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Color-coded perfluorocarbon nanodroplets for multiplexed ultrasound and Photoacoustic imaging

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Abstract

Laser-activated perfluorocarbon nanodroplets are an emerging class of phase change, dual-contrast agents that can be utilized in ultrasound and photoacoustic imaging. Through the ability to differentiate subpopulations of nanodroplets via laser activation at different wavelengths of near-infrared light, optically-triggered color-coded perfluorocarbon nanodroplets present themselves as an attractive tool for multiplexed ultrasound and photoacoustic imaging. In particular, laser-activated droplets can be used to provide quantitative spatiotemporal information regarding distinct biological targets, allowing for their potential use in a wide range of diagnostic and therapeutic applications. In the work presented, laser-activated color-coded perfluorocarbon nanodroplets are synthesized to selectively respond to laser irradiation at corresponding wavelengths. The dynamic ultrasound and photoacoustic signals produced by laser-activated perfluorocarbon nanodroplets are evaluated *in situ* prior to implementation in a murine model. *In vivo*, these particles are used to distinguish unique particle trafficking mechanisms and are shown to provide ultrasound and photoacoustic contrast for up to 72 hours within lymphatics. Overall, the conducted studies show that laser-activated color-coded perfluorocarbon nanodroplets are a promising agent for multiplexed ultrasound and photoacoustic imaging.

Graphical Abstract

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Multiplexed ultrasound and photoacoustic imaging is achieved through the use of color-coded perfluorocarbon nanodroplets. Specifically, distinct subpopulations of laser-activated perfluorocarbon nanodroplets produce optical wavelength-selective ultrasound and photoacoustic signals. The dynamic ultrasound and photoacoustic contrast produced by these agents allows the simultaneous acquisition and discrimination of quantitative spatial information from distinct biological targets, which were tested and demonstrated *in vivo*.

Keywords

multiplexed imaging; perfluorocarbon nanodroplets; photoacoustic imaging; ultrasound imaging; molecular targets

1 Introduction

For several decades, diagnostic agents have been judged on their ability to differentiate diseased versus healthy tissue. As knowledge regarding tissue heterogeneity and its implications has increased [1–3], so too has the demand for *in vivo* diagnostic platforms capable of conveying more detailed information. Multiplexed imaging, the ability to simultaneously visualize functional parameters and/or molecular targets using one or several imaging modalities, is well-suited to accomplish this task. To date, optical based modalities have offered the most robust multiplexing capabilities due to the availability of diverse imaging probes [4]. However, the limited penetration depth of optical modalities significantly restricts their clinical utility [4, 5]. Hence, great attention has been placed on increasing the multiplexing abilities of clinically relevant imaging modalities. Thus far, nuclear medicine has shown that it can effectively differentiate various phenotypes by selective targeting using various radioisotopes [4, 6]. However, the acquisition time associated with multiplexed positron emission tomography (PET) or single-photon emission computed tomography (SPECT) can be lengthy [4]. Additionally, since PET or SPECT are typically combined with magnetic resonance imaging (MRI) or computed tomography (CT) imaging [4], there are concerns of high-costs and/or safety regarding repetitive imaging [7]. To address these limitations, we have introduced a platform for multiplexed ultrasound (US) and photoacoustic (PA) imaging based on laser-activated perfluorocarbon nanodroplets (PFCnD).

Our innovative platform consists of color-coded laser-activated PFCnDs and US/PA imaging to noninvasively provide multiplexed, multimodal contrast. Laser-activated PFCnDs are the

