in post-processing, covering the image areas that do not belong to the sample phantom and the normalized signal intensities less than 0.2.

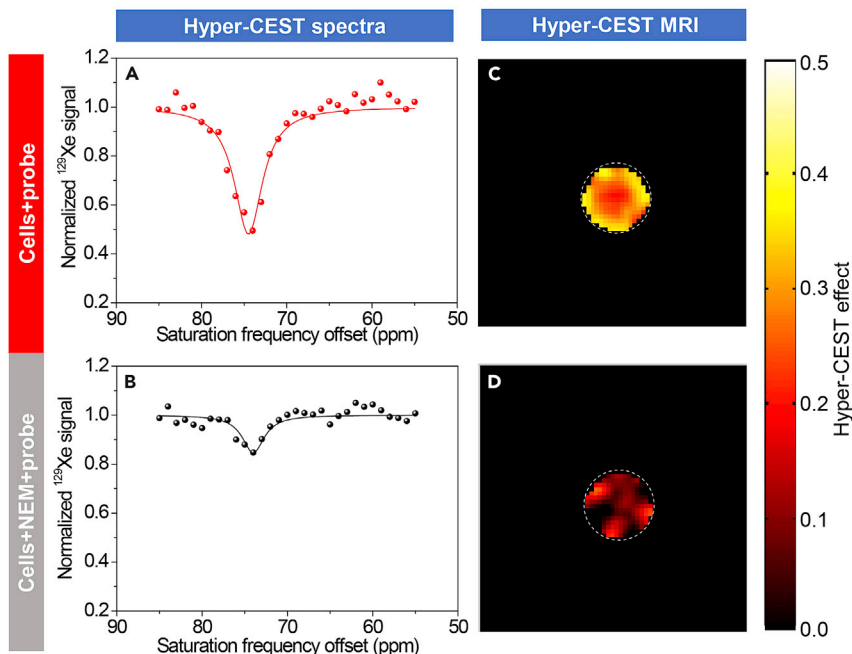
**△ CRITICAL:** The saturation power of  $B_1$  field should not exceed the limits of the NMR coil to avoid damaging the coil permanently. Before removing the NMR tube from the magnet, the output pressure should decrease to 0 psig. The control group and experimental group perform the same procedures.

### Shut down the system

⌚ Timing: 10–15 min

This main step is to shut down the hyperpolarized system. Before closing the system, the experimenter should read and understand the SOP.

9. Shut down the system according to the SOP.
  - a. Ensure the outlet valve of hyperpolarizer is closed.
  - b. Shut down the hyperpolarizer according to the SOP.
  - c. Turn off the  $\text{N}_2$  tank.
  - d. Turn off the Xe mixture tank.
  - e. Turn off the VTU of the spectrometer.



**Figure 4. Expected outcomes of Hyper-CEST experiments for living cells**

(A) Hyper-CEST spectra of A549 cells treated with cryptophane-based probe (30  $\mu$ M).

(B) Hyper-CEST spectra of A549 cells treated with NEM and cryptophane-based probe (30  $\mu$ M).

(C) Hyper-CEST MR phantom images for A549 cells treated with cryptophane-based probe (30  $\mu$ M).

(D) Hyper-CEST MR phantom images for A549 cells treated with NEM and cryptophane-based probe (30  $\mu$ M).

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**△ CRITICAL:** To avoid permanently damaging hyperpolarized system while shutting down the hyperpolarizer, all procedures should perform accurately as the description of the SOP.

## EXPECTED OUTCOMES

The expected outcomes of the Hyper-CEST experiments in this protocol were shown in Figure 4. After the A549 cells were incubated with the probe, a single signal appeared at 74 ppm corresponds to  $^{129}\text{Xe}$ @cryptophane-A in cells (Figure 4A). While the cells were treated with NEM (3 mM) followed by the probe, the Hyper-CEST effect decreased significantly (Figure 4B). For the Hyper-CEST MRI of the living cells, the experimental group treated with the probe showed a strong Hyper-CEST effect (Figure 4C), while the control group treated with both NEM and probe showed a weak Hyper-CEST effect (Figure 4D), which kept consistent with the Hyper-CEST spectra. These results unambiguously demonstrated that the probe responded well to biothiols in living cells, and the probe can be used to image the biothiols in living cells by hyperpolarized  $^{129}\text{Xe}$  MRI.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification and statistical analysis of CEST were performed by Origin version 2016 for Windows (Origin Software, USA), all results are expressed as mean  $\pm$  SD as indicated. The MR images were processed on MATLAB (R2014a, MathWorks, Natick, MA).

## LIMITATIONS

This protocol describes a method to detect and image the biothiols in living cells by hyperpolarized  $^{129}\text{Xe}$  NMR. First, it should be noted that this protocol is especial for cryptophane-based Xe probes.

If other Xe-based probes detect other molecules, the above mentioned parameters should be adjusted to a suitable range. Second, the bubble gas mixture might damage the viability of the cells. If necessary, the rate of the bubble should be well controlled and the Hyper-CEST experiments should be performed as soon as possible.

### TROUBLESHOOTING

#### Problem 1

The growth of the A549 cells is too slow (steps 1 and 2).

#### Potential solution

We suggest increasing the quantity of the FBS (the range of the percentages of FBS is 10%–15%) in the culture medium to solve this problem (steps 1 and 2).

#### Problem 2

The  $^{129}\text{Xe}$  NMR spectrum shows weak signal intensity or very distorted signals (step 6).

#### Potential solution

There are several reasons to induce the above issues including but not limited to the poor field homogeneity, the not collapsed gas bubble while acquiring the FID signal and the oxidized Rb of the hyperpolarized system.

We suggest three strategies to solve these problems (step 6):

Strategy 1, shim the magnet field to increase the field homogeneity.

Strategy 2, increase the waiting time after bubbling or add antifoam agents (such as L-81) into the solution to solve gas bubbles that have not collapsed while acquiring the signal.

Strategy 3, check the oxidation degree of Rb in the pumping cell and replace the Rb if necessary.

#### Problem 3

The Z-spectrum of the living cells shows no CEST response, or the  $^{129}\text{Xe}$  MRI of the living cells with on-resonant saturation does not show a significant decreased signal intensity (step 7).

#### Potential solution

There are several reasons to induce the above issues including but not limited to the too low concentration of the cryptophane based Xe probe, the living cells, the saturation power ( $B_1$ ) and the saturation time.

We suggest solving these problems by the following strategies:

Strategy 1, increase the concentration of the cryptophane-based Xe probe while incubating with living cells, or increase the concentration of the living cells (step 3).

Strategy 2, increase the saturation power of the  $B_1$  field or increase the saturation time (step 7).

#### Problem 4

The CEST response from the living cells is too broad (step 7).

#### Potential solution

The saturation power of the  $B_1$  field is too high may be led to the CEST response from the living cells is too broad. We suggest decreasing the saturation power of the  $B_1$  to solve this problem (step 7).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Prof. Dr Xin Zhou ([xinzhou@wipm.ac.cn](mailto:xinzhou@wipm.ac.cn)).

### Materials availability

This study did not generate new unique reagents. All reagents used in this study were commercially available and used without further purification.

### Data and code availability

This paper does not report original code. All data reported in this paper will be shared by the [lead contact](#) upon request.

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## AUTHOR CONTRIBUTIONS

X.Z. led the project. X.Z. and Q.G. designed the protocol. Q.Z., Q.G., Y.Y., W.J., and Y.Y. performed all the experiments. Q.Z. wrote the original manuscript; Q.G., L.Z., and X.Z. edited the paper. All the authors discussed and reviewed the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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