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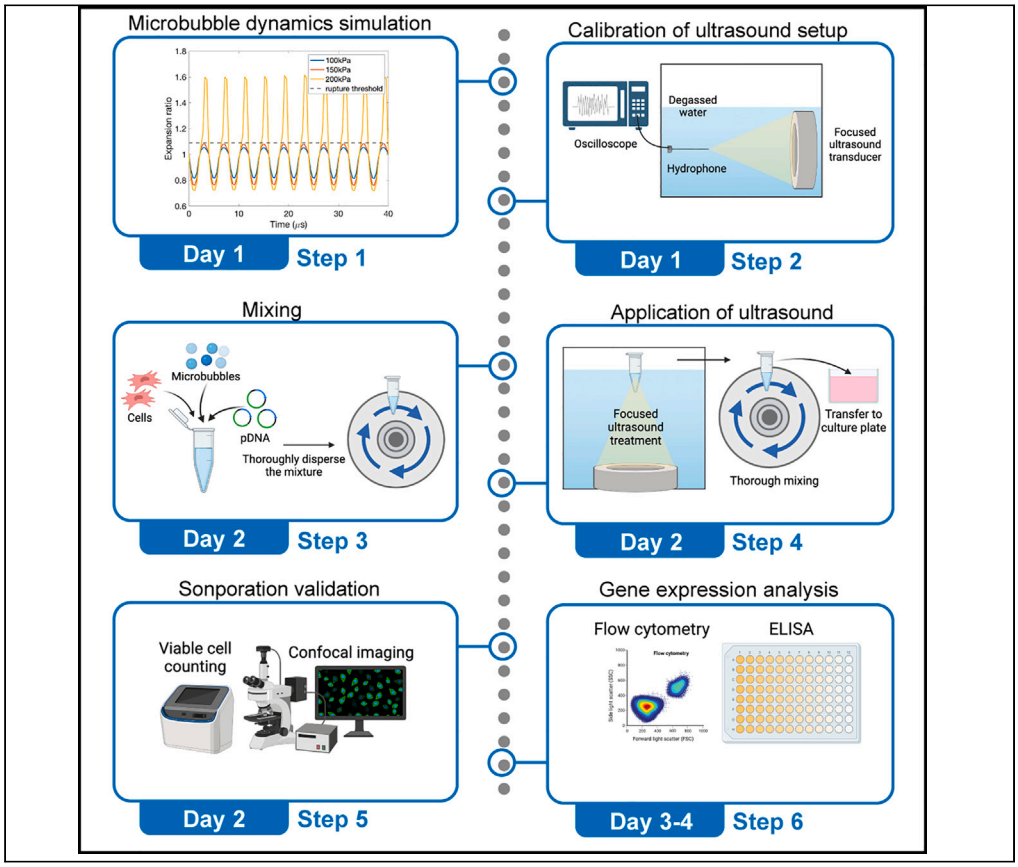
# Protocol for *in vitro* sonoporation validation using non-targeted microbubbles for human studies of ultrasound-mediated gene delivery




Microbubbles are currently approved for diagnostic ultrasound imaging and are under evaluation in therapeutic protocols. Here, we present a protocol for *in vitro* sonoporation validation using non-targeted microbubbles for gene delivery. We describe steps for computational simulation, experimental calibration, reagent preparation, ultrasound treatment, validation, and gene expression analysis. This protocol uses approved diagnostic microbubbles and parameters that are applicable for human use.

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

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## Highlights

Protocol for *in vitro* sonoporation validation using non-targeted microbubbles

Microbubble dynamics simulation for optimizing sonoporation conditions

Steps for ultrasound calibration and application for sonoporation

Steps to validate sonoporation and characterize plasmid transduction

Zhang et al., STAR Protocols 4, 102723
December 15, 2023 
https://doi.org/10.1016/j.xpro.2023.102723

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# Protocol for *in vitro* sonoporation validation using non-targeted microbubbles for human studies of ultrasound-mediated gene delivery

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https://doi.org/10.1016/j.xpro.2023.102723

## SUMMARY

**Microbubbles are currently approved for diagnostic ultrasound imaging and are under evaluation in therapeutic protocols. Here, we present a protocol for *in vitro* sonoporation validation using non-targeted microbubbles for gene delivery. We describe steps for computational simulation, experimental calibration, reagent preparation, ultrasound treatment, validation, and gene expression analysis. This protocol uses approved diagnostic microbubbles and parameters that are applicable for human use.**

**For complete details on the use and execution of this protocol, please refer to Bez et al. (2017).[1]**

## BEFORE YOU BEGIN

Through our protocol, we describe the specific steps for the optimization of a sonoporation regimen using a 250-kHz focused ultrasound transducer, microbubbles, plasmid DNA and human mesenchymal stem cells (hMSCs). The overall goal is to transfer genes to mesenchymal stem cells and assess the resulting protein production. In our previous studies, we achieved targeted bone regeneration via activation of resident stem cells. Sonoporation was applied to deliver bone morphogenetic protein 6 (BMP-6) plasmid to the recruited MSCs within bone fractures to induce bone regeneration and fracture healing.[1,2] This protocol is focused on *in vitro* sonoporation validation in support of translational studies of ultrasound-mediated gene delivery. With optimization of a few key parameters in the ultrasound treatment and cell culture conditions, we anticipate that this workflow is suitable for most *in vitro* experimental models.

### General preparation

1. Sterilize all required equipment and accessories by autoclave (e.g., pipette tips and Eppendorf tubes).
2. Prepare all chemical reagents and buffer solutions as described below.
3. Degas two liters of deionized (DI) water in a 2.5-L bottle and 10 mL of buffers in 50-mL conical flasks under vacuum for at least 12 h.

### Cell culture

Timing: 7–10 days

Thaw one cryo-tube of hMSCs approximately one week before conducting the sonoporation experiment. Seed approximately 5 × $10^5$ cells in one cryo-vial in a T75 flask and allow to adhere at least 12 h before passaging or collecting for the experiment. MSCs should have less than 6 passages at the time of the study.

4. Revive hMSCs from frozen stock.
   a. Prewarm complete medium in a 15-mL centrifugal tube in a 37°C water bath.
   b. Thaw one cryo-vial of frozen cells in a 37°C water bath for a few minutes. When thawed, add the prewarmed growth medium to the cells.
   c. Centrifuge cells (300 × *g*, 5 min), discard the supernatant, and add 10 mL of cell growth medium to resuspend cell pellet.
   d. Plate the cells in a T75 flask and add the 10 mL of additional growth medium to the cells.
   e. Incubate cells in a cell-culture incubator (37°C, 5% $CO_2$, 85%–95% humidity).
   f. The next day, use prewarmed phosphate buffered saline without calcium and magnesium (PBS−/−) to wash cells two times in, and add fresh complete medium (20 mL).

   *Note:* hMSCs should be tested for mycoplasma. For long-term storage, harvest hMSCs in medium with 20% FBS and 10% dimethyl sulfoxide (DMSO) for freezing. Slowly cool the cryo-vials to –80°C. The cryo-vial is then transferred to liquid nitrogen. One cryo-vial has approximately 5 × $10^5$ cells from a monolayer harvested from a T75 flask.

5. Passage hMSCs.
   a. Preheat PBS (−/−), TrypLE Express and complete medium to 37°C.
   b. Remove medium and wash cells two times in prewarmed PBS (−/−) (10 mL).
   c. Add 1 mL TrypLE Express and incubate cells in an incubator. Check the detachment of cells from the flask using a microscope. This usually requires 1–2 min.
   d. Add 2 mL of complete medium and transfer the cells to a 15-mL centrifugal tube.
   e. Centrifuge cells (300 × *g*, 5 min), discard the supernatant, and add 10 mL of cell growth medium to resuspend cell pellet.
   f. Transfer cells to a new T75 flask. For passaging, cells are split approximately 1:4 to reach 5 × $10^5$ cells per T75 flask on the day of the experiment. hMSCs usually require two days to adhere and actively grow.
   g. Fill the T75 flask with complete medium (20 mL) and incubate cells in incubator.

   *Note:* Cells should be 70%–80% confluent on the day that the *in vitro* sonoporation experiment starts.

   ⚠ CRITICAL: Keep the atmosphere sterile and perform all cell-related work in a biosafety cabinet.

### Microbubble activation and measurement

**Timing: 30 min**

6. Store the stock solution of Definity microbubbles in the refrigerator.

   *Note:* Allow the vial of Definity microbubble stock solution to warm to room temperature (RT) (20°C) before starting the activation procedure.

   *Note:* Definity is an injectable solution, while Definity RT is in a powder form and is stored in RT. If Definity RT is used instead of Definity, it requires VIALMIX RFID for activation and an additional injection of saline. Please follow the manufacturer's online protocol for Definity RT product.

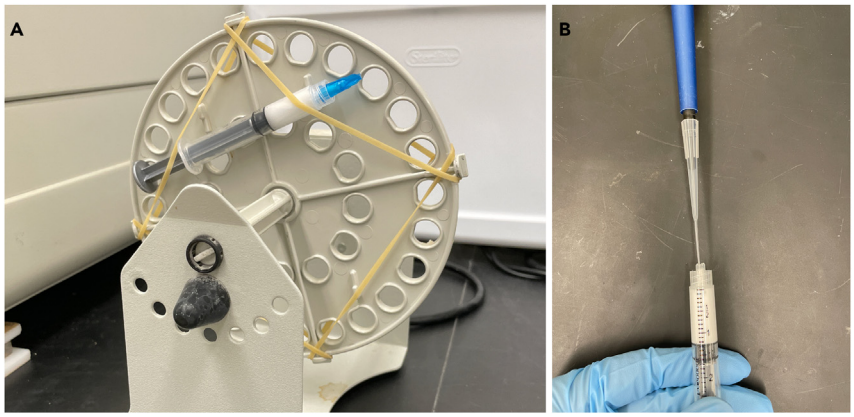



**Figure 1. Representative pictures for microbubble mixing and syringe access for aspiration**
(A) Fix the syringe containing microbubbles with rubber bands on a rotator for agitation and thorough mixing.
(B) Draw the microbubbles from the syringe using a 20 μL pipette with a gel loading tip.

