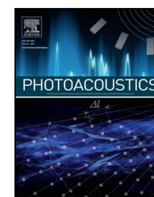
Photoacoustics 6 (2017) 16–25

Contents lists available at ScienceDirect

Photoacoustics

journal homepage: www.elsevier.com/locate/pacs



Photoacoustic flow cytometry for nanomaterial research

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ARTICLE INFO

Article history:
Received 29 September 2016
Received in revised form 31 January 2017
Accepted 14 March 2017
Available online 27 March 2017

Keywords:
Photoacoustic
Flow cytometry
Nanotoxicity
Graphene
Gold nanorods

ABSTRACT

Conventional flow cytometry is a versatile tool for drug research and cell characterization. However, it is poorly suited for quantification of non-fluorescent proteins and artificial nanomaterials without the use of additional labeling. The rapid growth of biomedical applications for small non-fluorescent nanoparticles (NPs) for drug delivery, image contrast and therapy enhancement, as well as research focused on natural cell pigments and chromophores, demands high-throughput quantification methods for the non-fluorescent components. In this work, we present an advanced novel photoacoustic (PA) fluorescence flow cytometry (PAFFC) platform that integrates NP quantification though PA detection with conventional sample characterization using fluorescence labeling. PAFFC simplifies high-throughput analysis of cell-NP interactions, optimization of targeted nanodrugs, and NP toxicity assessment by providing a direct correlation between NP uptake and characterization of toxicity markers for every cell.
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1. Introduction

Understanding the interactions between nanomaterials and cells is becoming increasingly important due to the rapid growth of biomedical research focused on the use of various nanoparticles (NPs) as drug delivery vehicles and imaging and therapy enhancers [1–4]. A variety of NP characteristics, including structure, surface chemistry, and physical properties, dramatically affects cell-NP interactions [3–7]. Recent nanotechnology advances in diagnosis and targeting of tumors, especially circulating tumor cells (CTCs) *in vitro* [8,9] and *in vivo* [10,11], have demonstrated that specificity and efficacy of cell targeting are essential for successful NP-assisted diagnosis and therapy [9,12,13]. However, traditional methods for the quantification of NPs at the single cell level have limited throughput (electron microscopy) [14], low sensitivity (light scattering detection) [15–17], and/or the inability to analyze low atomic weight elements (electron microscopy [2,18],and mass spectroscopy) [1,19]. For fluorescent NPs, conventional flow cytometry (FC) [20,21] and microscopy [22,23] may provide high throughput cell counting, sorting, and quantification of nanomaterials [24]. However, current FC systems, though compatible with fluorescent NPs, are not suited well for non- or weakly-fluorescent materials due to their low sensitivity of absorption detection [24] and the significant scattering/auto-fluorescence background [17,24]. FC quantification of NPs in cells using light scattering has been reported for gold nanorods (GNRs) [25], TiO_2 and ZnO particles [26,27], and some 80–100 nm gold NPs [17]. The major drawback of light scattering detection is the rather low light scattering of NPs smaller than 100 nm [28] and the strong light scattering background of cell structures. This limits detection sensitivity, even under ideal static conditions (e.g. in microscopy) [16]. Fluorescent labeling of non-fluorescent NPs using chemical attachment of fluorescent tags makes it possible to use conventional FC analysis for NP uptake quantification; [29] however, such chemical modifications may dramatically change NP properties, including *in vivo* toxicity and/or targeting specificity, and, thus, are not desirable in the analysis of an NP toxicity profile.

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http://dx.doi.org/10.1016/j.pacs.2017.03.002
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The high throughput quantification of non-fluorescent NPs, intrinsic cell chromophores, and pigments (*e.g.*, hemoglobin or melanin in melanoma cancer cells) [11,30–39] at a single cell level can be done using light absorption contrast as an alternative to fluorescent labeling. The level of absorption sensitivity required for NP detection is achievable using photoacoustic (PA) detection, which is based on detection of acoustic waves generated in the sample upon absorption of laser irradiation followed by non-radiative relaxation and sample heating (Fig. 1). PA contrast is common for most nanomaterials (including fluorescent quantum dots) [33], light-absorbing proteins, and dyes. PA detection can be performed in static conditions and in flow, even in the presence of significant light scattering and auto-fluorescent backgrounds [37,40,41]. We previously demonstrated high-speed PA detection in flow as well as the feasibility of PA detection for various NPs, including single cells and NPs at flow velocities up to 3 m/s [11,35,40]. PA detection of cells and NPs has also been demonstrated in mouse blood [11,34,35], lymph [42], tomato plants [43], and artificial vessels [35]. The PA phenomenon demonstrates remarkable spectral selectivity [36], high sensitivity (single particle level) [31,33], and label-free absorbance quantification [11,37]. Still, full integration of PA technology into conventional flow cytometry has not been reported yet. Current PA detection in flow either lacks speed (*e.g.*, 10–20 Hz laser pulse repetition rate [PRR]) [41,44,45] or does not provide multimodal (scattering, fluorescence, absorbance) analysis of single cells in fast flow [30,37,46].

Here, we report on the development of an integrated *in vitro* PA-fluorescence flow cytometry technique (PAFFC) that extends the range of application of conventional FC, allowing highly sensitive absorbance quantification and single NP detection sensitivity. PA detection serves as an additional source of data for complete cell characterization without compromising conventional fluorescence and light scattering detection of various cell biomarkers.

