

Ultrasound-induced release of micropallets with cells

Sijia Guo,¹ Yuli Wang,² Nancy Allbritton,^{2,3} and Xiaoning Jiang^{1,a)}

¹*Department of Mechanical and Aerospace Engineering, North Carolina State University, Raleigh, North Carolina 27695, USA*

²*Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA*

³*Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA and North Carolina State University, Raleigh, North Carolina 27695, USA*

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Separation of selected adherent live cells attached on an array of microelements, termed micropallets, from a mixed population is an important process in biomedical research. We demonstrated that adherent cells can be safely, selectively, and rapidly released from the glass substrate together with micropallets using an ultrasound wave. A 3.3-MHz ultrasound transducer was used to release micropallets ($500\ \mu\text{m} \times 500\ \mu\text{m} \times 300\ \mu\text{m}$) with attached HeLa cells, and a cell viability of 92% was obtained after ultrasound release. The ultrasound-induced release process was recorded by a high-speed camera, revealing a proximate velocity of $\sim 0.5\ \text{m/s}$. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4757648>]

Separation of selected cells from a mixed population is an important process in many biomedical research areas including genetic engineering, stem cell research, and regenerative medicine. For example, to study the function of individual genes and proteins, genetically engineered cells must be isolated from a mixed cell sample first. To understand the properties of undifferentiated stem cells, they must be selected from more differentiated cells. To develop human cell lines for personalized medicine, isolation and cloning of desirable cells from a patient's biopsy is required.¹⁻³ A number of technologies have been developed to separate live cells.⁴⁻⁶ Fluorescence-activated cell sorting (FACS) and limiting dilution with or without antibiotic selection are the two most common methods.⁷ However, both strategies require the cells to be detached from the cultured surface prior to the sorting process, which may impose cell stress or lead to programmed cell death, especially for sensitive cells such as stem cells and primary cells.^{8,9} As a result, it is desirable to develop a stress-free technology that can perform gentle cell isolation while cells remain attached on the cultured surface.

In recent years, microarray technologies were developed to isolate adherent cells by using an array of releasable microfabricated elements, termed pallets, or rafts. The pallets were fabricated from a biocompatible, transparent, 1002F epoxy photoresist on a microscope slide by standard photolithography.^{10,11} The pallets can be varied in size from tens to hundreds of microns to provide an adequate growth area for single cells or colonies. In addition, the pallet surfaces can be chemically modified with polymers, or proteins to enhance cell attachment and growth.¹² To select and sort cells using the pallet arrays, cells are initially placed in suspension and allowed to settle and grow on individual pallets of the array, and the targeted cells are released later together with the pallets. Over the last few years, release of pallets by a laser pulse¹⁰⁻¹⁴ and release of rafts by a microneedle¹⁵ have been investigated for isolation of adherent live cells.

However, the laser pulse was unable to detach large micropallets (e.g., $500\ \mu\text{m} \times 500\ \mu\text{m}$) from glass substrate because a high laser energy density is needed yielding poor cell survival.¹⁶ Needle-based mechanical release involved piercing the elastomeric substrate to dislodge the rafts, and this release process is relatively slow. The objective of this Letter is to investigate the strategy based on ultrasound waves to release pallets for isolation of adherent live cells with high cell survivability and potentially featuring high throughput cell release.

To investigate the interaction between an ultrasound wave and microfabricated elements, arrays of pallets were fabricated on glass substrates (pallet dimension: $L \times W \times H = 500\ \mu\text{m} \times 500\ \mu\text{m} \times 300\ \mu\text{m}$, spacing between two neighboring pallets = $200\ \mu\text{m}$). The pallet cassette dish and collection dish were fabricated from polycarbonate by Computer Numerical Control machine, and the micropallets were fabricated on a glass substrate with a thickness of 1 mm. Figure 1 shows the schematic of the experimental setup for pallet release using ultrasound. A 3.3-MHz ultrasound transducer (Blatek Corporation) was positioned in water with the focal plane at the pallet/glass interface and was used to deliver ultrasound waves. The micropallets with living cells were immersed in the culture medium on the other side of the substrate. This process of pallet release was recorded using a high-speed camera (Phantom 640, Ametek), which provided 0.5-ms-interval resolution for a close monitoring of pallet release. Two Light-Emitting Diode light sources were used to improve the quality of transmitted light imaging.