7. Place the vial of Definity microbubble stock solution in the microbubble shaker (VIALMIX) and shake for 45 s for activation.

   *Note:* This process ensures that the microbubbles are evenly distributed in the suspension. If a VIALMIX is not available, amalgamator is also recommended. Vortex should not be used to shake a glass vial. To activate and generate more homogeneous and stable microbubbles, a high speed VIALMIX is required.

   *Note:* Activation of microbubbles generates heat during shaking, and allowing the solution to cool to 20°C for several seconds improves the accuracy of microbubble measurement.

8. Once the microbubbles are activated, draw the solution from the vial into a syringe and seal it with a syringe cap for further use.

   *Note:* A 3-mL syringe with a 15-gauge blunt cannula is typically used for this step, as it accommodates the viscous solution of activated microbubbles. Using a larger needle (15-gauge cannula) reduces damage to microbubbles during the process of drawing them into a syringe. Microbubbles are damaged by shear stress, which occurs when they pass through a small needle or narrow tubing.

9. The syringe is rotated on a rotator at a fixed speed of 24 rpm to ensure that the microbubbles are thoroughly mixed in the solution.

   *Note:* This reduces clumping or settling of the microbubbles (Figure 1).

10. Draw 2 μL of microbubbles from the syringe using a 20 μL pipette with a gel loading tip. Use a Kimwipe to remove the microbubbles outside the tip and add the microbubbles to the flask of the particle-sizing device.

    *Note:* The device measures the concentration and size distribution of the microbubbles (Figure 2), which ensures their quality and suitability.

    *Alternatives:* There are other instruments available for these setups. For microbubble measurement, the Coulter counter (i.e., Beckman Coulter) is a practical option. Additionally, the Amalgamator, commonly used for vial mixing, is also used for microbubble shaking and

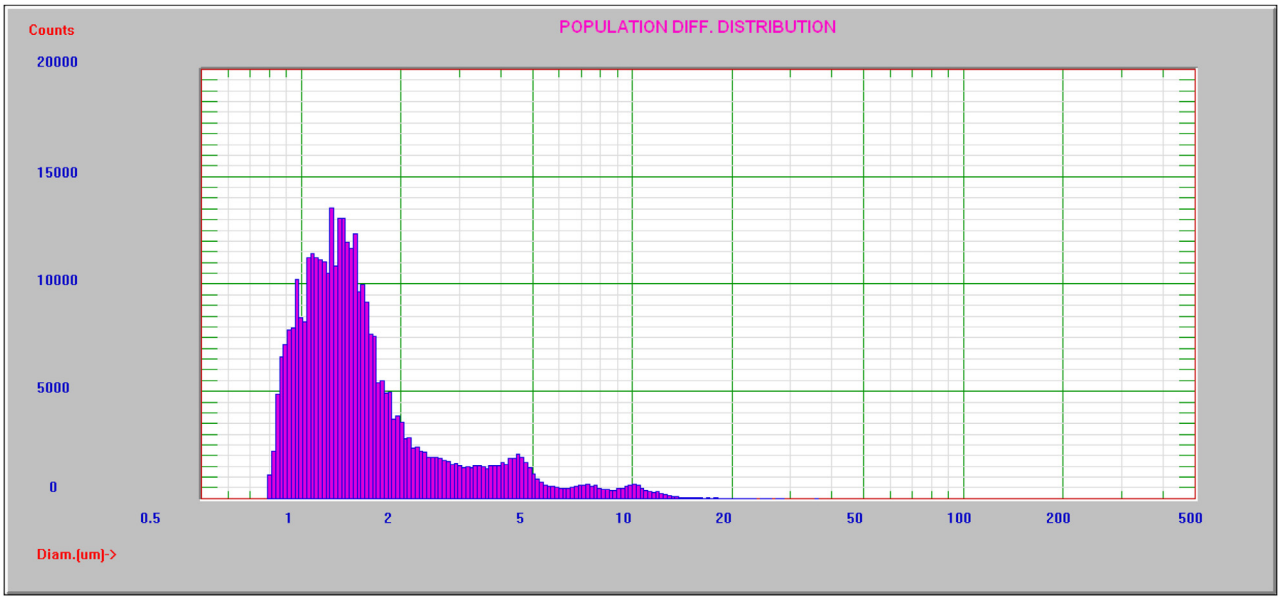



**Figure 2. Measurement for the size distribution and concentration of microbubbles using an AccuSizer or similar particle sizer**

The concentration of Definity microbubbles is about 1 × $10^{10}$/mL, and the average diameter is 1.5 μm. It is recommended to use the activated microbubbles promptly or calibrate them immediately before conducting an experiment. The activated microbubbles should be stored in the refrigerator and used within 12 h.

activation. Similarly, alternative brands with comparable functionalities can be employed for the rotator, hydrophone, and vacuum oven.

## Microbubble dynamics simulation

Timing: 30 min

11. If the ultrasound parameters are to be varied from those used here, the experimental work is informed by a theoretical simulation of microbubble dynamics during ultrasound application using MATLAB. This mathematical model captures the acoustically driven microbubble dynamics in an unbounded fluid and is used to select the acoustic pressure for a given frequency.
    a. Definition for Modified Rayleigh-Plesset (RP) equation:

```
function dy = RP_Marmottant(t, y, freq, tspan)

global k rho mu p0 pv R0 A ks ksi sigma c0

pa = A*sin(2*pi*freq*tspan);

pA = interp1( tspan, pa, t );

% Marmottant model

Rbuck = R0;         % Buckling Radius

Rbreak = min(Rbuck*sqrt(sigma/ksi+1), 2*R0);     % Break up Radius

Abuckle = 4*pi*R0^2;                % Buckling Area

sigma_m = @(R)(R <= Rbuck).*0+ ...

((R > Rbuck) && (R <= Rbreak)).*(ksi*((R./Rbuck).^(2) - 1))+ ...

(R > Rbreak).*sigma;
```

```
% second derivative of the bubble radius

R = y(1);

dR = y(2);

ddR = (1./R)*((1./rho)*((p0 - pv + 2*sigma_m(R0)/R0)*((R/R0)^(-3*k))*(1 - 3*k*dR/c0)...

- 2*sigma_m(R)/R - 4*mu*dR/R - 4*ks*dR/R^(2) - p0 - pA + pv) - 3/2*dR^(2));

% Return the state vector

dy = [dR; ddR];

end
```

*Note:* The microbubble oscillation is modeled using a modified RP equation that accounts for the effect of the microbubble (MB) shell,[3] as follows:

$$\rho\left(R\ddot{R}+\frac{3}{2}\dot{R}^2\right)=\left[P_0+\frac{2\sigma(R_0)}{R_0}\right]\left(\frac{R_0}{R}\right)^{3\kappa}\left(1-\frac{3\kappa}{c}\dot{R}\right)-\frac{2\sigma(R)}{R}-4\mu\frac{\dot{R}}{R}-4\kappa_s\frac{\dot{R}}{R^2}-P_{ac}(t)-P_0 \quad \text{(Equation 1)}$$

Where R is the MB radius, ρ is the fluid density of the surrounding environment, $P_0$ is the ambient pressure, κ is a polytropic gas constant, μ is the fluid viscosity, and $\kappa_s$ is the viscosity of shell surface. The acoustic pressure, $P_{ac}(t) = A\sin(\omega t)$, is considered as a continuous sinusoidal wave, where A is the amplitude of the acoustic pressure and ω is the angular frequency of the acoustic wave.

Equation 1 account for the non-linear effect of bubble oscillation by separating the shell motion into three regimes (buckled, elastic and ruptured region). The change of surface tension, σ, during bubble expansion and compression and at shell break-up is described as follows:

$$\sigma(R)=\begin{cases}0 & ,\ if\ R\le R_{buckling}\\ \chi\left(\dfrac{R^2}{{R_{buckling}}^2}-1\right) & ,\ if\ R_{buckling}\le R\le R_{break-up}\\ \sigma_{water} & if\ R\ge R_{ruptured}\end{cases} \quad \text{(Equation 2)}$$

where χ is the shell elastic modulus, $R_{buckling}$ is the MB radius at which the shell starts to buckle and is assumed to be the MB resting radius, and $R_{ruptured} = R_{buckling}\sqrt{\left(\frac{\sigma_{water}}{\chi}\right)+1}$ is the MB radius when the MB shell ruptures.

b. Define the parameters based on the experimental conditions:

```
clear all

close all

clc

global k sigma rho mu p0 pv R0 A ksi ks c0

% define parameters

k = 1.07;         % Polytropic index

sigma =         0.072; % Water Surface tension [N/m]

rho = 1000;       % water Density [kg/m^3]

mu = 0.004;       % Viscosity [Pa.s]
```

```
p0 = 1.046E5;       % Ambient pressure [Pa]
pv = 2.33E3;       % Vapor pressure inside bubble [Pa]
dR0 = 0;         % Initial bubble wall velocity [m/s]
R0 = 1E-6;       % Initial bubble radius [m]
A = 50E3;       % Acoustic pressure amplitude [Pa]
c0 = 1500;       % Sound speed [m/s]
freq = 0.25E6;     % Excitation frequency [Hz]
ncycle = 30;       % number of cycle
t0 = 0;
tf = ncycle/freq;
tspan = linspace(t0, tf, 200*ncycle);
% parameters (Marmottant model)
ksi = 0.38; % shell elasticity [N/m]
ks = 2.4E-9; % shell viscosity [kg/s]
```

