Direct measurement of neuronal ensemble activity using photoacoustic imaging in the stimulated Fos-LacZ transgenic rat brain: A proof-of-principle study

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ABSTRACT

Measuring neuroactivity underlying complex behaviors facilitates understanding the microcircuitry that supports these behaviors. We have developed a functional and molecular photoacoustic tomography (F/M-PAT) system which allows direct imaging of Fos-expressing neuronal ensembles in Fos-LacZ transgenic rats with a large field-of-view and high spatial resolution. F/M-PAT measures the beta-galactosidase catalyzed enzymatic product of exogenous chromophore X-gal within ensemble neurons. We used an ex vivo imaging method in the Wistar Fos-LacZ transgenic rat, to detect neuronal ensembles in medial prefrontal cortex (mPFC) following cocaine administration or a shock-tone paired stimulus. Robust and selective F/M-PAT signal was detected in mPFC neurons after both conditions (compare to naive controls) demonstrating successful and direct detection of Fos-expressing neuronal ensembles using this approach. The results of this study indicate that F/M-PAT can be used in conjunction with Fos-LacZ rats to monitor neuronal ensembles that underlie a range of behavioral processes, such as fear learning or addiction.

1. Introduction

Functional mapping of the complex neuronal activation patterns in both normal and pathologic processes can offer significant insight in understanding brain mechanisms [1]. Current neuroimaging methods such as functional magnetic resonance imaging (fMRI) [2] and functional connectivity mapping with optical intrinsic signal imaging [3,4] cannot easily be applied to small animals due to insufficient spatial resolution. Higher resolution modalities such as optical coherence tomography (OCT) [5] or two-photon microscopy [6,7] are invasive with a limited penetration depth and/or field-of-view (FOV). Most neuroimaging modalities measure neuronal activity indirectly, relying upon blood oxygen level-dependent response and changes in blood flow [2,4,8-11]. Contrast-enhanced methods, such as genetically encoded calcium indicators [12] and ex vivo tissue staining techniques [13,14], can measure neuronal activity directly. However, these techniques are either invasive, i.e., requiring implantation of fiber optic cannulas for fiber photometry [15] or GRIN lenses [6], lacking in sufficient FOV for...
whole brain imaging, or performed ex vivo, resulting in a terminal end point for the experiment.

Most imaging techniques assess the average activity of neurons in a given volume [9,16–18], even though most neurons in this volume do not contribute to the ongoing learned behavior [19,20]. Learned behaviors are thought to be encoded and mediated by specific patterns of sparsely distributed neurons called neuronal ensembles that correspond to the specific memory or behavior elicited. Only a small percentage of neurons (1–3 % or less) are activated enough to be part of this ensemble while the majority of surrounding neurons are less activated and do not contribute directly to the behavior. The surrounding neurons can be part of other neuronal ensembles that encode and mediate other learned behaviors, so that many different ensembles can intermingle in the same volume, even ensembles that encode learned behaviors that oppose each other. Thus, the measurement of neuronal activity that is most relevant to learned behaviors can be assessed only by measuring the activity of the neuronal ensembles specifically, and not by assessing activity in a volume where the less relevant non-ensemble neurons dilute measurements of activity in the behaviorally relevant ensemble neurons. This makes it critical to develop a procedure that isolates the activity of only the ensemble neurons in learned behaviors.

We have developed a functional and molecular photoacoustic tomography (F/M-PAT) system (see Fig. 1) that allows imaging of Fos-expressing neuronal ensembles, i.e., highly-active groups of neurons, in the transgenic rat brain with a large FOV and high spatial resolution adequate for subregional neuroimaging. Our F/M-PAT imaging is dependent on the LacZ reporter gene possessing the ability to selectively cleave an exogenously administered pro-chromogenic dye into a colored product within Fos-expressing cells. PA imaging of the brain is based on the acoustic detection of optical absorption from endogenous or exogenous chromophores within the tissue [21]. Here, the organic colorless pro-chromogenic compound X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) was used in an exogenous contrast agent system because the X-gal product dye (5′-dibromo-4′-dichloro-indigo) possesses a strong molar extinction coefficient (ε635 nm = 14,460 cmM−1) where natural chromophores of the body, such as hemoglobin, absorb light weakly (oxy-hemoglobin, ε635 nm = 488 cmM−1, deoxy-hemoglobin, ε635 nm = 3,796 cmM−1) [22,23]. The X-gal system has been shown previously to be an effective PA contrast agent upon conversion of the substrate X-gal to its PA-active X-gal product [24]. It works by β-D-galactosidase (β-gal) cleaving the galactose side chain from colorless X-gal, which then undergoes subsequent oxidation and dimerization to form the colored PA-active X-gal product dye [25].