A 3.3-MHz focused ultrasound transducer was used for pallet release experiments. According to the transducer simulation using Field II,¹⁷ the diameter of 6 dB focal beam is 0.51 mm. This transducer has a 29-mm-diameter aperture and 30-mm focal length, and the output power can be adjusted as high as 30 W under a continuous sinusoidal signal. The driving signal was generated from a function generator (AFG3101, Tektronix) and amplified by a RF power amplifier (3700, ENI). The input signal was set to match the center frequency of the transducer. The output acoustic

^{a)}Electronic mail: xjiang5@ncsu.edu.

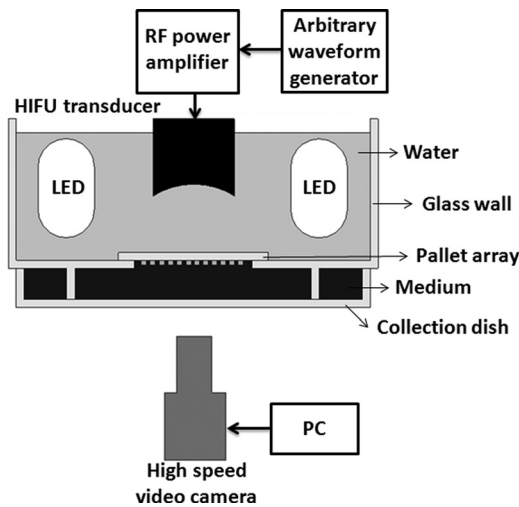


FIG. 1. Schematic of the experimental setup for pallet release.

power of the transducer was calibrated using an acoustic power radiation balance (UPM-DT-1AV, Ohmico) under different input electrical power.

For cell release experiments, HeLa cells were grown on the fibronectin-coated pallet array at 37°C for 24 h. Two hours prior to the release experiment, HeLa cells were co-stained with 5 μ M Calcein acetomethoxy (AM) and 5 μ M SYTO 17 (Invitrogen Corporation) for 30 min at 37°C. SYTO 17, a cell-permeant red fluorescent nucleic acid stain, which exhibits bright, red fluorescence upon binding to nucleic acids, was used to facilitate cell counting. Calcein AM is a cell-permeant molecule that is used as a cell-viability indicator. Immediately prior to use, the growth media was removed from the cell chamber and replaced with phosphate buffered saline (PBS). To determine cell viability, released cells co-stained with calcein AM and SYTO 17 were imaged by a transillumination and fluorescence microscopy (Nikon TE200) equipped with FITC and Texas Red filter sets. Viable cells show both calcein AM and SYTO 17 staining, while dead cells show only SYTO 17 staining.

Single-pallet release was achieved within a short ultrasonic exposure time (varies from 100 ms to 1 s) without affecting neighboring pallets. Figures 2(a) and 2(b) shows the array of pallets with living cells before and after the single pallet release, respectively. The targeted pallet is indicated by the arrow. To enable cell release, the target pallet was aligned to the acoustic axis of the ultrasound transducer. After the amplified signal to the ultrasound was triggered on at the output power of 10 W, the selected pallet disappeared in the field of view, indicating a complete release of the pallet (Fig. 2(b)). After single-pallet release, the ultrasound had no effect on the surrounding non-targeted pallets and the cells.

