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Evaluation of Arsenazo III as a Contrast Agent for Photoacoustic Detection of Micromolar Calcium Transients

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ABSTRACT

Elucidating the role of calcium fluctuations at the cellular level is essential to gain insight into more complex signaling and metabolic activity within tissues. Recent developments in optical monitoring of calcium transients suggest that cells integrate and transmit information through large networks. Thus, monitoring calcium transients in these populations is important for identifying normal and pathological states of a variety of systems. Though optical techniques can be used to image calcium fluxes using fluorescent probes, depth penetration limits the information that can be acquired from tissues *in vivo*. Alternatively, the calcium-sensitive dye arsenazo III is useful for optical techniques that rely on absorption of light rather than fluorescence for image contrast. We report on the use of arsenazo III for detection of calcium using photoacoustics, a deeply penetrating imaging technique in which an ultrasound signal is generated following localized absorption of light. The absorbance properties of the dye in the presence of calcium were measured directly using UV-Vis spectrophotometry. For photoacoustic studies, a phantom was constructed to monitor the change in absorbance of 25 μM arsenazo III at 680 nm in the presence of calcium. Subsequent results demonstrated a linear increase in photoacoustic signal as calcium in the range of 1 – 20 μM complexed with the dye, followed by saturation of the signal as increasing amounts of calcium were added. For delivery of the dye to tissue preparations, a liposomal carrier was fabricated and characterized. This work demonstrates the feasibility of using arsenazo III for photoacoustic monitoring of calcium *in vivo*.

Keywords: Contrast agent, photoacoustic imaging, arsenazo III, calcium flux

1. INTRODUCTION

Calcium is an important and versatile messenger involved in both intracellular and intercellular processes, from cellular proliferation and muscle contraction to neuronal plasticity [1]. Further, abnormal calcium levels are often associated with a number of disease conditions, some of which include dysfunction of the thyroid, renal system, pancreas, and liver, as well as osteoporosis and breast cancer [2]. Thus, identifying a technique to image Ca^{2+} levels *in vivo* is vital for determining tissue function and distinguishing diseased from healthy tissue.

Monitoring of calcium fluxes can be accomplished using high resolution optical techniques such as confocal or two-photon microscopy and applying a contrast agent that produces a fluorescence signal in the presence of calcium. However, with a maximum imaging depth of 1 mm, the use of optical techniques is largely restricted to tissue culture studies. Alternatively, photoacoustic imaging is a non-invasive, non-ionizing technique with an imaging depth of 2 – 3 cm. Unlike fluorescence methods, photoacoustic imaging relies on optical absorption to generate image contrast. Acquisition of a photoacoustic image can be simplified to a four-step process: first, the tissue is irradiated with a short pulse of light (nanosecond duration). Next, the deposition of light energy in the tissue causes localized heating followed by rapid thermal expansion and the generation of acoustic pressure transients. Finally, the acoustic waves are detected with an ultrasound transducer and post-processed to form an image of the spatial distribution of optical absorption. For clinical applications, photoacoustics is likely to be performed in the near infrared (NIR) region (600 – 900 nm), where the aggregate absorption by tissue components is minimal and maximum penetration depth can be achieved [3].

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Moreover, when ultrasound imaging is performed simultaneously, the photoacoustic images can be augmented with anatomical detail [4].

An appropriate contrast agent for photoacoustic imaging studies should be both sensitive and specific to physiologically relevant concentrations of Ca^{2+}, as well as exhibiting strong absorbance in the NIR. The suitability of arsenazo III as a reliable indicator of free Ca^{2+} is presented here. Arsenazo III (2,2′-(1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo)bisbenzenearsonic acid) is a chromotropic acid derivative that was initially synthesized for the detection of metal cations in solution using photometric techniques [5]. As shown in Figure 1, the mirror symmetry of arsenazo III forms a binding pocket lined with six active O^- sites for metal cation binding. Although arsenazo III interacts with a number of metal cations, it forms particularly stable chelates with Ca^{2+} in a 1:1 fashion with a reported dissociation constant (K_D) of 0.96 μM, nearly two orders of magnitude higher than that of Mg^{2+}, another physiologically relevant metal cation (K_D = 75.9 μM) [6].