liquid, submicrometer-sized version of the commonly used ultrasound contrast agent, gas microbubbles. PFCnDs' reduced size and increased stability makes them desirable diagnostic and/or therapeutic agents by allowing for prolonged imaging times and the potential for extravascular imaging [8, 9]. The essential component of laser-activated PFCnDs is an encapsulated photoabsorber. Upon pulsed laser irradiation at a wavelength tuned to the peak optical absorption of the photoabsorber, a PFCnD phase-change is initiated, which can be detected by both US and PA imaging to afford distinct spatiotemporal contrast [8, 10–12]. In the following work, we extend laser-activated PFCnD imaging capabilities by supplementing their unique multimodal US/PA imaging contrast with the ability for multiplexed US/PA imaging. The developed platform consists of color-coded PFCnDs (i.e., PFCnD subpopulations with distinct encapsulated photoabsorbers) for which user-specified laser irradiation activates selective subpopulations to induce US/PA contrast (Fig. 1(a)). Considering that PFCnDs are already studied in a myriad of both diagnostic and therapeutic agents [9, 13–15]. After successfully showing the US/PA multiplexed imaging platform's feasibility, we demonstrate its potential in a murine tumor-draining lymph node model. The multiplexed imaging capabilities of color-coded PFCnDs helped elucidate distinct particle trafficking mechanisms (i.e., intravascular versus intradermal) to lymph nodes in a single imaging session. Additionally, color-coded PFCnDs helped uncover *in vivo* PFCnD characteristics that significantly expand their potential applications.

2 Experimental

2.1 Synthesis of color-coded PFCnDs

UV-Vis spectrophotometry (Evolution 220; Thermo Scientific) confirmed that the selected dyes (Epolight 9151, Epolight 3832; Epolin, Inc.), referred to as 680-nm dye and 1064-nm dye respectively, had absorption spectra with minimal spectral overlap. Perfluoropentane (FluoroMed, L.P.) was used for the perfluorocarbon nanodroplet (PFCnD) core and a mixture of phospholipids was used for the stabilizing shell. To synthesize the color-coded PFCnDs, 20 μ L of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, 10 mg/mL; NanoCS Inc.) and 15 μ L of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG(2000), 25 mg/mL; Avanti Polar Lipids, Inc.) lipids in chloroform were added to a 50 mL pear shaped flask. Dye solutions were prepared in chloroform (1 mg/mL) and 100 μ L of the desired dye was added to the lipid solution. Two milliliters of chloroform were added to the flask to facilitate the production of a smooth lipid cake. The flask was placed under reduced pressure (Rotavapor; BÜCHI Labortechnik) to remove the chloroform, and the result was a thin, colored lipid cake. One milliliter of PBS was added to the flask and sonicated (VWR, 180 W) to resuspend the lipids and dye. The solution was transferred to an 8 mL scintillation vial and 75 μ L of perfluoropentane were added. The vial was vortexed and sonicated in ice-cold water until the solution became milky, and there was no visibly remaining perfluoropentane bolus. The resulting PFCnDs were washed to remove excess dye by placing the solutions in 1.5 milliliter microcentrifuge tubes and centrifuging them at 100 rcf for 1 minute. The supernatant was pulled off, leaving any aggregated dye behind. The same synthesis process was repeated with the other dye, and the two synthesized droplet sets were kept separately for characterization.

In studies requiring fluorescently labeled particles, PFCnDs were synthesized according to the same protocol but an additional 1.6 μL of fluorescent dye (DiI, 3mg/mL; Thermo Fisher) was added to the lipid-dye-chloroform mixture. Being highly lipophilic, DiI integrates within the lipid shell but does not influence PFCnD vaporization dynamics.

2.2 Dynamic light scattering and zeta potential measurements

A dynamic light scattering instrument (Zetasizer Nano ZS; Malvern Instruments Ltd.) was used to characterize the PFCnDs. For size measurements, one milliliter of a 1:100 stock dilution of PFCnDs in PBS was placed in a plastic cuvette. To study surface charge, 500 μL of a 1:100 PFCnD stock solution in nanopure water (Barnstead Smart2Pure, Thermo Scientific) was placed in a folded capillary zeta cell (DTS1070, Malvern).

2.3 US/PA image acquisition

All imaging experiments were conducted using a 40-MHz ultrasound and photoacoustic imaging probe (LZ-550; FUJIFILM VisualSonics Inc.) coupled to a combined ultrasound and photoacoustic (US/PA) imaging system (Vevo LAZR; FUJIFILM VisualSonics Inc.). The tunable Nd:YAG laser operated at either a 680 nm or a 1064 nm wavelength producing 5–7 ns laser pulses at a 20 Hz pulse repetition frequency. The fluences were measured to be between 12–15 $\text{mJ}\cdot\text{cm}^{-2}$ at 680 nm and 6–9 $\text{mJ}\cdot\text{cm}^{-2}$ at 1064 nm, well below the American National Standards Institute limits. Ultrasound and photoacoustic images were captured at 5 frames per second.