All released pallets settled down to the collection dish by gravity force. The viability of cells attached to targeted pallets after ultrasound release was examined by the fluorescence. Examples of one released pallet with live cells were shown in Figures 2(c)–2(e), where the pallet had 95 live cells (both green and red colors) and 14 dead cells (red color). 92.2% of the cells remaining on the pallets (number of pallets counted $N = 12$) were found to be viable after release.

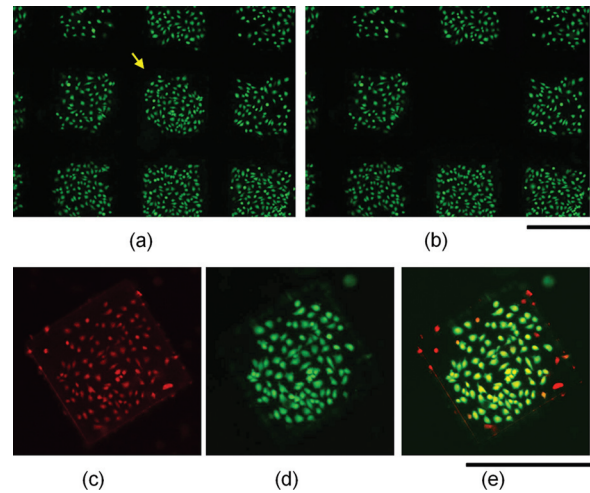


FIG. 2. Ultrasound induced pallet release with HeLa cells. (a)–(b) Selective release of a single 500 μ m micropallet with HeLa cells. HeLa cells were stained with Calcein AM, a cell-permeant dye that can be used to determine cell viability. Pallet array before the single pallet release (arrow points to the pallet to be released) (a), and after release (b). (c)–(e) Collected 500 μ m micropallet with HeLa cells. The cells were co-stained with SYTO 17 (c) and Calcein AM (d) to compare live and dead cells. (e) Merged image. 95 cells from a total number of 109 were alive. Scale bar = 500 μ m.

To analyze the mechanism of ultrasound-enabled micropallet release, we monitored the process of pallet release at 0.5 ms intervals using a high-speed camera (Fig. 3). Unlike laser-based pallet release,¹⁸ no plasma formation was identified during the ultrasound-enabled detachment as observed from the images recorded by the high-speed camera. The releasing velocity of the micropallet was estimated to be 0.5 m/s, considering the length of the focal zone of the high-speed camera and the time needed for the released pallet passing the focal zone. This relatively smooth separation, compared to the release speed of 45~60 m/s in laser-based pallet release,¹⁸ may contribute to the high viability of cells after ultrasonic pallet release. Rotation of the pallets after detachment was observed similar to that found in laser-based pallet release.¹⁴

Ultrasound has been used in global population studies of cells.^{19,20} In this Letter, ultrasound was used to enable single pallet/cell release and selective release of micropallets with cells. Acoustic radiation force was discussed to show the effect while an ultrasound beam acting on a target, which is caused by the acoustic pressure and the momentum

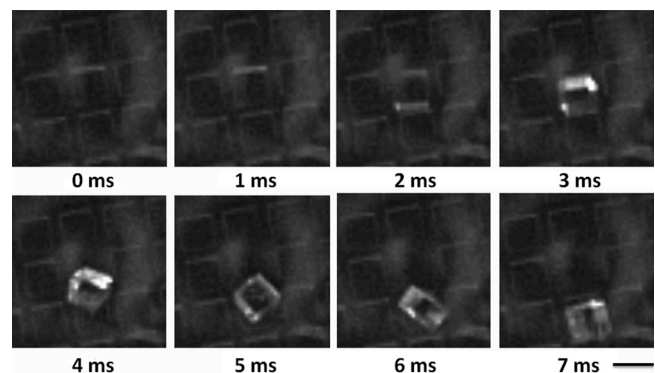


FIG. 3. Photograph pictures from a movie (7 ms) of pallet release process recorded using a high-speed camera. Scale bar = 500 μ m.