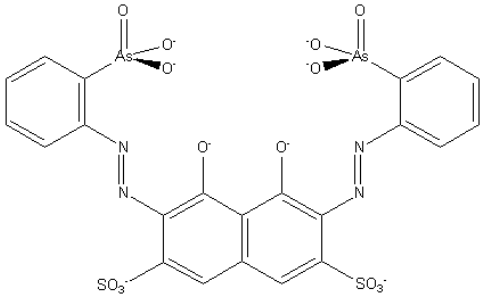

Figure 1. The structure of Arsenazo III. The dye forms a 1:1 complex with Ca^{2+}, with the ion interacting with the six oxygen atoms in the pocket formed by the arsono and phenol groups [6].

Arsenazo III is membrane impermeant, requiring a mechanism for introduction into the cytosol. Although the dye has been widely used in single cell preparations by direct microinjection [7-8], this technique is clearly impractical for studies involving large numbers of cells. Rather, it is necessary to encapsulate the dye within a stable, biocompatible carrier for delivery to the intercellular space. In addition, the carrier should achieve high local concentration of dye, prevent loss or degradation of the dye prior to delivery, and have a fabrication method that results in a monodisperse size distribution. To address these requirements, a liposomal carrier was synthesized which meets these criteria and prevents the dye from interacting with free Ca^{2+} prior to cellular delivery.

This study examines the feasibility of detecting micromolar quantities of calcium upon complexation with arsenazo III using a photoacoustic imaging platform. We demonstrate that this technque is sensitive to calcium concentrations ranging from 1 – 500 μM upon complexation with 25 μM arsenazo III.

2. MATERIALS AND METHODS

2.1. Characterization of Arsenazo III

The absorbance of 25 μM arsenazo III titrated with Ca^{2+} in the range of 1 – 500 μM was measured directly using a DU-640 spectrophotometer (Beckman Coulter Inc., Brea, CA). Arsenazo III (>99% pure) was obtained from Sigma-Aldrich (St. Louis, MO), and was used without further treatment. HEPES was purchased from Sigma-Aldrich while all other chemicals were obtained from Fisher Scientific (Hampton, NH). Arsenazo III was dissolved in 18 MΩ water to give a 10 mM solution and protected from light at 4°C. The dye was further diluted to 25 μM in HEPES buffered saline, pH 7.4, and 1 mL was pipetted into plastic cuvettes of 1 cm light-path, containing the desired calcium concentration dissolved in 18 MΩ water.

2.2. Photoacoustic imaging setup

For photoacoustic experiments, an imaging phantom was constructed that consisted of a plastic, water-filled tank, through which a length of tygon tubing (OD = 2.29 mm, ID = 1.29 mm) was threaded for sample injection (Figure 2). An optical window on the front of the tank permitted irradiation of the tubing containing the sample. The phantom was irradiated at 680 nm (7 ns pulse duration, 10 Hz repetition rate) with a tunable OPO laser pumped by a Q-switched, 532 nm Nd:YAG laser (Opotek, Carlsbad, CA). An unfocused, single element ultrasound transducer (Panametrics-NDT, Waltham, Massachusetts) with a center frequency of 1 MHz was submersed in the tank above the tubing. Samples were prepared by titrating 25 μM arsenazo III with $CaCl_2$ in the range of 1 – 500 μM. 1 mL of each sample to be analyzed was then injected into the tube using a syringe whose flow rate was controlled with a syringe pump. Following each experiment, the tubing was flushed with EDTA followed by 18 MΩ water to ensure removal of residual calcium and dye.

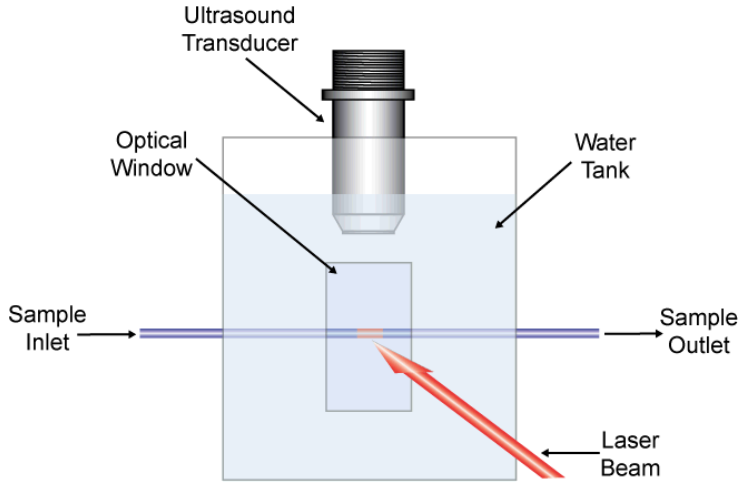

Figure 2. Phantom used for photoacoustic assessment of arsenazo III – Ca^{2+} absorption.