2.4 Flow phantom construction

A flow phantom was used to demonstrate that each PFCnD subset provides selective photoacoustic and ultrasound contrast at its respective absorption wavelength. To construct the phantom, the face of a clear plastic container was drilled with three equally spaced holes. This process was repeated on the opposite face of the container, and silicone tubing (HelixMark; Freudenberg Medical, ID 0.250" OD 0.374") was threaded from one side to the other. Solutions (1:100 PBS stock dilution) of 1064-nm colored PFCnDs, 680-nm colored PFCnDs, and blank PFCnDs (i.e., no dye) were prepared and loaded into separate 10 milliliter syringes. An 18-gauge needle and an accompanying 10 milliliter sample-filled syringe were attached to one side of the tubing, enabling flow through the tubes within the cuvette. A syringe pump (Fusion 400; Chemyx Inc.) controlled the flow rate of samples through the tubes at either 0 $\text{mL}\cdot\text{min}^{-1}$ (i.e., stationary) or at 2 $\text{mL}\cdot\text{min}^{-1}$. The phantom container was filled with degassed water and the imaging probe was placed perpendicular to the tubes such that the optical focus of the integrated laser fibers was aligned with the cross-sectional area of the tubes. Photoacoustic and ultrasound data were collected for approximately 30 seconds at 680 nm and 1064 nm laser irradiation.

2.5 Quantitative US/PA imaging of a color-coded PFCnDs

Different ratios of 1:100 dilutions of 680-PFCnDs to 1064-PFCnDs (1:3, 2:2, and 3:1) were mixed together and placed in three separate 10 mL syringes. Samples were monitored to ensure that PFCnDs did not settle during the imaging process. Using the previously described flow phantom, the samples were irradiated at 680 nm or at 1064 nm either while

flowing through the tubes at $2 \text{ ml} \cdot \text{min}^{-1}$ or while stationary. For both wavelengths, a region of interest within the center of each tube was selected, and the vaporization-associated photoacoustic signal from each wavelength was determined ($n = 10$ photoacoustic frames). A time period ($n = 10$ photoacoustic frames) from the stationary baseline photoacoustic signal (i.e., photoabsorber-associated photoacoustic signal) was averaged and subtracted from the averaged vaporization-associated photoacoustic signal. The result allowed for comparison of vaporization-associated photoacoustic signal of each excitation wavelength across the differing ratios.

2.6 *In vivo* US/PA imaging

The subsequently described small animal studies adhered to protocols approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology. During imaging, mice were anesthetized with a combination of isoflurane (3% induction, 2% maintenance) and O_2 ($0.8 \text{ L} \cdot \text{min}^{-1}$), and placed on an animal heating pad regulated at 37°C .

2.7 *In vivo* US/PA spleen imaging

To ensure the *in vivo* efficacy of the color-coded PFCnDs, a healthy nu/nu mouse model was utilized. A 1:1 cocktail of the two PFCnD subpopulations was prepared from stock solutions and $150 \mu\text{L}$ were injected intravenously via the jugular vein. The particles were allowed to circulate for 30 minutes, and then the spleen was imaged at 680 nm and again at 1064 nm. Photoacoustic and ultrasound images were collected until no more PFCnD-associated ultrasound or photoacoustic signal was observed. After imaging sessions, mice were either kept for observation or euthanized via primary CO_2 asphyxiation followed by secondary cervical dislocation.