*Note:* In future human studies, the ultrasound pressure and frequency may differ from those used here. A protocol should be designed to produce an expansion ratio similar to that in Figure 3.

c. Solve the modified RP equation using ODE45 function in MATLAB, and then plot the microbubble expansion ratio during sonication and for different acoustic pressures.

```
% RP_Marmottant solution
[T, R] = ode45(@(t, y) RP_Marmottant(t, y, freq, tspan), tspan, [R0; dR0]);
R_normalised = R/R0;
T=T*1E6;
%plot
figure(1)
plot(T,R_normalised(:,1),'-','LineWidth',1)
xlabel('time [\mus]','FontSize',16)
ylabel('Oscillation Amplitude R/R0','FontSize',16)
ax = gca;
ax.FontSize = 16;
```

12. Theoretical predictions of the distribution and the proximity of cells and microbubbles using MATLAB.
    a. Define the estimated properties of microbubbles and cells. For example, if a 125-μL sample is scaled down by a factor of 1000, the total number of microbubbles (8000) and cells (200) are in a 0.125 μL volume, with a microbubble diameter of 2 μm and a cell diameter of 20 μm.

```
% Set the number of MBs and Cells

n_MBs = 8000;

n_Cells = 200;

ratio = 40;

% Set the size of MB and cell

MB_size = 2 % 2 um in diameter

Cell_size = 20 % 20 um in diameter

% Set the size of cube, a cube root of total volume in tube

cube_size = 500; % 500 um in length, and the volume is 0.125 uL
```

b. Plot a random distribution of microbubbles and cells in a cube (Figure 4A).

```
% Generate N points in cube

xyz1 = cube_size*rand(n_MBs,3);

xyz2 = cube_size*rand(n_Cells,3);

close all

% Plot microbubbles

scatter3(xyz1(:,1),xyz1(:,2),xyz1(:,3), MB_size, [0 0.4470 0.7410]);

hold on

% Plot cells

scatter3(xyz2(:,1),xyz2(:,2),xyz2(:,3), Cell_size,[0.9290 0.6940 0.1250], 'filled');

axis equal
```

c. Create a distribution curve showing the distances between the cell and its nearest 40 microbubbles in a cube. Calculate the density at each distance interval (in this example, the interval size is 1 μm) by dividing the number of cells in the interval by the volume of the interval, and then calculate the standard deviation of the densities (Figure 4B).

```
% Set the distance interval size

inr_size = 1;

% Generate random coordinates for the dots of MBs and Cells

dots_MBs = rand(n_MBs, 3) * cube_size;

dots_Cells = rand(n_Cells, 3) * cube_size;

% Calculate distances between all Cells and their nearest MBs

dist_all = zeros(n_Cells, ratio);

for i = 1:n_Cells

  dist = vecnorm(dots_MBs - dots_Cells(i,:), 2, 2);

  [~, idx] = sort(dist);

  nearest_MBs_dots = idx(1:ratio);

  nearest_MBs_dist = dist(nearest_MBs_dots);

  dist_all(i,:) = nearest_MBs_dist;
```

```
end
% Calculate the density at each distance interval for each Cell
max_dist = max(max(dist_all));
dist_inr = 0:inr_size:max_dist;
density = zeros(n_Cells, length(dist_inr)-1);
for i = 1:n_Cells
  for j = 1:length(dist_inr)-1
    mask = dist_all(i,:) >= dist_inr(j)& dist_all(i,:) <dist_inr(j+1);
  den(i,j) = sum(mask) / ((4/3)*pi*((dist_inr(j+1))^3 -(dist_inr(j))^3));
  end
end
% Calculate the mean density and standard deviation across all Cells at each distance interval
mean_density = mean(density, 1);
std_density = std(density, 1);
% Plot the density curve with error bars
figure;
errorbar(dis_inr(1:end-1), mean_density, std_density./sqrt(n_Cells-1), 'b');
xlabel('Distance from cell to its nearest 40 MBs (\mum)');
ylabel('Average density');
```

⚠ CRITICAL: Some functions will not work in older versions of MATLAB. If an error is received, install the latest version. On the Home tab, click Help > Check for Updates > Products. Restart the updated MATLAB version and repeat the simulation.

## Calibration of focused ultrasound transducer

Timing: 2 h

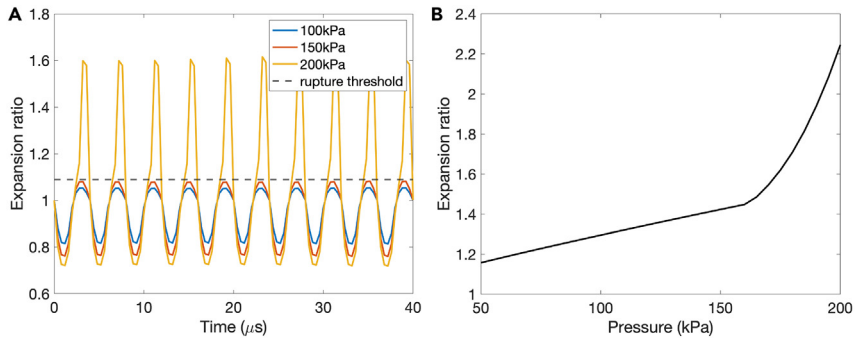



**Figure 3. Theoretical predictions of a 1 μm radius Definity MB oscillation at 250 kHz using the RP equation**

(A) Definity MB oscillation at 250 kHz is modeled by a modified RP equation with the Marmottant model, which takes into account the change of surface tension during oscillation. When the acoustic pressure is 200 kPa, the MB expansion ratio is approximately 1.6, which is above a presumed rupture threshold.

(B) The expansion ratio of MB as a function of pressure from 50 to 200 kPa.

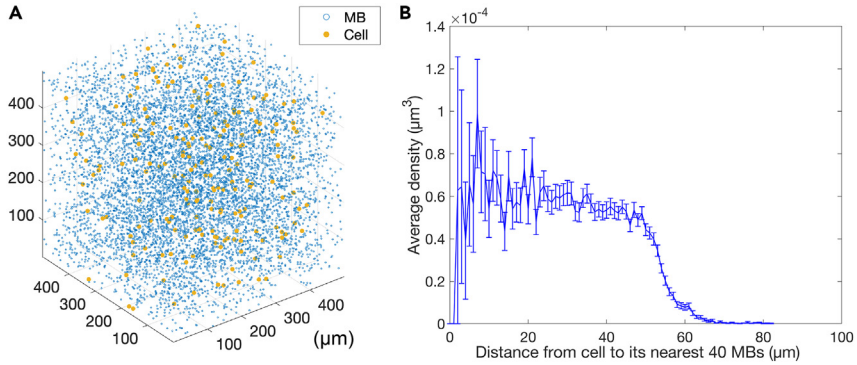



**Figure 4. Theoretical predictions of the distribution and the proximity of hMSCs and Definity MBs**

(A) MBs and cells are randomly distributed in a certain size of cube with a ratio of 40:1 and a density of 8000 MBs per 0.125 μL. Assuming the diameter of the MBs is 2 μm and the size of the cells is 20 μm, the graph displays MBs (represented by blue dots) and cells (yellow dots) in a cube.

(B) The average density of MBs and cells is plotted against the distance between a cell and its nearest 40 MBs, revealing that most cells are located within 5–40 μm of a MB. Given that the size of a hMSC and a MB is approximately 20 μm and 2 μm, respectively, the proximity is adequate for inducing sonoporation during sonication. This suggests that the MBs oscillate and induce sonoporation in close proximity to cells.

13. Fill the degassed DI water in the water tank. The water should be free of air bubbles to prevent interference with the ultrasound waves.
14. Connect the ultrasound transducer to its radio frequency (RF) impedance matching network, which ensures that the transducer is properly matched to the RF drive amplifier (Sonic Concepts System).
15. Fix the hydrophone in a holder that is controlled by a micrometer actuator and connect the hydrophone to an oscilloscope.
16. Place the hydrophone at a fixed distance from the transducer. Both hydrophone and transducer are submerged in the water. Adjust the positions of the hydrophone to the center of the ultrasound focus (Figure 5).

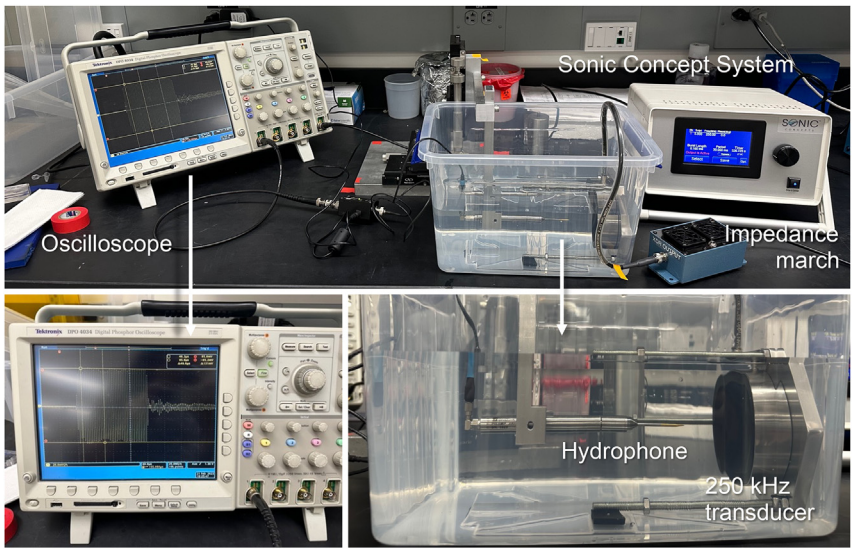
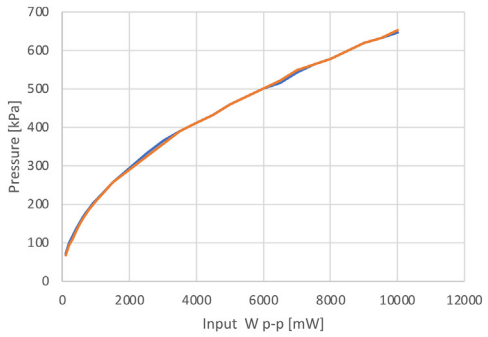