The photoacoustic imaging system is illustrated schematically in Figure 3. Acquired acoustic signals were amplified with an ultrasound pulser/receiver (5073PR, Panametrics-NDT) and digitized with a 200 MHz bandwidth, 2 GS/sec. oscilloscope (TDS2022B, Tektronix Inc., Beaverton, OR) triggered by the laser's Q-switch. The acquired signals were averaged 128 times to reduce noise in the photoacoustic signal and then sent to a computer for processing offline.

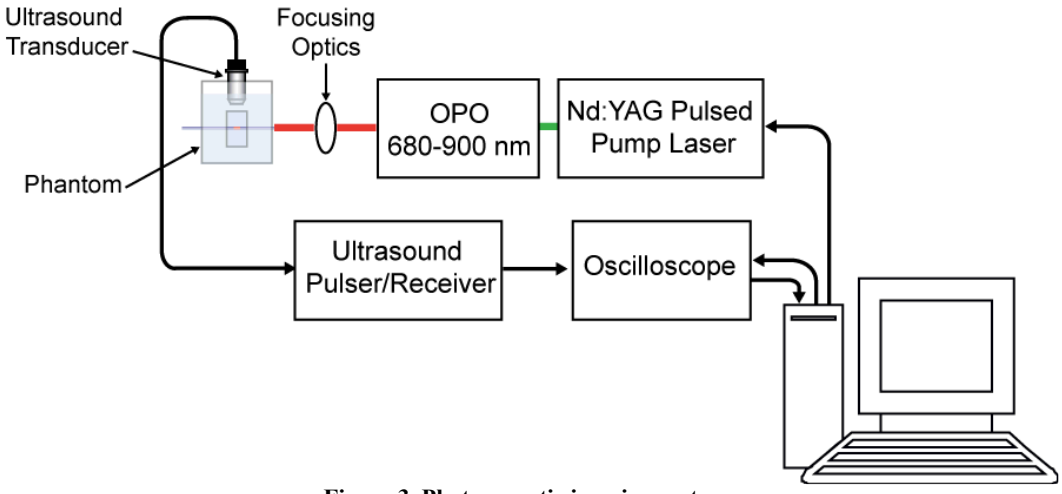

Figure 3. Photoacoustic imaging system.

2.3. Liposome preparation

Liposomes were synthesized following the formulations of Brailoiu et al. [9]. Egg phosphatidylcholine (egg-PC) and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (DOPC) dissolved in chloroform were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Briefly, egg-PC and DOPC were combined in a 9:1 molar ratio followed by removal of the solvent using rotary evaporation. The lipids were rehydrated at 7 mg/mL in either buffer (HEPES buffered saline, pH 7.4) alone or buffer containing arsenazo III (3 mM). To form unilamellar liposomes, the lipid suspension was extruded through a polycarbonate membrane with a 100 nm pore size (Mini-extruder, Avanti Polar Lipids, Inc.). Unincorporated arsenazo III was removed using a Slide-a-Lyzer® dialysis cassette, with a 10 kDa molecular weight cutoff (Thermo Scientific, Waltham, MA) against HEPES buffered saline, pH 7.4, for 24 hours at 4ºC. Size distribution was verified using a Zetasizer Nano dynamic light scattering instrument (Malvern Instruments Ltd., Worcestershire, UK). Absorbance spectra were collected for the liposome only suspension and for the liposome suspension containing arsenazo III. To verify the structural integrity of the liposomes containing dye, a saturating concentration of Ca^{2+} (3 mM) was added to the suspension and the absorbance spectrum measured.