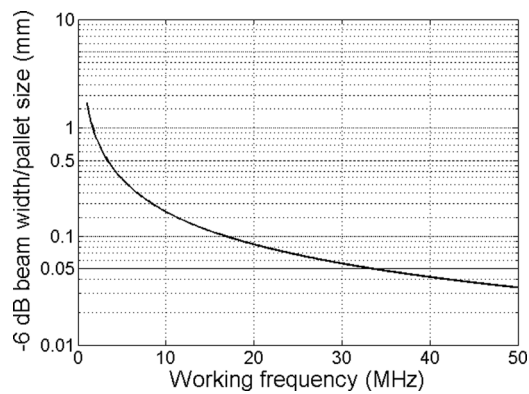


FIG. 4. The relationship between working frequency and -6 dB ultrasonic beam width at a fixed F-number (F-number = 1.03). From the experimental finding in this Letter, a single pallet can be released if its size is about the -6 dB beam width.

transported by the beam.^{21,22} The acoustic radiation force provides the major influences on the particles inside the acoustic field, and drives them towards the direction of acoustic streaming.²³ The acoustic radiation force acted on micropallets could be estimated when it is assumed to be a plane field propagating along the vertical direction,²⁰ since the length of the -6 dB focal zone of this 3.3-MHz transducer is as long as 4.34 mm based on the acoustic field simulation. The radiation force is estimated following the formalism derived by Gor'kov.^{24,25}

$$F(z) = \frac{\pi}{2\rho_0 c_0^3} \left(f_1 + \frac{3}{2} f_2 \right) \cdot V \cdot P_0^2 \cdot v \cdot \sin \left(2\pi \frac{z}{\gamma_0 l^2} \right),$$

where z is the distance from the transducer surface along its acoustic axis, v is the frequency, V is the particle volume in the acoustic field, p_0 is the pressure amplitude, and γ_0 is the acoustic wavelength in the medium. The dimensionless factors $f_1 = 1 - \frac{\rho_0 c_0^2}{\rho c^2}$ and $f_2 = \frac{2(\rho - \rho_0)}{2\rho + \rho_0}$ are determined by the density and sound velocity of the medium around the pallets. Based on those assumptions, the radiation force acting on the $500 \mu\text{m}$ -size, $300 \mu\text{m}$ -height pallet at 10 W output power of the transducer is calculated to be $14.9 \mu\text{N}$, which has been proved to be safe for HeLa cells attaching on those pallets according to the experimental results. For transducers with similar F-number (e.g., 1.034, same as the transducer used in the tests), the relationship between the working frequency and -6 dB beam width can be simulated as shown in Fig. 4. Assuming that the radiation pressure required to release a pallet is the same as the one found in our experiments ($14.9 \mu\text{N}/(\pi \times (0.25 \text{ mm})^2)$), the size of a pallet that can be released is about the -6 dB beam size and can then be estimated as a function of ultrasound frequency (Fig. 4). One can find that the pallets with larger culture surface require lower-frequency ultrasound, while high frequency ultrasound may be used for micro-pallet release. For example, a 34 MHz transducer may be required to release a single $50 \mu\text{m}$ pallet since the -6 dB beam width of the transducer is about the pallet size under the above assumptions. Pallet height, -6 dB beam length, and the total acoustic path length may also be considered in ultrasonic pallet release. Further-

more, the increased acoustic attenuation at high frequencies may be the limiting factor of using high frequency ultrasound in pallet release.

In summary, ultrasound-enabled microfabricated pallets release with adherent cell attached on them has been demonstrated. This highly selective pallet/cell release method yields significantly higher cell viability after single-pallet release, comparing with other existing methods, largely resulting from the relatively slow release speed and low radiation forces. The demonstrated selection, separation, and collection of specific adherent cells from a mixed cell population using the ultrasonic method holds great potential for biomedical research, including genetic cell engineering and stem cell engineering toward effective drug discovery and therapy exploration.

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