**Figure 5. Calibration setup for the 250-kHz transducer with the RF drive amplifier**

The Sonic Concepts RF drive amplifier is used to drive the transducer to generate acoustic power. The RF impedance matching box is connected between the RF drive amplifier and the transducer to optimize the transducer acoustic output. The hydrophone receives the generated acoustic signals from the transducer to measure the ultrasound acoustic field. The oscilloscope displays the waveforms received by the hydrophone as a two-dimensional plot of one or more signals as a function of time, showing the acoustic output of the transducer.




| Electrical power (mW) | PNP (kPa) |
|---|---|
| 200 | 98.61 |
| 500 | 150.00 |
| 900 | 201.39 |
| 2000 | 293.06 |
| 4000 | 411.11 |
| 6000 | 500.00 |
| 8500 | 597.22 |

**Figure 6. Calibration curves for the 250-kHz transducer with the RF drive amplifier**

The acoustic peak negative pressure (PNP) is plotted as a function as the input power setting on the amplifier in milliwatts (mW). We recommend using ~200 kPa, which corresponds to 900 mW for the amplifier applied here.

*Note:* The hydrophone is carefully adjusted to receive signals from the center of the ultrasound focus.

17. Set the output power of the RF drive amplifier and start sending signals to the transducer to generate ultrasound waves. The output power is varied systematically to obtain a range of measurements.
18. As the ultrasound waves propagate through the water and are measured by the hydrophone, record the received signals with the oscilloscope.

*Note:* To correctly visualize the signals on the oscilloscope, adjust the settings to display the ultrasound waveform. This includes adjusting the timebase (horizontal scale), voltage scale, and trigger settings. Adjust the settings as needed to refine the display and capture the data.

19. Use the hydrophone calibration to convert the measured voltage into acoustic peak negative pressure (PNP).
20. Make a correlation curve to plot the PNP as a function of input electrical power, which characterizes the performance of the ultrasound transducer with the RF drive amplifier.

*Note:* We calibrated the 250-kHz transducer (H-115) with the Sonic Concepts RF drive amplifier. The input power was ranged from 0.1 to 10 W, and the calibration curve (Figure 6) and the corresponding output pressures are shown in the embedded table.

Based on the MB dynamics simulations, we will test three PNPs in the sonoporation experiment: 200 kPa (with the largest microbubble oscillation amplitude and beyond the rupture threshold), 150 kPa (at the rupture threshold of the microbubble), and 100 kPa (smaller than the rupture threshold). According to the calibration curve, and as shown in the table, the electrical input power settings of the RF amplifier for these conditions will be 900, 500 and 200 mW, respectively.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Critical commercial assays | | |
| DuoSet ELISA Ancillary Reagent Kit 2 | R&D Systems | DY008B |
| Human BMP-6 DuoSet ELISA | R&D Systems | DY507 |
| LIVE/DEAD Fixable Blue Dead Cell Stain Kit, for UV excitation | Thermo Fisher Scientific | L34962 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| GFP BrightComp eBeads Compensation Bead Kit | Thermo Fisher Scientific | A10514 |
| MSCGM Mesenchymal Stem Cell Growth Medium BulletKit | Lonza | PT-3001 |
| Experimental models: Cell lines | | |
| Human mesenchymal stem cells (hMSCs), adherent cells | Lonza | PT-2501 |
| Recombinant DNA | | |
| pGFP: GFP plasmids with cytomegalovirus promoter in a lentiviral (pCCLc) backbone, pCCL-CMV-GFP | From the UC Davis Stem Cell Vector Core[4] | NA |
| pBMP: BMP-6 plasmid with cytomegalovirus promoter | GenScript | pCMV-BMP6 |
| pmNG: mNeonGreen plasmids with CAG promoter in a AAV backbone, pAAV-CAG-mNeonGreen | Addgene | 99134 |
| Chemicals, peptides, and recombinant proteins | | |
| Deionized (DI) water | Millipore | NA |
| Definity (perflutren lipid microsphere) | Lantheus | Definity |
| Dulbecco's modified Eagle's medium (DMEM) | HyClone | SH30243.01 |
| Fetal bovine serum (FBS) | Omega Scientific | FB01 |
| Penicillin-Streptomycin solution (PS) | Caisson Labs | PSL01-6X100ML |
| TrypLE Express (100 mL) | Thermo Fisher Scientific | 12604-039 |
| PBS (+/+): Dulbecco's phosphate-buffered salt solution 1X (calcium and magnesium) | Corning | 21030CV |
| PBS (−/−): Dulbecco's phosphate-buffered salt solution 1X (no calcium, no magnesium) | Corning | 21031CV |
| PFA: 4% paraformaldehyde solution in PBS | Santa Cruz Biotechnology | sc-281692 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D2650 |
| 4′,6-Diamidino-2-phenylindole (DAPI) | Sigma-Aldrich | D9542 |
| Propidium iodide (1.0 mg/mL solution in water) (PI) | Sigma-Aldrich | P4864 |
| Cal Green 1, AM (Calcium Green-AM) | AAT Bioquest | 20501 |
| Trypan blue, 0.4% (w/v) | Corning | 15250061 |
| Software and algorithms | | |
| MATLAB R2023a | MathWorks | https://www.mathworks.com/products/matlab.html |
| FlowJo v10.9 | BD | https://www.flowjo.com/solutions/flowjo |
| GraphPad Prism 9 | GraphPad | https://www.graphpad.com/scientific-software/prism/ RRID: SCR_002798 |
| ImageJ 1.54d | National Institutes of Health | https://imagej.nih.gov/ij/ |
| Other | | |
| 250 kHz transducer | Sonic Concepts | H-115 |
| Transducer power output system, RF drive amplifier | Sonic Concepts | Transducer Power Output (TPO) |
| Waterproof BNC to UHF cable | Olympus | BCU-58-6W |
| Vial holder stage | Sahar Ltd., Israel | Custom designed |
| Microbubble shaker | Lantheus | VIALMIX |
| Hydrophone | Onda | HNP-0400 |
| Kimwipe delicate task wipes | Kimtech Science | 34155 |

## MATERIALS AND EQUIPMENT

### Accessories for microbubble processing and sonoporation

- 0.5 mL microcentrifuge tube.
- 200 μL Round Gel-Loading Pipette Tip.
- 3 mL syringe with Luer Lock Tip.
- Sterile syringe caps for Luer Lock syringes.
- Cannulas: 15 Gauge, 1-1/2 Inch, blunt.
- Rubber bands.

*Alternatives:* Accessories from any brands are available if they have the same specifications.

### Reagents for cell culture

- Media supplemented with DMEM with 10% of FBS and 1% of PS.

[Keep the complete media at 4°C for up to 6 months.]

### Accessories for characterization of plasmid transduction

- Glass slide: non-treated surface, 75 mm × 38 mm.
- Cover glasses: 12 mm Circles.
- Glass antifade mountant.
- Cell counting chamber.
- 96-well plates.

*Alternatives:* In this protocol, we use Countess Automated Cell Counter (Invitrogen). Other cell counters or cell counting methods are available, such as hemocytometer or cell counting chamber. 96 well plates with V-bottom or U-bottom, or flow tubes from other brands are also available if they meet the sampling requirements for flow cytometer.

### Equipment

- Vacuum oven.
- Rotator: a fixed speed of 24 rpm, a rotisserie for microcentrifuge tubes and syringes.
- Particle counter.

*Alternatives:* In this protocol, we use AccuSizer (Particle Sizing System) for microbubble measurement. Alternatives, such as Coulter counter (Beckman) or Bubble counter (GAMPT), are also available.

- Confocal microscope.
- Flow Cytometer.
- Microplate reader.

*Alternatives:* Microscope, flow cytometer, and microplate reader equipped with suitable lasers and filters are used to characterize plasmid transduction.

## STEP-BY-STEP METHOD DETAILS

### Part I: Mixing cells with microbubbles and plasmids

Timing: 30 min

These steps prepare the mixture of cells, activated microbubbles and PI/Cal Green or plasmids (i.e., pGFP, pmNG or other plasmid of interest) for sonoporation. Thorough mixing of the components improves the sonoporation efficiency.

1. Assembly of sonoporation apparatus (Methods video S1):
   a. Fix the transducer on the holder of the tube rack, and place in the water tank.
   b. Adjust the height of the stage according to the focal length of the transducer and ensure the focal point covers the sample tube.
   c. Connect the transducer with impedance match that is connected to the Sonic Concepts system using a waterproof BNC-UHF cable.

d. Fill degassed DI water in the tank and top off the tube stage.

2. Prepare cell solutions for validation of the sonoporation parameters (for Part III):
   a. Generate cell pellets of 2 × $10^5$ cells in 0.5-mL Eppendorf tubes and suspend in 100 μL degassed PBS (+/+).

   *Note:* Calcium and magnesium in PBS (+/+) is important in many cellular processes, such as signaling, adhesion, and proliferation. The addition of calcium and magnesium ions to PBS maintains the normal physiological functions of cells *in vitro* and improves experimental outcomes.

   b. Mix with 2 μL of PI, 0.2 μL of Cal Green and 5 μL of activated microbubbles, and add them to the cell suspensions.
   c. Add degassed PBS (+/+) to achieve a total volume of 125 μL in the sample tube.
   d. Thoroughly mix the solution but avoid splashing the liquid into the tube lid.