3. RESULTS

3.1. Spectrophotometry Studies

Representative absorbance spectra of 25 µM arsenazo III as well as arsenazo III – calcium complexes are presented in Figure 4. In the absence of calcium, arsenazo III exhibits an absorbance peak at approximately 560 nm. Following the addition of 20 µM Ca^{2+}, a spectral red shift is evident, along with the emergence of peaks at 600 and 650 nm. Addition of 500 µM Ca^{2+} results in a further red shift of the dye and a sharp increase in the peaks at 600 and 650 nm.

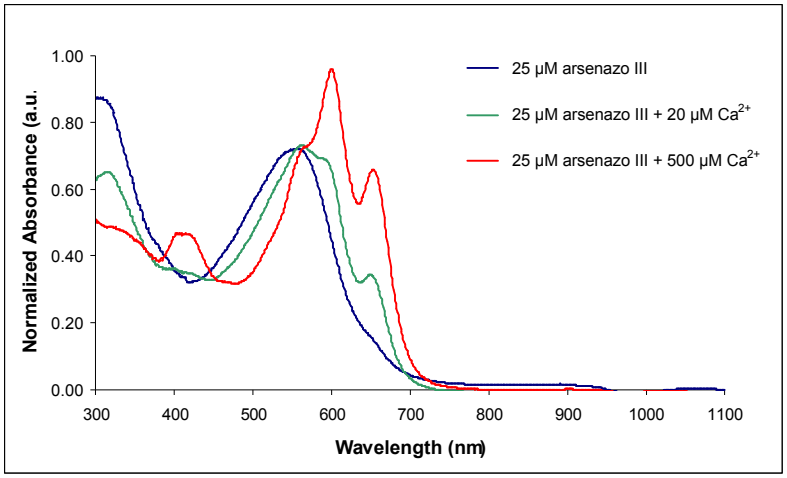

Figure 4. Absorbance spectra of arsenazo III dye and arsenazo III-calcium complex. In the absence of calcium, the dye exhibits an absorbance peak at 560 nm. When complexed with calcium, the absorbance shifts to longer wavelengths with peaks at 600 and 650 nm.

The results of the spectrophotometry study were specifically evaluated at 680 nm, the lowest accessible wavelength using the OPO laser described previously. At 680 nm, the absorbance of arsenazo III as a function of Ca^{2+} added shows a linear increase in absorbance in the range of 1 – 20 µM Ca^{2+}, with an apparent saturation of the dye as the Ca^{2+} concentration reaches 500 µM (Figure 5).

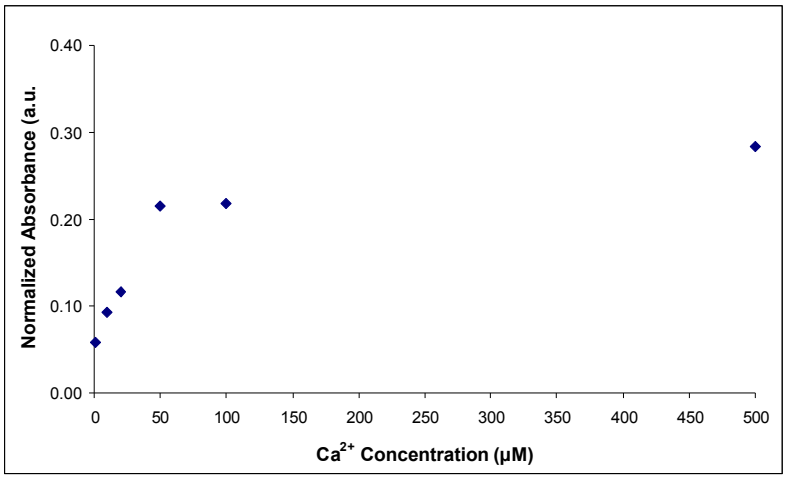

Figure 5. Absorbance at 680 nm vs. Ca^{2+} added to 25 µM arsenazo III dye.

3.2. Photoacoustic Studies

A representative A-line scan collected from a photoacoustic imaging experiment is shown in Figure 6. The peak-to-peak magnitude was measured for each sample, which was indicative of the absorption of light by the arsenazo III – Ca^{2+} complex.