To specifically image Fos-expressing neurons, we utilized the transgenic Fos-LacZ reporter system which relies on the expression of the LacZ gene to produce β-gal in Fos-expressing neurons. X-gal is administered in these animals during the peak of Fos expression, which drives β-gal protein expression. The X-gal enters cells indiscriminately, but the galactose side chain is only able to be hydrolyzed within cells expressing β-gal, thus selectively tagging these Fos cells with the PA-active X-gal product dye. We chose Fos because it is widely used as a marker for identifying regions of high activity following behaviors and Fos-expressing neurons have been causally linked to specific behavior [13,14,26–29].

The X-gal staining of Fos-expressing neuronal ensembles offers unique advantages when compared to other existing targeted brain PA imaging methods. The relative stability of the X-gal product dye allows it to persist after the behavior has concluded. This allows us to obtain a measurement of the most active (Fos-expressing) neurons during a stimulus of interest that can be measured after the presentation of the chosen stimulus. Persistence of the product dye allows the Fos-provoking stimulus and the imaging session to happen at separate times. Thus, an anesthetized animal can be imaged after the experiment or stimulus presentation, while still allowing us to measure Fos-based neuronal activity that occurred during the event of interest. A similar method using X-gal has been performed previously in preserved and sectioned brains with light and immunofluorescence microscopy ex vivo [13,30]. The X-gal contrast agent selected here for F/M-PAT is highly stable when compared to current PA functional imaging approaches such as voltage sensitive dyes, calcium sensitive dyes, and blood oxygenation changes where the brain activity changes are much more transient and temporary [17,18,31]. The stability of F/M-PAT allows the staining of Fos-expressing neurons to persist into an anesthetized imaging session, allowing visualization of Fos-expressing ensembles from a previous behavior.

To develop and provide initial proof-of-concept data for F/M-PAT, we conducted a series of experiments to develop the method and apply it in a rat model. First, we measured the absorption spectrum of X-gal product and compared it to that of oxy- and deoxy-hemoglobin. Next, we tested the sensitivity limit of PA imaging for detecting X-gal product in a brain-like phantom ex vivo at depths up to 15 mm. We then measured the PA signal obtained from neuron concentrations of X-gal product that were intracranially administered in the rat brain ex vivo. Finally, we exposed Fos-LacZ transgenic rats to one of two stimulation paradigms, including acute cocaine injection or a shock-tone pairing similar to that used in conditioned fear paradigms, to detect (activated) Fos-expressing neuronal ensembles in the medial prefrontal cortex (mPFC) compared to control conditions [14].

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Fig. 1. A schematic of the F/M-PAT system; high energy short pulse laser light transverses brain tissue and in Fos-expressing and β-gal-expressing (Fos+ and β-gal+) nuclei the X-gal is converted to the product dye generating PA waves detected by an ultrasound transducer. B. Chemical conversion of pro-chromogenic substrate X-gal to colored X-gal product via β-gal. X-gal first undergoes hydrolysis assisted by the β-D-galactosidase followed by auto-oxidation and dimerization resulting in the highly conjugated colored X-gal product (5′-dibromo-4′-dichloro-indigo).
2. Materials and methods

2.1. Spectrophotometry of X-gal product

The concentration of the X-gal product was determined via measuring the substrate concentration using the molar ratio (2:1 substrate to product). First, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Biotium) underwent exhaustive enzymatic digest (15 units/mL) in phosphate buffered saline (PBS) containing 10 mM MgCl₂ and 0.1 mM 2-mercaptoethanol [32,33]. After digest, there was a 24-h wait time for oxidation and dimerization into the blue colored X-gal product dye (Fig. 1B). A BioMate™ 3S Spectrophotometer (Thermo Scientific™, USA) was then used to measure the absorption spectrum of X-gal product, at 5, 50, or 500 μM dissolved in PBS, in the range of 500 nm–900 nm.