   *Note:* To ensure thorough mixing of microbubbles, plasmids and cell suspension in the solution in steps 2d and 3d below, gently rotate the Eppendorf tube on a rotator at a fixed speed of 24 rpm. Additionally, flick the tube to dislodge any components adherent to the lid (Methods video S2).

   e. Immediately transfer the tube containing the cell mixture to the sonoporation apparatus, which applies ultrasound to the cells.

3. Prepare cell solutions for *in vitro* plasmid transduction (for Part IV):
   a. Generate cell pellets of 2 × $10^5$ in 0.5-mL Eppendorf tubes and suspend in 100 μL degassed PBS (+/+). Troubleshooting 1.
   b. Mix 9 μg of plasmid and 5 μL of activated Definity microbubbles, and add them to the cell suspensions.

   *Note:* The plasmid concentration can vary in different preparations. Plasmids are typically stored in glycerol stock at –20°C or –80°C for long-term storage, where the shortage concentration should range from 0.5 to 10 μg/μL. Since cryoprotectant impacts cell function and transfection, it is recommended to concentrate plasmids through lyophilization and reconstitution in either PBS or glycerol stock during storage to achieve the above range.

   c. Add degassed PBS (+/+) to achieve a total volume of 125 μL in the sample tube.
   d. Thoroughly mix the solution (as above) but avoid liquid splashing into the tube lid.
   e. Immediately transfer the tube containing the cell mixture to the sonoporation apparatus, which applies ultrasound to the cells.

⚠ CRITICAL: It is important to perform sonoporation as quickly as possible after mixing the components to ensure the best results. If there is a delay, rotate the tube a few times to keep the components mixed and prevent settling.

### Part II: Application of ultrasound for sonoporation

Timing: 1 h

These steps induce sonoporation within cells in the Eppendorf tube. Thorough mixing of the components improves the consistency of gene transduction. This protocol is suitable for regular application in cell transfection testing as well as for immediate use in human studies.

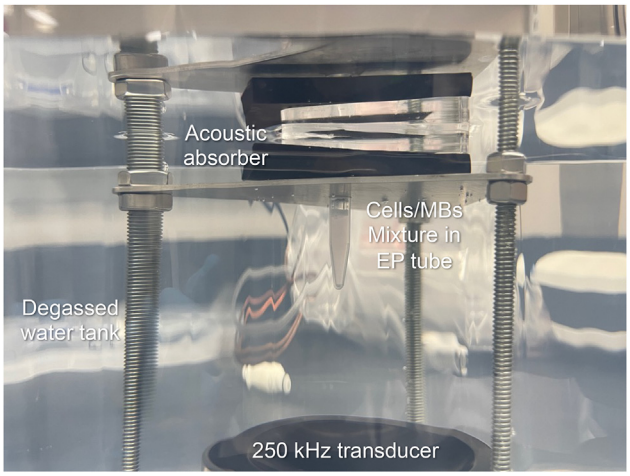



**Figure 7. Setup for *in vitro* sonoporation**

The tube to transducer distance is adjusted to align with the focal point of the 250-kHz ultrasound transducer.

4. Enter the ultrasound parameters through a computer ('Remote' mode) or input them directly from the front panel ('Local' mode).

   ***Note:*** The parameters include the frequency of 250 kHz, the burst length of 33 ms, the period of 500–4000 ms, the total treatment time of 180 s, and the PNPs of 100–200 kPa which correspond to a setting of 200–900 mW on the system (Methods video S3).

   ***Note:*** It is possible to visualize the ultrasound burst without the sample tube present (Methods video S4) and the effect on the microbubbles during ultrasound application is visualized with the Eppendorf tube present (Methods video S5).

5. After thorough mixing of the solution (Step 3d in Part II), insert the Eppendorf tube into the place holder at the center of the ultrasound focus.

   ***Note:*** It is important to ensure that the tube is not floating and is properly positioned at transducer focal region (Figure 7).

6. Place an acoustic absorber on the top of the tube to ensure that the ultrasound waves do not escape into the surrounding environment.
7. Press the 'START' button on the RF drive amplifier and trigger the ultrasound application on the tube. Troubleshooting 2.

   ***Note:*** In Methods video S5, we first show the effect in an Eppendorf tube with a fluid volume that is too large and second show the effect with the proper fluid volume. Visualize the tube after the experiment to ensure that insonation occurred (Figure 8).

8. Take off the tube from the sonication apparatus and rotate for 5 min at 20°C. Fix the tube using rubber bands on a rotator for thorough mixing of the sonoporated cells and plasmids. Troubleshooting 3.

   ***Note:*** To serve as controls, samples treated without ultrasound or without microbubbles are mixed on a rotator for the same duration.

   ***Note:*** For cell samples to be used in Part IV, rinse the tube with growth media and then transfer the cells to the appropriate well of a 6-well cell plate (see Part IV, Step 14 for details).


**Figure 8. Representative pictures of the solutions before and after sonication**

Based on observation, after shaking the cell-microbubble mixture, the larger microbubbles (MBs) tend to float quickly. The MBs with smaller sizes disperse in the mixture solution. The MB concentration in the infranatant was approximately $5 \times 10^4$/mL. Without ultrasound exposure, the mixture was stable for 3 min. The solution in the tube is turbid before sonication but is clear after sonoporation, indicating that the microbubbles in the infranatant are destroyed due to the ultrasound bursts.

## Part III: Validation of cell viability and sonoporation

Timing: 2 h

These steps validate the ultrasound parameters predicted based on microbubble dynamics simulation for efficient sonoporation *in vitro*. Propidium iodide (PI) is normally an indicator of dead cells, but due to the increased cell permeability induced by sonoporation, here PI is used as an indicator of sonoporated cells. Cal Green is used as an indicator of live cells.[5]

9. Following the sonoporation process, add 375 μL of PBS (+/+) to the sample to achieve a total volume of 0.5 mL. Troubleshooting 4.
10. Cell viability measurement.
    a. Take out 10 μL of sample and thoroughly mix with 0.4% (w/v) Trypan Blue solution (10 μL) using a pipette.
    b. Draw 10 μL of mixture and add to the cell counter. The cell counter will provide a count of viable cells in the sample.
    c. Calculate the total number of viable cells per sample by multiplying the number of viable cells per mL by the total sample volume (0.5 mL).
    d. Analyze the data and compare the cell viability between the experimental and control groups to determine the significant differences after sonoporation under different ultrasound conditions.
11. Centrifuge the cells in the tube (300 × *g*, 5 min, 4°C) and discard the supernatant.
12. Prepare the samples for confocal imaging:
    a. Resuspend the cells in 4% PFA (100 μL), and incubate for 10 min at 20°C in the dark.
    b. Centrifuge the cells in the tube (300 × *g*, 5 min, 4°C) and discard the PFA.
    c. Wash the cells with PBS (−/−) and centrifuge again (300 × *g*, 5 min, 4°C). Discard the PBS.
    d. Resuspend the cells in 30 μL of DI water.
    e. Add the cell suspension to a glass slide and spread the cells in a circle within a 12 mm diameter.
    f. Let the cell suspension dry for at least 30 min at 20°C.
    g. Add a drop of antifade mount reagent on the dried cell samples and carefully place the coverslip on top for further confocal imaging.
13. Confocal imaging of PI and Cal Green (Figure 9):

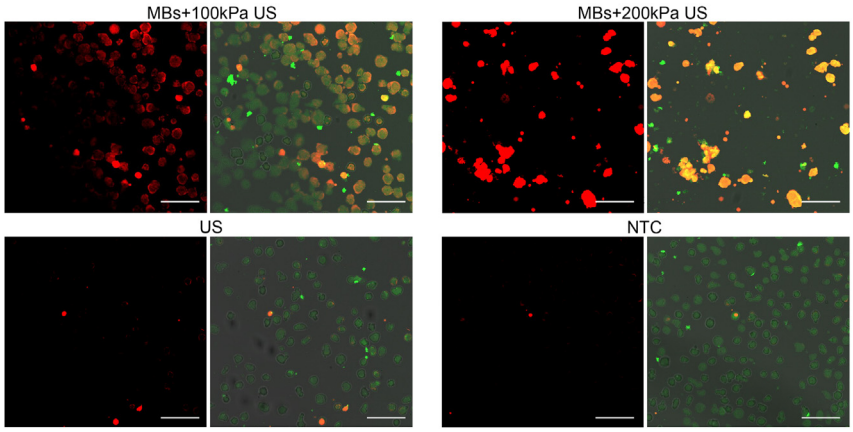



**Figure 9. Confocal images of cells treated with PI (red) and Cal Green (Green)**

Upon treating the sample with microbubbles (MBs) and PNP of 200 kPa ultrasound, a substantial amount of cell debris was observed, while the remaining viable cells exhibited highly saturated PI uptake. Similarly, the use of MBs with lower ultrasound pressure (100 kPa) induced sonoporation, but the PI uptake was lower than that of the MBs+200 kPa ultrasound treatment. Conversely, the samples treated with ultrasound alone or the no-treatment control (NTC) showed low PI penetration, indicating no sonoporation in cells. Scale bar = 50 μm.

a. Set up the microscope with the appropriate imaging settings, including the objective lens (40x oil lens), laser intensity, emission filters, and motorized stage for precise movement. PI: excitation peak (Ex) = 566 nm, and emission peak (Em) = 574 nm; Cal Green: Ex = 506 nm; Em = 531 nm.
b. Use the microscope to image the sample. Each image is acquired with specific imaging parameters such as the focal plane, exposure time, and laser intensity.