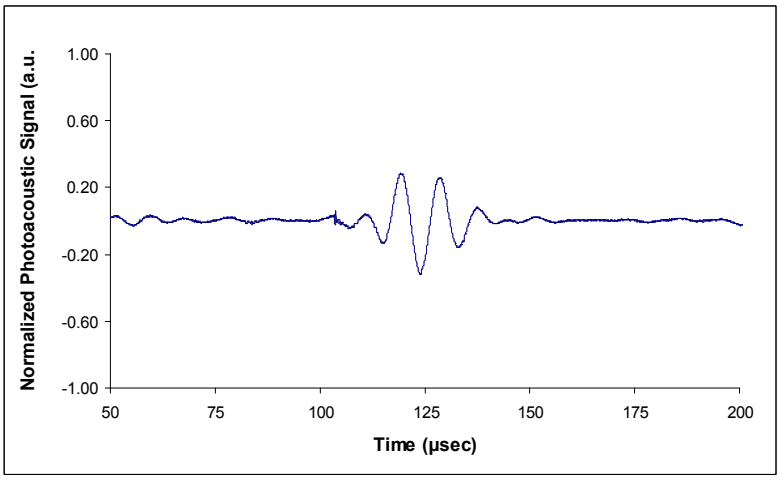

Figure 6. A-line scan acquired from photoacoustic experiment. The peak to peak magnitude was measured for each sample.

The measured peak-to-peak magnitudes of the photoacoustic experiments are plotted in Figure 7. At 680 nm, the photoacoustic signal as a function of Ca^{2+} added to arsenazo III shows a linear increase in absorbance in the range of 1 – 20 µM Ca^{2+}, with an apparent saturation of the signal at higher Ca^{2+} concentrations. This result is in good agreement with the direct absorption measurements presented in Figure 5.

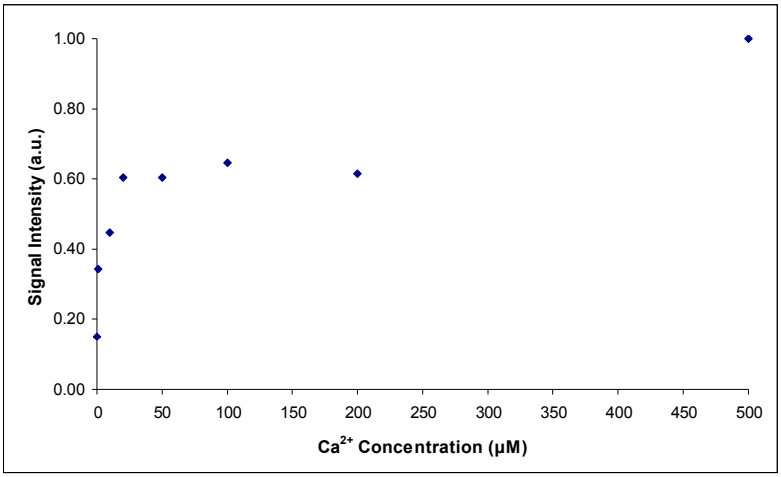

Figure 7. Photoacoustics signal intensity at 680 nm vs. Ca^{2+} added to 25 µM arsenazo III dye.

3.3. Arsenazo III Carrier Synthesis

Dynamic light scattering measurements of synthesized egg PC – DOPC liposomes revealed a monodispersed size distribution with a peak diameter at 122 nm (Figure 8).

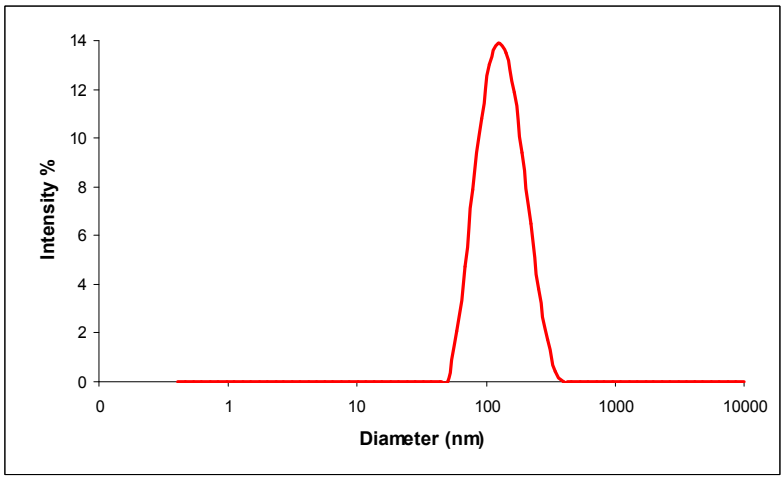

Figure 8. Size distribution of Egg PC – DOPC liposomes extruded through a 100 nm polycarbonate filter.