2.2. Photoacoustic imaging setup

We developed a linear array-based ultrasound/photoacoustic (US/PA) imaging system for this study. The components of the US/PA system can be seen in Fig. 2. A Q-switched Nd:Yag Opotek Phocus HE MOBILE laser (OPOTEK, LLC, USA) with a pulse width of 5 ns and a repetition rate of 10 Hz was used. The laser uses an optical parametric oscillator (OPO), which allows selection of wavelengths in the range of 690–950 nm with fast switching between any two wavelengths across the wavelength range. The laser energy was controlled with an internal attenuator with the maximum energy of the laser being 100 mJ at 690 nm; at this wavelength, the maximum energy reaching the tissue was recorded at 20 mJ. We made sure that the maximum energy deposited to the samples was always below the American National Standards Institute (ANSI) limit; 20 mJ/cm² for 690 nm and 40 mJ/cm² for 850 nm [34,35]. The samples were illuminated by laser light through a bifurcated fiber bundle (with 130 cm length) and a numerical aperture of 0.55. The bundle on the laser side had an aluminum ferrule with 7 mm diameter, and on the object side it had two 22 mm bifurcated plastic plates with an active aperture of 20 mm × 6 mm). Following manufacturing by Fiberoptics Technology Inc. (Pomfret, CT, USA), the fiber bundle was attached to a 3D-printed housing. The fiber bundle had the highest transmissivity in the wavelength range of 400 nm–1300 nm. For US pulse/echo, as well as PA signal acquisition and image generation, we used a high frequency Vantage 128 system (Verasonics Inc., Kirkland, WA, USA). We used an L22-14v US transducer (Vermon Inc., Tours, France) with 128 elements, a pitch size of 0.1 mm, central frequency of 18.5 MHz and fractional bandwidth of 67 %, axial lateral, and elevational (Supplemental Fig. 1) resolutions of 250 μm, 480 μm, and 620 μm, respectively [36]. The probe imaged an area of 1.3 cm transversely by 2.2 cm depth. The data acquisition was triggered by the laser. Data acquisition and processing were performed using a customized MATLAB code. A fast photodetector (DET10A2, Thorlabs, USA) was used for pulse-to-pulse laser fluctuation compensation. The output of the US/PA system were: (i) US image, (ii) PA image at 690 nm and (iii) PA image at 850 nm. The US images provided anatomical landmarks, the 690 nm provided the X-gal concentration map, and the 850 nm provided images for background correction. The 690 nm was used instead of 635 nm (peak absorption of X-gal product) because X-gal product has a broad absorption peak from 625 nm to 700 nm and our laser operated in the wavelength range from 690 nm to 950 nm [24].

2.3. Intralipid brain-like phantoms

We constructed our brain-like phantoms with open-top, cubic, transparent-plastic boxes (Urban Outfitters, USA) in which we drilled two opposing holes to secure a thin capillary (3/32″ inner diameter and 1/32″ wall thickness). A 0.25 % intralipid solution (MilliporeSigma, Burlington, MA, USA) diluted with deionized water was used to mimic the brain composition and account for the optical properties of the brain tissue (i.e., reduced scattering coefficient of 12 cm⁻¹) reported in the literature [37]. The optical properties of the solution were measured using previously detailed methods [38]. The samples were prepared by dissolving the X-gal product in PBS. We imaged the samples from depths 5 mm–15 mm with 1 mm intervals. These depths were chosen to encapsulate the full imaging depths needed to contain the entire rat brain. The capillary tube was filled with different concentrations (1, 10, or 100 μM) of X-gal product (MilliporeSigma, Burlington, MA, USA). The capillary tube was rinsed three times separately with 70 % ethanol and distilled water between imaging sessions. The saline-filled capillary images were used for background correction by image subtraction, i.e., to remove the effect of the tube in the produced PA signals. During the imaging session, the probe was held by a stereotaxic frame (American Standard Instruments (ASI), Michigan, USA), and suspended in the intralipid phantom 1 cm superior to the chromophore-filled capillary.