*Note:* Before imaging the experimental samples, adjust the confocal parameters using control samples of cells stained with only PI or Cal Green, as well as unstained cells.

## Part IV: Characterization of plasmid transduction

Timing: 24–48 h

These steps aim to assess whether the ultrasound treatment induces sonoporation in cells and determine if the resulting sonoporation effectively enhances plasmid transduction.

14. After sonoporation, add the plasmid-treated cell suspension to a well of a 6-well plate containing 875 μL cell growth media. The final total volume for each sample is 1 mL. Put the plates into the cell-culture incubator. Troubleshooting 4.
15. Collect supernatant samples and cell samples.
    a. Following a 24-h or 48-h incubation, collect the supernatant from the wells using a sterile pipette and transfer to sterile tubes. Keep the tubes at 4°C to store the samples until further analysis.
    b. Gently wash the cells with PBS (−/−) to remove any residual media. Aspirate the solution.
    c. Add 0.5 mL of TrypLE Express to cover the cells and incubate at 37°C for the recommended time (usually 5–10 min for hMSCs).
    d. Check the cells under a microscope to ensure they have detached from the bottom of the well and are in a single-cell suspension.
    e. Gently mix the cells with a pipette and transfer them to sterile tubes.

*Note:* To prepare for confocal microscopy analysis of adherent cells, the cell samples are seeded in wells containing uncoated 12-mm coverslips and allowed to adhere to the coverslip during culture. Do not use TrypLE Express to harvest the cells on the coverslips.

*Note:* The duration of incubation depends on the properties of the plasmids used and the cell types under investigation. For example, the optimal incubation time for observable GFP expression in hMSCs is typically about 24 h, whereas other plasmids (i.e., BMP-6) require up to 48 h to achieve optimal transfection efficiency.

*Note:* TrypLE Express is a trypsin-like enzyme that is commonly used for detaching cells from culture surfaces. Compared to Trypsin, TrypLE limits the effect on cell viability and yield by reducing damage to the cells during detachment. Additionally, TrypLE does not contain animal-derived components, making it more suitable for applications that require animal-free culture conditions. This is particularly important for research involving human cells for cell therapy or regenerative medicine purposes.

*Alternatives:* Trypsin with different concentration of ethylene diamine tetraacetic acid (EDTA) is also available for cell detachment, depending on the cell line and its behavior.

16. Flow cytometry for analyzing GFP expression levels.
    a. Centrifuge the cell samples in a TrypLE-treated cell tube (300 × *g*, 5 min, 4°C) and discard the supernatant.
    b. Resuspend the cells in 150 μL of PBS (−/−) and add the samples to the wells of a U-bottom 96-well plate.
    c. Centrifuge the plate (300 × *g*, 5 min, 4°C). Invert, flick and dab the plate to ensure all the liquid is removed.
    d. Resuspend the cells in 100 μL Live/Dead Blue mix (1:500 dilution in sterile PBS (−/−)), and incubate for 20 min at 4°C in the dark.
    e. Add 100 μL of PBS (−/−), and centrifuge the plate (300 × *g*, 5 min, 4°C). Invert, flick and dab the plate to remove PBS (−/−).
    f. Resuspend the cells in 200 μL of PBS (−/−).
    g. Prepare compensation controls by using a well with unstained cells, a well with Live/Dead stained cells, and a well with a drop of GFP compensation beads in 175 μL of PBS (−/−).

    *Note:* Alternatively, the cell samples can be fixed (refer to Step 12e) for future analysis. To maintain the accuracy of quantification, we recommend performing measurements on live cells or as soon as possible after fixation to minimize the impact of autofluorescence. Typically, fixed cells samples are stored at 4°C (in a refrigerator) for up to one week.

    h. Examine the samples using a Guava EasyCyte Flow Cytometer, and process the data with FlowJo Software for analysis. The gating strategy for quantification of the $GFP^+$ cell population is shown in Figure 10.

    *Note:* For further information, please see the product brochure of the Guava flow cytometer.

17. Confocal microscopy for imaging mNG expression in cells.
    a. Wash the cells on the coverslips in the 6-well culture plates with 1 mL PBS (−/−). Repeat this aspiration/wash step twice.
    b. Fix the cells with 500 μL of 4% PFA for 10 min at 20°C in the dark. Aspirate the PFA, and repeat this aspiration/wash step.
    c. Stain the cell nuclei with 500 μL DAPI working solution for 10 min at 20°C in the dark.
    d. Mount the coverslip with the cell samples onto a glass slide using a drop of antifade mount reagent.
    e. Set up the microscope with the appropriate imaging settings, including the objective lens (20x dry lens or 40x oil lens), laser intensity, emission filters, and motorized stage for precise movement.

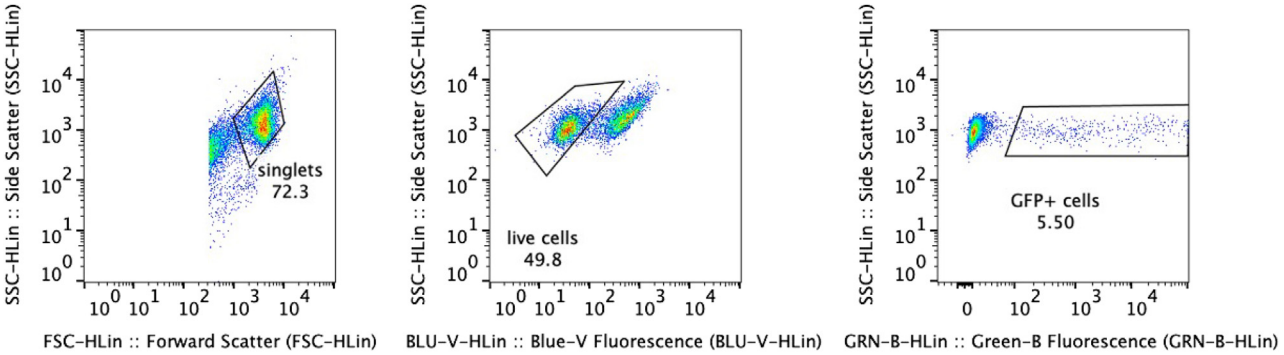



**Figure 10. Gating strategy for flow cytometry analysis**

It is important to carefully optimize and validate the gating strategy to ensure accurate and reliable data analysis. First, based on the forward scatter (FSC) and side scatter (SSC), create a gate for single cells. Next, set a live/dead discrimination gate based on Live/dead blue stain to exclude dead cells. Finally, identify the $GFP^+$ cell population relative to the negative control samples.

f. Use the microscope to capture multiple images of adjacent regions of the sample, ensuring complete coverage of the specimen with a grid or pattern. Each image is acquired with consistent imaging parameters, including the focal plane, exposure time, and laser intensity. DAPI: Ex = 355 nm, Em = 461 nm; mNG: Ex = 506 nm; Em = 517 nm.

18. ELISA for measuring the expression levels of the proteins of interest.

*Note:* Follow the manufacturer's specific online protocol for each ELISA kit. A general outline is described below which has been customized.

a. Prepare the working solution of Capture Antibody using PBS (−/−) to dilute the stock solution. Add 100 μL of Capture Antibody in the 96-well plate. Seal the plate and incubate for at least 12 h at 20°C.
b. Aspiration/wash step: Discard the solutions and wash the wells with Wash Buffer (repeat the washing process twice).
c. For plate blocking, add Reagent Diluent (300 μL) to each well. Incubate at 20°C for at least 1 h.
d. Add 100 μL of the sample collected in Step 15a above per well. Add 100 μL of the standard samples. Seal the plate and incubate 2 h at 20°C. After incubation, repeat the Step 18b. Troubleshooting 5.
e. According to the manufacturer's protocol, add Detection Antibody, Streptavidin-HRP, Substrate Solution, and stop solution to each well. Repeat Step 18b when changing the solution.
f. Immediately measure the optical density of each well at 450 nm with a microplate reader. Use a reference wavelength of 570 nm to correct for any background signal. Record the readings and use them to calculate the concentration of the analyte in the sample.

## EXPECTED OUTCOMES

In our protocol on gene delivery through *in vitro* sonoporation, we compared different ultrasound conditions (100–200 kPa) and the presence of Definity microbubbles (MBs). We found that MBs+200 kPa resulted in the highest transfection efficiency. Despite a decrease in cell viability to 51.0% after treatment with plasmids+MBs+200 kPa ultrasound (Figures 11 and 12), successful delivery of plasmids to cells and induction of transfection were observed. The population of pGFP-transfected cells increased to 3.09%, whereas for the other groups, the $GFP^+$ cells were less than 0.5% (Figure 13). The confocal images of pmNG expression also showed consistent results (Figure 14). To validate the transfection of the gene of interest, BMP-6 reached the expression level of 40.64 pg per million cells, compared to the group treated with MBs+150 kPa US at 15.75 pg per million cells. Ultrasound alone, sonoporation with 100 kPa ultrasound and microbubbles alone did not induce sufficient pBMP delivery (Figure 15).

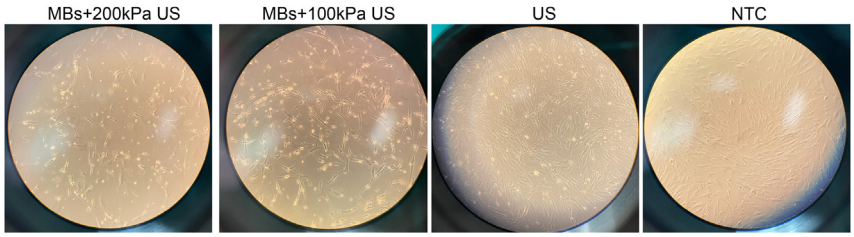



**Figure 11. Microscopy images showing cell morphology and confluency after sonoporation treatment**

Cell viability decreased when exposed to a PNP of 200 kPa, whereas the damage was reduced in the group treated with 150 kPa ultrasound. There was no significant damage in the group treated with microbubbles (MBs)+100 kPa ultrasound compared to the no treatment control (NTC) group.

