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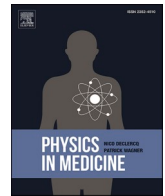
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## A brief note on experimental setups for in-vitro optical observations of HeLa cells and phospholipid-shelled microbubbles subjected to ultrasound

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### ABSTRACT

In a recent issue of *Physics in Medicine*, Akashi et al. demonstrated increased mortality of HeLa cells in combination with phospholipid-shelled microbubbles under high-amplitude sonication in an in-vitro experimental setup [1]. The authors attributed the increased mortality to cell damage caused by microbubble dynamics and mentioned the term sonoporation early in their paper. Their findings are a most valuable contribution with regards to ultrasonic imaging safety standards. However, the findings would be even more beneficial to the drug delivery community, provided they could be linked to previous outcomes on in-vitro experimental sonoporation. The purpose of this paper is to briefly underscore previous works with similar setups, highlighting the many agreements between early and recent work.

In a recent issue of *Physics in Medicine*, Akashi et al. demonstrated increased mortality of HeLa cells in combination with phospholipid-shelled microbubbles under high-amplitude sonication in an in-vitro experimental setup [1]. The authors attributed the increased mortality to cell damage caused by microbubble dynamics and mentioned the term sonoporation early in their paper. Their findings are a most valuable contribution with regards to ultrasonic imaging safety standards. However, the findings would be even more beneficial to the drug delivery community, provided they could be linked to previous outcomes on in-vitro experimental sonoporation. The purpose of this paper is to briefly underscore previous works with similar setups, highlighting the many agreements between early and recent work.

Sonoporation is the transient permeation of cell membranes by means of ultrasound, typically assisted by the presence of microbubbles. Since the earliest observations of this phenomenon, sonoporation has been associated with the increased uptake of drugs and genes by cells under sonication [2]. Most of these early studies were carried out with cell cultures, under the assumption that the conditions of successful sonoporation in a culturing chamber could eventually be scaled up to sonoporation-based treatment in humans. An overview of such early sonoporation studies was published in 2011 [3, Chapter 9]. Cancer cells were the obvious choice for sonoporation experiments, as to investigate the feasibility of ultrasound-assisted treatment. Before sonication, an ultrasound contrast agent, typically comprising

phospholipid-shelled microbubbles, would be added to the cell culture.

Following basic *before-sonication* — *after-sonication* studies, the interest arose to study sonoporation in real-time. Given the microscopic sizes of the cells and microbubbles involved, and given their ultra-fast dynamics, such observations required high-numerical-aperture microscopy and high-speed photography. This in itself presented a major challenge, namely how to avoid acoustic interference from a microscope objective at a distance of less than a wavelength from the cells under observation. Around the world, setups were designed with long-distance objectives and large-aperture transducers [4–6].

The now famous experiments by Van Wamel et al. on endothelial cells mixed with phospholipid-shelled microbubbles sonicated at 1 MHz and observed at ten million frames per second [7] were followed by experiments under the same conditions, but now also including fluorescence microscopy [8]. Despite the high frame rates of these groundbreaking works, the total observation duration was limited to a few sonication periods.

Delalande et al. incorporated confocal fluorescence microscopy in their setups, as well as a colour camera that recorded at frame rates up to 10 kHz [9]. Aware of destructive effects at higher acoustics amplitudes, they subjected a mixture of HeLa cells and phospholipid microbubbles to low-amplitude ultrasound and observed microbubbles travelling through the HeLa cell membranes into the cells [9,10]. The low-amplitude acoustic settings used in these in-vitro experiments were

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translated to those chosen for the successful clinical studies on sonoporation treatment of human pancreatic cancer [11,12].

Still, the question remains why oscillating microbubbles interact with cells and why some cells respond to ultrasound, even at moderate acoustic amplitudes and without microbubble presence [13]. The sonophore hypothesis proposed rectified diffusion into cell membranes, supposedly causing the membranes to dynamically respond to ultrasound [14]. This hypothesis has not been confirmed nor refuted. As the number of groups working on cell sonoporation is growing, we are anticipating an answer to this important question.

#### Declaration of competing interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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