2.4. Ex vivo PA imaging of X-gal product in the rat brain

Six naive male Sprague Dawley rats (Charles River Laboratories, OH, USA) were used for PA imaging of X-gal product in the rat brain ex vivo. These animals were pair-housed at Wayne State University Division of Laboratory Animal Resources (DLAR) until use. All procedures incorporating the use of animals were approved by the Institutional Animal Care and Use Committee at Wayne State University. Brains were extracted from non-anesthetized, decapitated rats (10–12 weeks of age) and frozen by submersion in isopentane (at −40 °C cooled with dry ice). At the time of experimentation, these brains were thawed and suspended in 8% porcine gelatin (Sigma Aldrich, St. Louis, MO, USA) approximately 5 mm below the top surface of the gelatin. Temperature of the gelatin was monitored and was not allowed to encounter brain tissue at temperatures above 40 °C to prevent experimental interference from heat-induced protein denaturation. Brains were injected with 10 μL
volumes of various concentrations of X-gal product, 5, 50, 500 µM, at +3.0 mm anterior-posterior (AP), -1.5 mm medial-lateral (ML), and -3.0 mm dorsal-ventral (DV) relative to bregma using a Quintessential Stereotoxic Injector (qsITM, Stoelting, Wood Dale, IL, USA) controlled by a stereotaxic frame (American Standard Instruments (ASI), Michigan, USA) (see Fig. 3A). According to published methods [39], we used the coronal plane that encompasses the posterior edges of the right and left hemispheres as our reference point (-6.27 mm AP to bregma). Once brains were injected, as shown in Fig. 3B, the US/PA probe was positioned above the brain phantom using the aforementioned ASI stereotaxic frame. The brain was elevationally scanned at 100-micron steps along the AP axis.

2.5. Ex vivo PA imaging of X-gal in the stimulated transgenic rat brain

Six male Fos-LacZ transgenic Sprague Dawley (SD-TgFos-LacZ) 1Otcc) rats, originally developed by Dr. Tom Curran at The Children’s Mercy Hospital in Kansas City, Missouri, were pair-housed at the National Institute of Drug Abuse Intramural Research Program (NIDA-IRP) animal research laboratory and used for PA imaging of X-gal product ex vivo following their stimulation in vivo. Handling and behavioral procedures took place during the dark (active) phase of the rats’ light cycle under dim red room and shock-tone pairing chamber light. This procedure was approved by the Institutional Animal Care and Use Committee of the NIDA Intramural program.

2.5.1. Cocaine injection stimulus

We used a single injection of cocaine paired with exposure to a novel environment to induce Fos in the mPFC. Fos-LacZ rats (n = 2) were injected intraperitoneally with a single dose of 20 mg·kg−1·(-) cocaine HCl (NIDA Drug Supply Program) and placed in a round bowl with bedding for 90 min [40].

2.5.2. Shock-tone pairing stimulus

We induced Fos in the mPFC in a second group of rats using a repeated sequence of shock-tone pairings typically used for fear conditioning studies [41]. Fear conditioning chambers (Med Associates Inc, St. Albans, Vermont, USA) consisted of modular aluminum side wall panels, transparent front and back walls, and an electrifiable grid floor. Fear conditioning chambers were enclosed in sound-attenuating cubicles. Animals were housed overnight in the conditioning chambers, with ad libitum access to food and water, to acclimate them to the chambers before testing. On test day, after a 180-second pre-trial period, the rats were exposed to three 10-second tones, co-terminating with a one-second duration shock at 1.0 mA. Shock-tone pairings were separated by a 50-second intertrial interval (Fig. 4). Behavioral data were not recorded. Rats were left in the fear conditioning chamber for 90 min.