Therefore, the reported outcomes of this *in vitro* sonoporation protocol on gene delivery demonstrate the feasibility of significant gene delivery through *in vitro* sonoporation using Definity microbubbles and optimized ultrasound conditions (center frequency = 250 kHz, PNP = 200 kPa, burst length = 33 ms, burst period = 4 s, treatment time = 3 min).

## LIMITATIONS

First, Definity microbubbles and other clinically-used microbubbles are non-targeted and have lower cell-binding efficiency compared to targeted microbubbles, as they are not specifically designed to interact with target cells or tissues.[6]

Additionally, *in vitro* sonoporation experiments are limited in their ability to fully replicate the complex physiological environment found *in vivo*, and the suitability of this method will vary depending on the specific application. *In vivo* sonoporation, for example, involves cells and microbubbles within a relatively confined and controlled ultrasound-treated region, resulting in more predictable proximity between cells and oscillating microbubbles, which improves transfection efficiency.[7]

Although this protocol is straightforward to implement and useful for initial validation of clinical ultrasound gene transfection, the resulting transfection efficiency may not be fully representative of the *in vivo* environment. Therefore, additional studies and optimization are necessary to confirm the effectiveness of *in vivo* sonoporation.

## TROUBLESHOOTING

### Problem 1

If there is no streaming observed in the sample during ultrasound application, and the cell viability after treatment remains as high as the non-treated samples, it suggests that interaction between cells and microbubbles was insufficient during insonation, and therefore limited the opportunities for sonoporation and plasmid transfer (Figure 16).

### Potential solution

- For a different transducer, consider adjusting the location of the Eppendorf tube relative to the transducer.
- To increase the proximity between cells and MBs and enhance the likelihood of sonoporation, decrease the total volume for each sample or increase the densities of cells and MBs.

### Problem 2

Microbubbles, particularly those with larger sizes, tend to float rapidly, resulting in a lower density of MBs in the infranatant and a lower MB to cell ratio. Inadequate microbubble-to-cell ratios limit the efficiency of plasmid transfection via sonoporation.

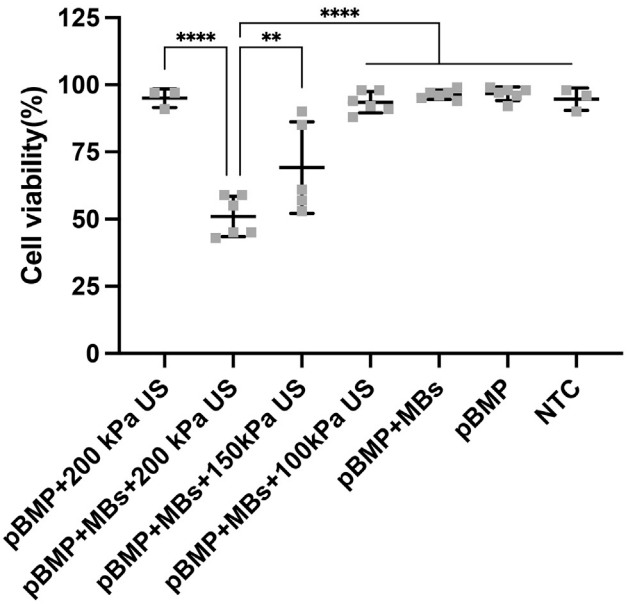



**Figure 12. Cell viability in the sample groups exposed to different sonoporation treatments**

After treatment of cells with microbubbles (MBs) with 200 kPa ultrasound, cell viability decreased to 51.0%, compared with 69.2% and 93.5% in groups treated with MBs and 150 or 100 kPa ultrasound, respectively. Notably, plasmid transfection with ultrasound (without MBs) did not induce significant damage to cells, as the cell viability was 95.0%, similar to that in groups of plasmid+MBs (96.0%), plasmid only (96.3%) or NTC (94.7%). Data are represented as mean ± SEM. Statistical analysis method: one-way ANOVA followed by Tukey's multiple comparisons test. **: $p < 0.01$, ****: $p < 0.0001$.

### Potential solution

- Prepare a blank tube with a volume of buffer equivalent to that of the experimental samples, add a defined number of microbubbles to the buffer, and thoroughly mix the contents. Let the sample stand for 3 min and measure the concentration of microbubbles in the infranatant using a particle counter (i.e., AccuSizer system). Based on the microbubble concentration in the infranatant, calculate the microbubble to cell ratio to match the predicted value.
- If the peaks of larger MBs (peaks at >4 μm) are higher in the size distribution curve (refer to Figure 2), the MB solution should be replaced.

### Problem 3

The solution remains turbid after ultrasound treatment, which can result from low acoustic pressure, high microbubble density or insufficient treatment time.

### Potential solution

- If the acoustic pressure is too low to destroy MBs, please return to the section of 'before you begin'. Here, repeat the transducer calibration procedure, measure the microbubble size, and repeat the microbubble dynamics simulation. These steps are essential for ensuring that the acoustic pressure exceeds the bubble rupture threshold.
- If the MB density is excessively high, MB interactions reduce oscillation. In this scenario, consider reducing MB concentration and adjust cell density accordingly. Recalculate their proximity to establish an appropriate concentration.
- Typically, MBs are disrupted after several pulses. However, if the solution is still turbid, extend the treatment time. The recommended duration for *in vitro* sonoporation is 3–5 min.

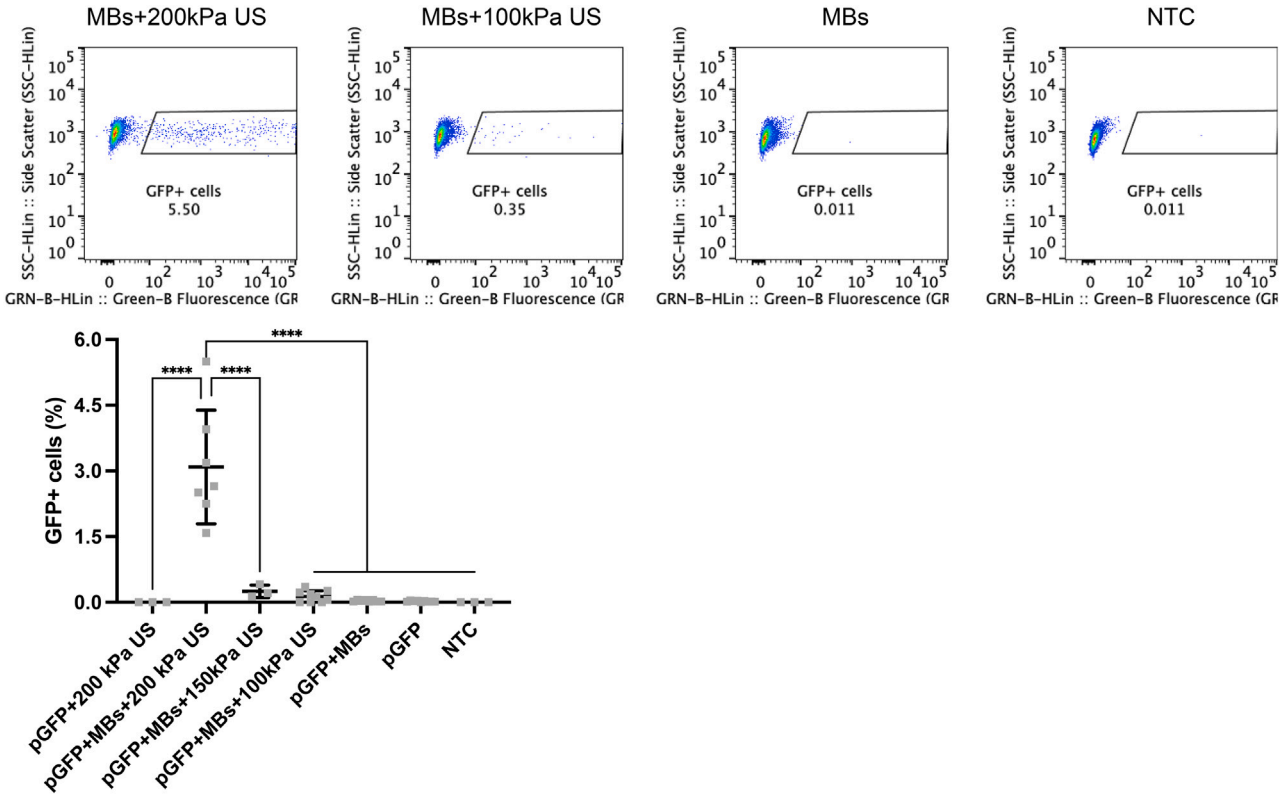



**Figure 13. Flow cytometry analysis of GFP$^+$ cell populations**

Based on the dot plots of flow cytometry, the population of GFP$^+$ cells increased with sonoporation. After the treatment of microbubbles (MBs) with 200 kPa ultrasound, the GFP$^+$ cell population was significantly enhanced, with an average percentage of 3.09%, whereas for the other sample groups, GFP$^+$ cells made up less than 0.5% of the total cell population. Data are represented as mean ± SEM. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test. ****: $p < 0.0001$.

### Problem 4

If the cell viability is measured to be extremely low (<20%) after ultrasound application, it could result from excessively high ultrasound pressure, high MB density or high MB-to-cell ratio.

### Potential solution

- Please return to the section of 'before you begin'. Recalibrate the transducer and reduce the acoustic pressure, ensuring that the acoustic pressure is suitable for sonoporation.
- Increase the cell density of the sample and adjust the doses of MB and plasmids accordingly.