2.8 *In vivo* US/PA imaging of particle trafficking mechanisms in a breast cancer model

4T1 murine breast cancer cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Nu/nu mice were injected with $50 \mu\text{L}$ of 4T1 cells (1×10^7 cells/mL) into the mammary fat pad. Tumor sizes were monitored for several days, and ultrasound imaging was conducted to observe any changes in the draining inguinal lymph node. To study different lymphatic trafficking capabilities of PFCnDs, intradermal and intravascular injections were conducted as follows. At 5–6 days post-inoculation, $100 \mu\text{L}$ of one subpopulation of color-coded PFCnDs (e.g. 1064-nm colored PFCnDs) were injected intradermally at the tumor site, while mice were under anesthesia. The particles were slowly injected to prevent drastic pressure changes at the injection site to reduce the risk of premature droplet vaporization or altering particle drainage. At the respective time points (24 or 72 hours), mice were anesthetized again, and the inguinal lymph node was located with ultrasound imaging. Then, $100 \mu\text{L}$ of the other subpopulation of PFCnDs (e.g. 680-nm colored PFCnDs) were injected intravenously through the jugular vein. After 1 minute, US/PA images of lymph nodes were obtained using first 1064 nm and then 680 nm irradiation. After US/PA imaging was complete, the animals were sacrificed via primary IP Euthasol injection (150 mg/kg , Henry Schein Medical) and secondary cervical dislocation.

2.9 Data processing

To obtain background-free images in the spleen and lymph node, a previously published algorithm was employed [12]. Briefly, we performed pixel-wise linear regression on either the ultrasound or photoacoustic signal over time. For ultrasound-based analysis, pixels that had a drastic rise in ultrasound signal amplitude over time corresponded to PFCnDs phase transitioning into microbubbles. Pixels with ultrasound signal amplitude increase rates above a certain threshold were identified, and a map of the above-threshold pixel rates is displayed over a co-registered ultrasound image, highlighting the location of PFCnDs within the image. For photoacoustic-based processing, pixels containing rapidly decreasing photoacoustic amplitude signals over time, also corresponding to vaporizing PFCnDs, were localized and overlaid on a co-registered ultrasound image. Therefore, PFCnDs' multimodal image contrast can be produced using either the ultrasound method, photoacoustic method, or both. Given that the location, concentration of droplets, and *in vivo* duration all play a role in affecting the ultrasound and photoacoustic signal produced by PFCnD vaporization, the type of processing (ultrasound or photoacoustic) was chosen on a per-case basis.

2.10 Trafficking of PFCnDs *in vivo* and verification via histology

After showing that intradermal PFCnDs successfully trafficked to lymph nodes, additional studies were conducted to better understand the transport mechanism. Healthy nu/nu mice ($n = 3$) were injected with 100 μL of fluorescently labeled 680-PFCnDs on both the left and right sides within the mammary fat pad. At 72 hours after injection, the left lymph node was imaged to verify PFCnD presence. Both the right and left inguinal lymph nodes were extracted for histology. After resection, lymph nodes were placed in a 4% solution of paraformaldehyde in PBS for 24 hours and kept at 4°C. Lymph nodes were then transferred to 30% sucrose solution in PBS for 2–3 days at 4°C. Organs were placed into cryomolds (Tissue-Tek, Sakura), filled in with cryotek, and frozen over dry ice. Frozen organs were sectioned in slices of 20 μm using a cryostat (Leica CM 1860, Leica Biosystems). Immunohistochemistry was performed using CD11c antibody (Millipore Sigma MABF374 Lot TN16071301). Then, a ZeissLSM 700 confocal microscope was used to image the samples and determine where the DiI-labelled PFCnDs were in relation to the dendritic cell marker, CD11c.

2.11 Functionalization of PFCnDs and *in vitro* study

To demonstrate the feasibility of color-coded PFCnDs for use as eventual diagnostic molecular imaging tools, the efficacy of functionalized PFCnDs was studied. A protocol for directional conjugation of antibodies to gold nanoparticles was adapted for PFCnDs to produce HER-2 targeted PFCnDs [11, 16].

SK-BR-3, a HER2-expressing breast cancer cell line, was used for studying HER2-targeted PFCnDs. Cells were cultured in 2-chamber well chamber slides (ThermoFisher). After cells had reached 50–70% confluence, they were stained with CellTracker Green dye (ThermoFisher). Then, 15 μL of either HER2-functionalized or PEGylated PFCnDs (both DiI labeled) were incubated with the cells (1.5 mL media per well). After two hours, cells were washed with PBS several times. Cells were then fixed with 10% formalin for 10

minutes, washed with PBS and coverslip mounted. A Zeiss LSM 700 confocal microscope was used to image the samples.