2.5.3. Undisturbed naive, control animals

Naive animals (n = 2) served as a behavioral control for measurement of baseline Fos expression compared to cocaine injection or shock-tone pairing stimuli. Rats were taken directly from their home cage without exposure to any novel context and immediately anesthetized for X-gal administration as described in Section 2.5.4.

2.5.4. Intracranial administration of X-gal in naive, cocaine, or shock-tone paired animals

Either X-gal or vehicle was injected intracranially to the rats 90 min after cocaine, shock or control stimuli to label Fos-expressing cells. The Fos-LacZ transgenic rats used in this study express a chimeric Fos-β-gal protein that peaks in expression with naive Fos at approximately 90 min post-stimulation [42]. This protein is key to the selective digestion of colorless (PA-inactive) X-gal to the colored X-gal product (PA-active) only within the cells expressing it. Rats were anesthetized with isoflurane (5% induction and 1–3 % maintenance in air), shaved, and secured in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The X-gal solution was composed of 5.9 mg/ml of X-gal dissolved in 7% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO USA), 9% Tween 80 (Sigma-Aldrich, St. Louis, MO USA), and sterile PBS (Sigma-Aldrich, St. Louis, MO USA). Potential interference from the use of isoflurane on F/M-PAT is minimized because of the temporal separation between the time of behavior, the Fos-LacZ protein expression, and the use of the anesthetic. Vehicle injection solution was composed of 7% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO USA), 9% Tween 80 (Sigma-Aldrich, St. Louis, MO USA), and sterile PBS (Sigma-Aldrich, St. Louis, MO USA). Two small burr holes were drilled into the rats’ skulls at AP: +3.0 mm, ML: ±1.5 mm relative to bregma. NanoFil syringes and needles (NF33BL-2 needle with NANOFIL injection system from World Precision Instruments, Sarasota, FL, USA) were used to administer 1 µL each of the X-gal solution (or corresponding vehicle) to the mPFC sub-regions including the prelimbic cortex (PL, -3.0 mm DV, 10 degrees) and infralimbic cortex (IL, -5.0 mm DV, 10 degrees) subregions of the mPFC through the previously drilled bilateral holes. X-gal was infused over 10 min at each injection point with a 2-minute wait period following each injection and slow removal of the needle. Following injections, the burr holes were filled with bone wax and the scalp sutured. Animals were then kept in the induction chamber under 1% isoflurane anesthesia for an additional 30 min to allow for the diffusion of X-gal solution. This step ensured further isolation of stimuli-induced Fos-expressing cells temporally, by delaying the onset of undesirable post-surgical Fos expression. After recovery, the animals were returned to their home cages.

Twenty-four hours following intracranial X-gal injection, which provided time for formation of X-gal product, rats were anesthetized with isoflurane. Within each stimulation condition, each rat either underwent transcardial perfusion followed by brain extraction and post-
fixation (perfused group) or their brains were removed and immediately frozen using isopentane maintained at -40 °C by addition of dry ice (fresh-frozen group). Deeply anesthetized rats were perfused through the left ventricle with PBS at a flow rate of 30 mL/min for 5 min and then with 2% paraformaldehyde (PFA; ~500 mL) in PBS for 15 min. Brains were removed, post-fixed in 2% PFA for 24 h, transferred to PBS with 0.01 % sodium azide, stored at 4 °C. Estimated time for perfusion was <20 min per rat. All brains were shipped to Wayne State University, where they were then PA imaged in a water bath (fresh-frozen brains were thawed in the water) as depicted in Fig. 5. Coronal cross-sectional scans were used to create 3-D reconstructions. PA imaging was done with the perfused group to compare the background-free F/M-PAT images to the fresh-frozen group’s images with hemoglobin background signal.