### Problem 5

If the sonoporation reduces cell count, subsequent characterization is compromised.

### Potential solution

- After sonoporation, instead of using a 6-well culture plate, transfer the cell suspension from the Eppendorf tube to a smaller well (i.e., 12-or 24-well plate) with a reduced volume of growth media. The final characterization readouts should be adjusted based on the cell count and the total volume of the sample.
- If the expression level of a secreted protein is too low to be detected in ELISA assay, add a higher volume of supernatant or concentrate supernatant using an ultra-centrifugal tube, and calibrate the readout after measurement. For example, BMP-6 is concentrated using an ultra-centrifugal tube with a molecular weight cutoff (WMCO) of 3 k.

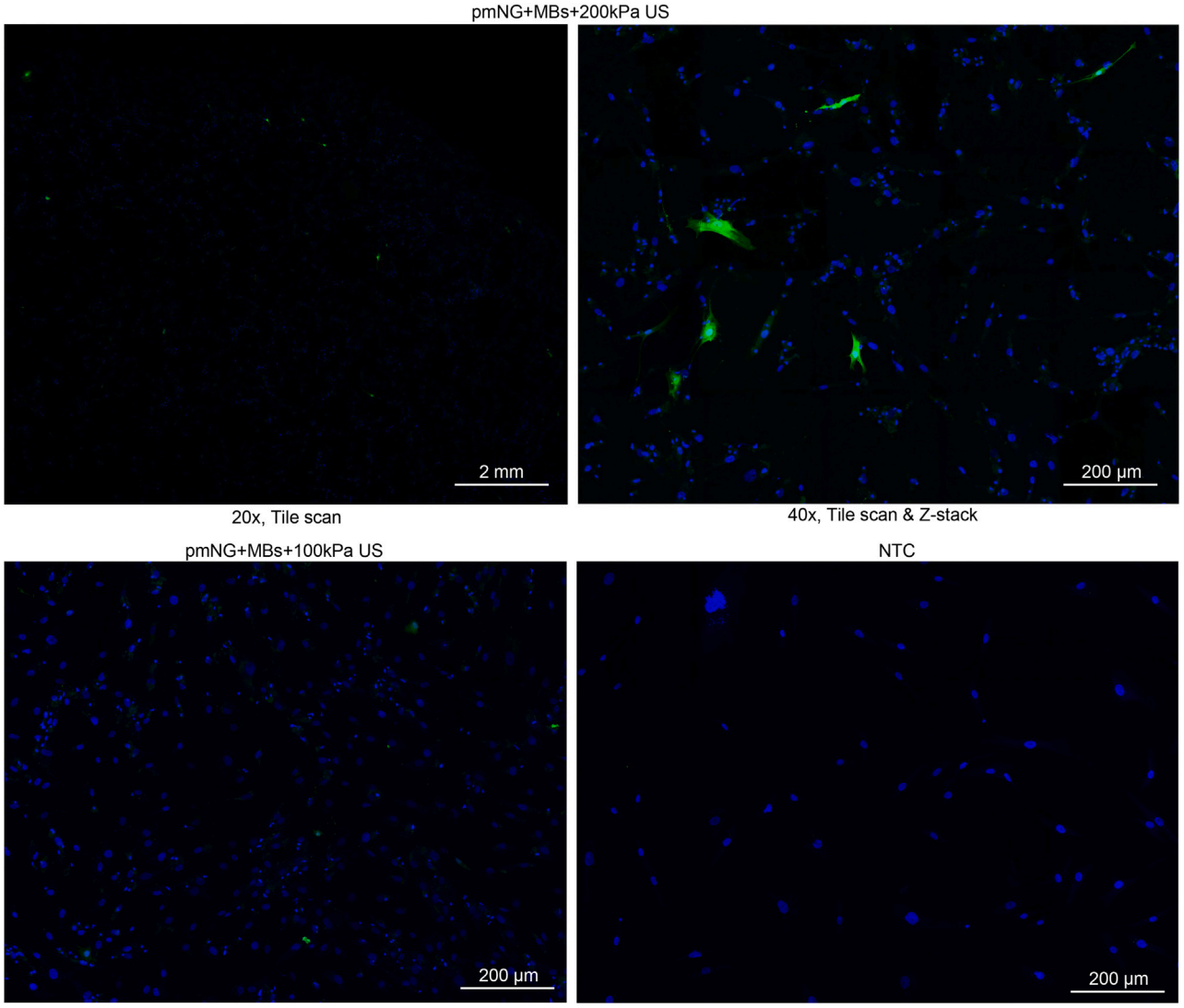



**Figure 14. Confocal microscope images showing pmNG expression in cells after sonoporation**

Following sonoporation treatment and incubation for 24 h, pmNG is expressed in hMSCs. To capture the entire region, the sample is initially scanned using a 20x dry lens to capture the entire region. After that, the microscope is switched to a 40x lens for scanning the regions of interests (ROIs) using z-stacking. The mNG signal is represented by green fluorescence, while cell nuclei are stained blue. As quantified by ImageJ, the overall images showed approximately 4% of cells exhibit mNG fluorescence signal in the pmNG+MBs+200 kPa US cell group, which was consistent with the results obtained from flow cytometry analysis.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Katherine W. Ferrara (kwferrar@stanford.edu).

### Materials availability

This study did not generate new unique reagents. The human BMP-6 plasmids used in this study and other relevant plasmids will be made available if possible on request from National Center for Advancing Translational Sciences (NCATS).

### Data and code availability

The software programs that were used in this study are listed in the key resources table.

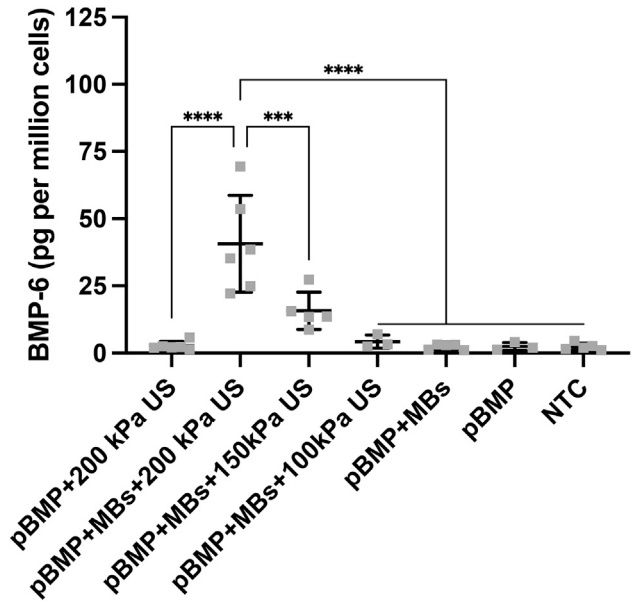



**Figure 15. BMP-6 expression levels in different treatment groups using ELISA quantification**

After sonoporation and 48-h incubation, pBMP was expressed and the cell supernatant was collected for ELISA analysis to quantify BMP-6 expression level. The readouts were normalized to live cell counts and the total volume of media. The group of cells treated with MBs+200 kPa US showed significantly higher BMP-6 secretion levels (40.64 pg per million cells), compared to the group treated with MBs+150 kPa US (15.75 pg per million cells). Ultrasound alone, sonoporation with 100 kPa ultrasound and microbubbles alone did not induce sufficient plasmid delivery. Data are represented as mean ± SEM. Statistical analysis: one-way ANOVA followed by Tukey's multiple comparisons test. ***: $p < 0.01$, ****: $p < 0.0001$.

Any additional information required to reanalyze the data reported in this study is available from the lead contact upon reasonable request.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102723.

## ACKNOWLEDGMENTS

The authors would like to thank Dr. Gadi Pelled from SonoStem Technologies, Inc. for providing expert advice on the study design and Alon Shani for the design of the vial holder stage. This work was supported by the Intramural Research Program of the National Center for Advancing

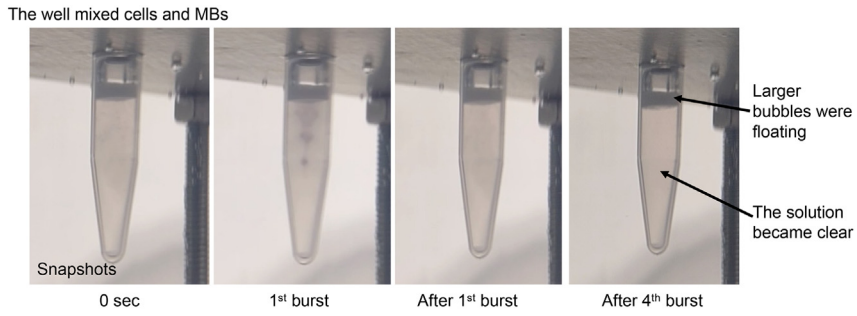



**Figure 16. Example snapshots of the cell-microbubble mixture during ultrasound treatment**

The cell-MB mixture was sonicated immediately after shaking, and we observed the streaming within the tube along with the destruction of microbubbles after each pulse. By the fourth pulse (approximately 16 s), the mixture became completely clear, indicating successful destruction of microbubbles in the infranatant. Larger microbubbles present in the floating cake, however, remained intact.

Translational Sciences (NCATS) at the National Institutes of Health (NIH). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services (DHHS), nor does mention of the trade names, commercial products, and organizations imply endorsement by the United States government. The Graphical Abstract was prepared with BioRender.com.

## AUTHOR CONTRIBUTIONS

K.W.F. and N.Z. designed the study. Y.G. and N.Z. performed the computational simulation. S.K.T. cultured the cells. N.Z., Y.G., and J.F. performed sonoporation experiments. R.P. performed flow cytometry. N.Z. analyzed the data. N.Z. and K.W.F. wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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