Spectrophotometry of the liposome solution revealed a broad scattering spectrum (Figure 9). The solution of arsenazo III encapsulated liposomes had a broad peak with a maximum at 560 nm, characteristic of arsenazo III dye in the absence of Ca^{2+}. Following the addition of a saturating amount of Ca^{2+}, no significant change in the peak or shape of the absorbance shift was detected.

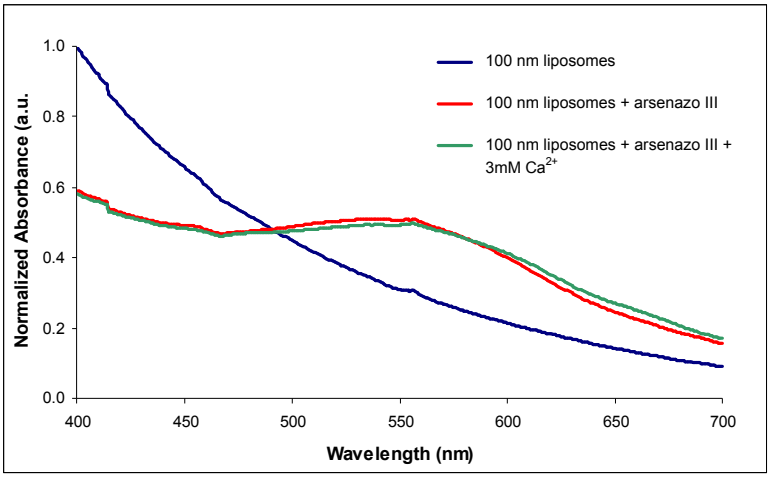

Figure 9. Absorbance spectra of synthesized liposomes. The spectrum of the liposome preparation containing arsenazo III is not significantly altered by the addition of 3 mM Ca^{2+}.

4. DISCUSSION AND CONCLUSIONS

The absorbance of arsenazo III dye was characterized using spectrophotometry. Unique spectral properties of the dye in the presence of calcium made it suitable for photoacoustic measurements at 680 nm. Future work with this dye will assess the potential cytotoxicity for *in vivo* preparations.

Detection of micromolar concentrations of calcium complexed with arsenazo III was demonstrated using photoacoustic imaging. In our experiments, a laser tuned to 680 nm was used to interrogate various arsenazo III – Ca^{2+} complexes. The practical limitation of the laser's tunability notwithstanding, a stronger photoacoustic signal could be obtained by measuring the change in absorbance of arsenazo III – Ca^{2+} complexes at the primary peak of 600 nm or the secondary peak at 650 nm. Further, by applying a spectroscopic technique, the absorbance spectra of the arsenazo III – Ca^{2+} complexes could be traced directly using multiple laser interrogation wavelengths. Temporal resolution is determined by the laser repetition rate, in this case, 10 Hz. The system described here is capable of generating a photoacoustic signal every 100 ms, although a laser with a higher repetition rate would be employed to image Ca^{2+} transients occurring on a shorter time scale. High spatial resolution will be achieved in future studies by using a high bandwidth, focused ultrasound transducer.

Finally, arsenazo III was successfully encapsulated within a liposomal carrier. This approach prevents loss of the dye prior to cellular delivery. The absorbance spectrum of liposome-encapsulated dye in the presence of Ca^{2+} indicates that the synthesized unilamellar liposomes prevented diffusion of either dye or Ca^{2+} across the lipid membrane. Additional studies will validate the cellular uptake of liposomes and the ability to deliver relevant concentrations of dye to the cytosol.

In conclusion, sensitivity of the photoacoustic technique for detection of arsenazo III – Ca^{2+} complexes has been demonstrated. Moreover, the results of this study suggest that this approach may be applied for imaging of calcium transients *in vivo*.

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