3 Results and discussion

3.1 Characterization of color-coded PFCnDs

Lipid-shelled PFCnDs were synthesized by encapsulating one of the two photoabsorbing dyes with distinct optical absorption peaks and minimally overlapping absorption spectra (Fig. 1(b)). As a result, two color-coded PFCnD subpopulations were produced. PFCnDs in each subpopulation optically absorb and, therefore, phase-change at a particular wavelength. To vaporize the color-coded PFCnDs, we used 680 nm and 1064 nm laser wavelengths – these two wavelengths were selected to ensure minimal spectral leakage between the droplets of different color. Therefore, the synthesized color-coded PFCnDs will subsequently be referred to as 680-PFCnDs and 1064-PFCnDs. For multiplexing purposes, it is essential that the PFCnD subpopulations have nearly identical characteristics, excluding the encapsulated photoabsorber, to ensure a fair comparison regarding image contrast, pharmacokinetics, and stability for downstream *in vivo* experiments. Accordingly, characterization studies indicated that the subpopulations had a similar hydrodynamic size, 350 nm (Fig. 1(c)), and zeta potential, -58.3 ± 7.2 mV (Fig. S1 in the ESM).

3.2 Imaging color-coded PFCnDs in a flow phantom

PFCnD subpopulations selectively responded to tuned pulsed-laser irradiation wavelengths (i.e., 680 nm and 1064 nm). This phenomenon was shown by studying the color-coded PFCnD groups (680-PFCnDs and 1064-PFCnDs) alongside blank PFCnDs (i.e., no dye) as a negative control in a flow phantom. The flow phantom ensured that, by controlling the flow rate, PFCnDs could enter or remain within the imaging plane for each laser pulse, thus allowing comprehensive study of the spatiotemporal US/PA contrast of the unique PFCnD groups (Fig. 2(a)). The phantom was imaged with a 40-MHz US/PA imaging probe and a Vevo 2100/LAZR imaging system. Maximum photoacoustic contrast of laser-activated PFCnDs occurred immediately after the vaporization-inducing laser pulse, which was typically the first laser pulse. The optical energy is absorbed by the encapsulated photoabsorber, producing a local temperature increase and pressure aberrations [8]. These events subsequently cause the nanodroplets to undergo a phase change (i.e., vaporize into microbubbles) and emit an intense photoacoustic signal [8, 17]. Hence, when the PFCnD solutions are flowing through the tubes, there should only be an increased photoacoustic signal of the PFCnD subpopulation containing the photoabsorber that absorbs at the irradiated laser wavelength.

At 680 nm laser irradiation, the 680-PFCnDs vaporized, resulting in the expected intense photoacoustic signal (Fig. 2(b,d)). There was no observable photoacoustic signal from either the blank PFCnDs or 1064-PFCnDs. During flow conditions, the photoacoustic signal of vaporized PFCnDs is increased and relatively stable because new, unvaporized liquid PFCnDs are constantly flowing through the laser-irradiated imaging plane and being immediately phase-changed, giving off a steady level of vaporization-associated photoacoustic signal (Fig. 2(b,d)). Conversely, ultrasound signal is not enhanced because the

phase-changed PFCnDs do not exhibit ultrasound enhancement until the laser-activated microbubbles reach a certain size and stability, which occurs out of the imaging plane due to the flow rate utilized. When the flow is stopped (i.e., from 12 to 22 seconds, Fig. 2(d-g)), there are no unactivated PFCnDs to vaporize and thus no new phase-change events occur. This results in a rapid decrease in 680 nm photoacoustic signal down to a baseline (Fig. 2(d)). On the other hand, ultrasound signal rapidly increases under stationary conditions, as the vaporized PFCnDs, now effectively gas microbubbles *in situ*, persist in the imaging plane (Fig. 2(c,f)). Upon irradiation at a wavelength of 1064 nm, an analogous trend in US and PA signals was observed for the 1064-PFCnD population (Fig. 2(b-c,e,g)).