2. Materials and methods

2.1. PAFFC

The PAFFC system was built on the basis of an upright microscope (Nikon Eclipse E400, Nikon Instruments, Inc., Melville, NY, USA) with an acoustic transducer (V316-SM, 20 MHz, 12 mm focal distance, 150 μm focal area, Olympus) mounted over flow cells on an XY positioning stage (Fig. 2). The flow module of the cytometer was built using a quartz capillary (Molex Inc., Phoenix, AZ) with a 100 × 100 μm square cross-section placed on the bottom of a water-filled chamber. The microscope condenser was replaced with a custom laser delivery and fluorescence collection optics featuring 20× micro-objective (PlanFluor, NA 0.4; Nikon Instruments, Inc.). The setup was equipped with an 820-nm diode-pumped pulsed laser (for PA excitation) with a maximal energy of 35 μJ, pulse duration of 8 ns, and pulse rate of 10 kHz (LUCE 820, Bright Solutions, Italy). Fluorescence was excited using a continuous wave (cw) diode 488 nm laser (IQ1C45 (488-60) G26, Power Tech., Alexander, AR, USA) providing 7 mW power in the sample. Laser beams were shaped using cylindrical lenses and focused inside the capillary. Both lasers formed 5 × 150 μm lines across the main capillary axis. Fluorescence was collected through the same objective and separated from excitation light using several dichroic mirrors and a bandpass filter (FF01-520/15, Semrock Inc., Rochester, NY). A photomultiplier tube (R3896, Hamamatsu Co., Bridgewater, NJ) connected to a high-voltage pre-amplifier (C6271, DC to 10 kHz bandwidth, Hamamatsu Co., Bridgewater, NJ) was used to measure the intensity of collected fluorescent light. PA signals from the transducer were amplified (preamplifier 5678; bandwidth, 200 kHz–40 MHz; gain 40 dB; Panametrics NDT) and digitized (PCI-5124, 12-bit, 200 MSPS, National Instruments Inc.). Custom-developed software recorded amplitudes of PA signals for each laser pulse, along with the second channel data for recording signals from photomultiplier tube (PMT) signal voltage. Both traces were displayed in real time and saved for later off-line peak detection and other statistical analysis. All the data acquisition and analysis were performed using custom LabView-based software.

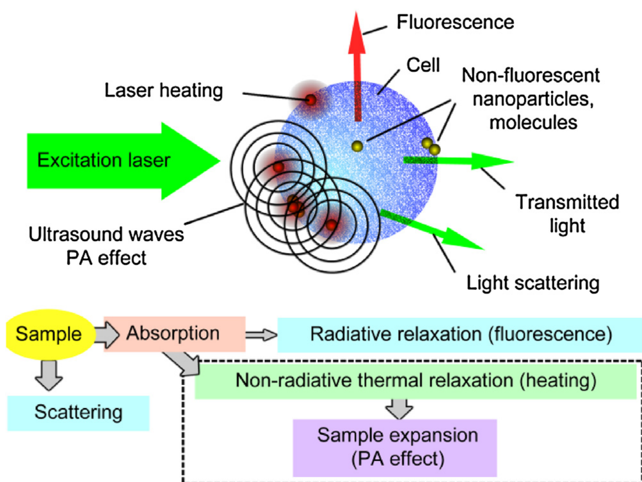

Fig. 1. Interaction of light with cells, NPs, chromo- and fluorophores, and corresponding phenomena.<!——!>

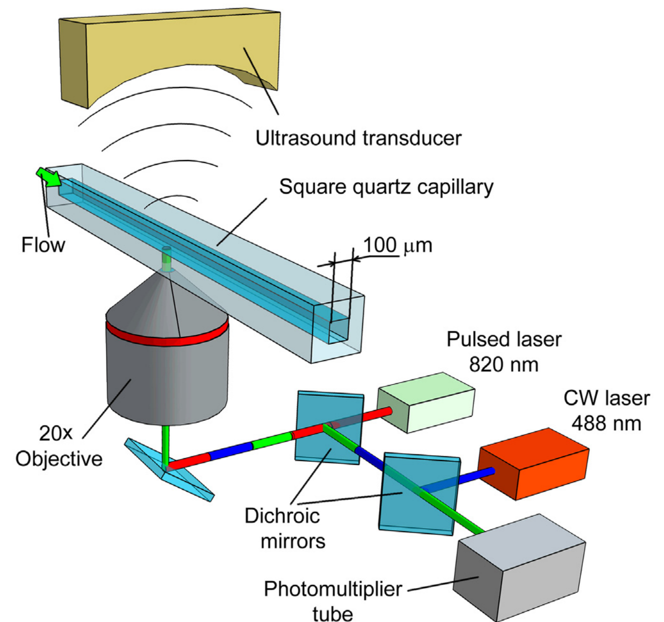

Fig. 2. General schematics of PAFFC system.

2.2. Enhanced dark-field microscopy

Dark-field imaging using light scattering contrast of cells incubated with NPs was performed using an enhanced illuminator, CytoViva 150 (CytoViva Inc., Auburn, AL), and Solarc 24W metal halide fiber light source (Welch Allyn, Skaneateles Falls, NY). Images were taken using a 100× objective (Olympus UPlanAPO fluorite, N.A. 1.35–0.55) with a high-resolution color camera (DP72, Olympus America Inc.).

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