Due to the high laser pulse energy at 680 nm and marginal optical absorption of the 1064 nm dye at this wavelength, there was a slight ultrasound enhancement from 1064-PFCnDs (Fig. 2(f)). However, the ultrasound crosstalk between different color-coded PFCnDs was minimal and not accompanied by any measurable photoacoustic signal increase. Nonetheless, to avoid any crosstalk in later *in vivo* studies, imaging was performed at 1064 nm first and then at 680 nm to mitigate this effect.

To study the ability of US/PA to quantify the subpopulations of color-coded PFCnDs, the flow phantom described above was used to assess the photoacoustic signal at 680 nm and 1064 nm pulsed-laser irradiation of three different mixtures with varying ratios of 680-PFCnDs to 1064-PFCnDs. The photoacoustic intensity from each wavelength linearly corresponded to the relative quantities of phase-changed PFCnDs present in the samples. At increasing proportions of 680-PFCnDs to 1064-PFCnDs, the photoacoustic signal at 680 nm laser irradiation increased, while the photoacoustic signal at 1064 nm decreased. Analogously, higher relative concentrations of 1064-PFCnDs corresponded to increased 1064 nm photoacoustic signal and decreased 680 nm signal (Fig. 2(h-j)). Combining this ability to quantify the signal present from distinct subpopulations with the ability to functionalize PFCnDs against cancer biomarkers (Fig. S2 in the ESM) signifies that color-coded PFCnDs have the potential to provide comprehensive diagnostic information in the context of cancer.

The selective, dual-modality US/PA imaging contrast of color-coded PFCnDs is extremely valuable for future diagnostic and therapy monitoring applications of color-coded PFCnDs, where measuring relative biomarker expression provides important prognostic information and dictates the appropriate treatment [18, 19]. The current clinical standard for diagnosing various solid cancers (e.g., breast cancer) is to conduct a biopsy followed by immunohistochemistry to assess the presence and abundance of cancer-specific biomarkers [20]. However, these procedures are invasive and prone to sampling error, which can limit their repetitive use for monitoring applications as well as their accuracy, respectively [20]. If color-coded PFCnDs can relay comparable information *in situ*, they could address the discussed limitations associated with biopsies. Differently colored PFCnDs could target unique bioreceptors to noninvasively convey their relative quantities. In addition, color-coded PFCnDs could monitor a patient's response to treatment without the need for invasive or costly procedures [20].

3.3 *In vivo* US/PA imaging and tracking of color-coded PFCnDs

The dual contrast of laser-activated PFCnDs enhances the robustness of the platform via versatile image processing techniques. Laser-activated PFCnDs have unique spatiotemporal photoacoustic and ultrasound signals; therefore, either, or both, data sets can be processed to form background-free images, which localize US/PA signals produced by the distinct PFCnD subpopulations at their respective peak optical wavelengths [12]. Often, either photoacoustic or ultrasound signal is superior due to factors such as PFCnD location, concentration, and competing endogenous signal. To demonstrate this, we used a mouse model and intravenously injected a 1:1 cocktail of 680-PFCnDs and 1064-PFCnDs. Upon systemic injection, PFCnDs are predominantly filtered into interendothelial gaps within the spleen and subsequently captured by red pulp macrophages [21]. This phenomenon makes the spleen an attractive organ for studying color-coded PFCnD characteristics *in vivo* [12, 21]. After allowing 30 minutes for PFCnDs to circulate, the spleen was imaged at 680 nm and 1064 nm, and US/PA data was collected. Following pulsed 680 nm laser irradiation, the 680-PFCnD group exhibited US/PA contrast. Since the PFCnDs are stationary in spleen, they enhance ultrasound signal as microbubbles are formed. In contrast, the photoacoustic signal of PFCnDs is at a maximum following the first laser pulse and then rapidly decreases [12]. In addition to PFCnD photoacoustic signal, endogenous photoacoustic signal is present, resulting in reduced PFCnD localization due to background noise (Fig. 3(a)). However, by processing the ultrasound data, background-free images of the 680-PFCnDs can still be produced to more clearly see their underlying distribution (Fig. 3(b-c)). The resulting image produced by evaluating the ultrasound data over time results in the localization of PFCnDs within the spleen (Fig. 3(c)).

Next, irradiation at 1064 nm activated the 1064-PFCnD group. Due to the high baseline ultrasound signal from the previously produced microbubbles from irradiation of the 680-PFCnD subpopulation, isolating PFCnD phase-change based on ultrasound signal alone was not possible; however, the PFCnD-vaporization-associated photoacoustic signal remains largely unaffected, and after using the developed algorithm to distinguish it from endogenous signal/noise, a similar background-free image is ultimately produced (Fig. 3(d-f)). Thus, multiple sets of colored PFCnDs can be readily distinguished from one another based on user-controlled inputs and processed with data from the best-suited modality (i.e., ultrasound or photoacoustic signal).

To showcase pertinent applications of color-coded PFCnDs, distinct particle trafficking mechanisms were studied in murine tumor-draining lymph nodes (TDLNs, Fig. 4). PFCnDs have demonstrated utility in wide-ranging applications; but until now, most PFCnD studies have focused on synthesis [22], improving imaging performance or processing algorithms [22, 23], and are typically conducted in tissue phantoms, *in vitro* [24], *ex vivo* [22], or in preliminary *in vivo* models [11]. Additionally, most of the *in vivo* studies focused on short-term applications, on the order of minutes to few hours and primarily used systemic injections to deliver PFCnDs [14, 25, 26]. Given the reported potential of PFCnDs, it is necessary to more thoroughly explore their *in vivo* capabilities. The established color-coded PFCnD platform presents an effective method for studying particle dynamics because experiments can be performed within a single imaging session and animal model. This

allows for higher throughput, more uniform comparison, and facilitates discoveries. In studying the TDLN, we demonstrate the potential of color-coded PFCnDs as well as uncover important *in vivo* characteristics relevant to all PFCnDs. The TDLN is of paramount importance in both treating cancers and diagnosing disease state [27]. In addition to being the main dissemination organ for metastatic cells, TDLNs are an important site of immune system regulation [28–30]. With the increasing interest in and successes of immunotherapy, the TDLN represents an attractive organ where PFCnDs can add immense value.

A murine model of breast cancer, in conjunction with color-coded PFCnDs, was used to uncover the intrinsic differences between intradermal and intravenous injections in PFCnD trafficking to the TDLN. Mice bearing a 4T1 tumor in the mammary fat pad were injected intradermally with 1064-PFCnDs at the tumor site. After 24 or 72 hours, 680-PFCnDs were injected intravenously. Subsequently, the inguinal lymph node (i.e., TDLN) was imaged, first at 1064 nm and then at 680 nm, followed by photoacoustic-based analysis to localize PFCnD subpopulations (Fig. 4). By using the color-coded PFCnD platform and background-free imaging processing techniques, the unique locations of nodal PFCnDs due to different biological trafficking mechanisms (i.e., lymphatic versus vascular) is easily discernable. As hypothesized, the 680-PFCnDs were located throughout the majority of the lymph node, given its extensive vascular bed (Fig. 4) [12, 31]. On the other hand, the intradermally injected PFCnDs were confined to one area of the lymph node, specifically to the side draining proximally to their initial injection. Surprisingly, the intradermally injected 1064-PFCnDs provided photoacoustic contrast up to 72 hours after injection, a significantly longer timeframe than previous *in vivo* studies have reported [9, 10, 14, 25]. Based on the timescale in which intradermally injected PFCnDs traffic to the lymph node and their location within the lymph node, it is likely that PFCnDs are trafficked to TDLNs via cellular-mediated transport [32, 33], which could explain their persistence relative to PFCnDs delivered through systemic vasculature. Antigen presenting cells, specifically dendritic cells, have been shown to transport various nanoparticles via afferent lymphatic vessels [33, 34]. To test our hypothesis, we used immunohistochemistry and found that intradermally injected PFCnDs were co-localized with dendritic cells (Fig. S3 in the ESM). Altogether, the use of color-coded PFCnDs helped to assess unique particle transport mechanisms within the same animal and simultaneously visualize the unique resultant spatial distributions of these particles within TDLNs, which would have been challenging to accomplish using just a single nanoparticle platform within a single imaging session. Notably, we also discovered that PFCnDs remain stable up to 72 hours after intradermal injection, much longer than what has been reported thus far in literature.

4 Conclusion

In summary, we have developed a novel imaging platform for multiplexed US/PA imaging and demonstrated its versatility in a TDLN mouse model. Biological specimens are often multifaceted; therefore, diagnostic agents must convey the probed tissue's intricacies accordingly. Color-coded PFCnDs present an effective way of accomplishing this, and they do so with non-invasive, non-ionizing imaging modalities. These multiplexed PFCnDs are capable of providing spatial, temporal, and quantitative information regarding distinct subpopulations, which enables their use in a wide variety of applications. Additionally, there

is no need for baseline imaging due to the specificity of the ultrasound and photoacoustic signals provided by laser-activated PFCnDs, which can be processed to localize PFCnDs within the tissue and produce background-free images. Furthermore, the fact that PFCnDs traffic to lymph nodes via dendritic cell uptake and persist for several days greatly increases PFCnDs' potential applications. Dendritic cells are known to have an instrumental role in inducing the adaptive immune system [30, 35, 36]. As a result, color-coded PFCnDs trafficked via dendritic cells have the potential to provide diagnostic and therapeutic information regarding the immune system. Future work will focus on continuing to develop color-coded PFCnDs to further demonstrate their diagnostic and therapeutic advantages by targeting PFCnD subpopulations towards different receptors to provide comprehensive anatomical and molecular information of diseased tissue. Overall, the described work advances PFCnD technology and demonstrates the potential these particles have as comprehensive diagnostic and therapeutic agents in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Cartoon depicting the delivery and vaporization of selectively activated color-coded PFCnDs. (b) UV-Vis-NIR spectrum of selected dyes with minimal optical absorption overlap. (c) Size distributions of the color-coded PFCnDs showing similar average size. Insert shows synthesized color-coded PFCnDs.

Figure 2.

(a) Flow phantom design to allow for simultaneous imaging of different PFCnD subpopulations. (b) Photoacoustic signal at either 680 nm or 1064 nm only elicits signal from the appropriate PFCnD subpopulation (1: 680-PFCnDs, 2: blank PFCnDs, 3: 1064-PFCnDs; flow is $2 \text{ ml} \cdot \text{min}^{-1}$). (c) Ultrasound signal during $0 \text{ ml} \cdot \text{min}^{-1}$ flow (i.e., no flow) shows accumulation of produced MBs (1: 680-PFCnDs, 2: blank PFCnDs, 3: 1064-PFCnDs). (d-g) Photoacoustic signal and ultrasound signal during the 30 seconds of laser irradiation. Flow starts at 0 seconds and is stopped from 12 to 22 seconds, indicated on graph (d) by the green (flow on) and red (flow off) regions. During the flow off time, photoacoustic signal drops and ultrasound signal increases in the activated subpopulations. (h) Mixtures of different 680-PFCnD to 1064-PFCnD ratios were imaged in a flow phantom. (i) Images of the first photoacoustic frame show how the signal changes as PFCnD subpopulations concentrations are changed (blue: 680 nm photoacoustic signal, green: 1064 nm photoacoustic signal). (j) Photoacoustic signal at either 680 nm or 1064 nm was correlated to the amount of PFCnD subpopulation present.

Figure 3.

(a) Photoacoustic imaging of the spleen at 680 nm overlaid on an ultrasound image. (b) Ultrasound signal of an individual PFCnD pixel in (a), showing the increase in ultrasound signal over time. (c) Upon processing the ultrasound data, a background-free image is produced that more clearly conveys the location of vaporized PFCnDs (in blue). (d) Photoacoustic imaging of the spleen at 1064 nm overlaid on an ultrasound image. (e) Photoacoustic signal of an individual PFCnD pixel in (d), showing a rapid decrease in photoacoustic signal over time. (f) Photoacoustic-based image processing of the 1064 nm irradiated sample reduces endogenous signal and noise resulting in localization of vaporized PFCnDs (in green).

Figure 4.

Photoacoustic-based processed images showing the different locations of intradermal versus intravenously injected PFCnDs. The 24 or 72 hour time point refers to the intradermally injected PFCnDs (1064-PFCnDs, green), whereas intravascular PFCnDs (680 PFCnDs, blue) were injected moments before US/PA imaging sessions.