2.6. Data processing and statistical analysis

We used MATLAB 2016b (on a PC with a Core-i7 6700 K CPU and 32 Giga bytes of memory) for data processing. We calculated the signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) from the images derived from ex vivo PA imaging of X-gal product experiment. The SNR, \( S_{X-gal}/\sigma_{back} \), was calculated as the averaged pixel intensity values from the injected region of interest (ROI), \( S_{X-gal} \), divided by the standard deviation of the background pixels’ intensity values calculated from the pixels outside of the ROI, \( \sigma_{back} \); and CNR, \( |S_{X-gal}-S_{non-injected}|/\sigma_{back} \), was calculated as the average of the pixel intensity values within the injected ROI subtracted from those on the contralateral non-injected side divided by the standard deviation of the intensity values of background pixels. 3-D image rendering was performed using FIJI distribution of ImageJ software [43]. One-way ANOVA with Bonferroni Post-Hoc tests were used to determine statistical differences (threshold set to \( p < 0.05 \)) in the average value of the voxels contained either within the X-gal or vehicle volumes of the mPFC using groups composed of repeated imaging scans. Voxel boundaries were determined anatomically using ultrasound images. Recorded PA image pixel values were adjusted proportionately to the laser energy recorded at the time of image acquisition to ensure cross-comparability of PA images. Image quantifications were performed by defining a volume of interest and taking the average of the PA voxel values within the ROI.

3. Results

3.1. Spectrophotometry of X-gal product and PA profile of X-gal product in an intralipid phantom

The spectrophotogram of X-gal product, obtained using the method explained in Section 2.1, is shown in Fig. 6A. In Fig. 6A, we overlaid the spectra of oxy- and deoxy-hemoglobin regenerated from [44] for comparison. The profile shows that the absorption peak of X-gal product \( \sigma_{max} = 14,460 \text{ cm}^{-1} \text{ M}^{-1} \) is at 635 nm, compared to that of oxy-hemoglobin at 556 nm and deoxy-hemoglobin at 576 nm.

We determined the sensitivity limit of the PA imaging to detect different concentrations of X-gal product (i.e., 1 μM, 10 μM, or 100 μM). We imaged the samples in an intralipid brain-like phantom using the setup explained in Section 2.3 from depths 7 mm–15 mm with steps of 1 mm. A picture of various concentrations of X-gal product is shown Fig. 6B with the PA signal results graphed in Fig. 6C. The increase of the PA signal with increasing concentration and the decrease of the PA signal with depth can be seen in this figure.

3.2. PA imaging of X-gal product in rat brain ex vivo

We imaged the rat brains prepared as described in Section 2.4. PA and US overlaid images of brains unilaterally injected with various concentrations of X-gal product (i.e., 5 μM, 50 μM or 500 μM) are shown in Fig. 7A–C. X-gal products were visible at 5 μM (Fig. 7A).

We conducted a simulation study to demonstrate the impact of laser energy (or fluence) on the photoacoustic imaging penetration depth. We used a simplified rat head model with an isotropic voxel size of 1 mm, and the same light illumination scheme used experimentally was simulated. Two wavelengths, i.e., 690 nm and 850 nm, were simulated. The absorption coefficient and scattering coefficient of skin and brain tissues at each wavelength were obtained from [37]. The refractive index and anisotropy factor were set to 1.35 and 0.9, respectively. Using Monte Carlo (MC) simulations, \( 10^7 \) photons were simulated for each wavelength utilizing MCX software [45]. Having normalized fluence, \( F \),
the initial pressure, $P_0$ can be calculated by $P_0 = \Gamma \mu_0 F_{\text{PE}}$, where $\Gamma$ is the Gruneisen parameter, set to 0.2. $E_p$ is the laser pulse energy limited to the tissue wavelength-dependent maximum pulse energy. The light intensity decay profile was then obtained. The inverse light intensity decay was used as an intensity decay compensation curve. All of the pixel values in depth were corrected and Fig. 7A–D were reproduced. We then calculated the SNR, CNR and normalized PA signal values from 15 PA images across the injection site., The results are shown in Fig. 7D. We found significant differences between each tested concentration using separate one-way ANOVAs for PA intensity: $F(2, 44) = 3074.61$, $p < 0.001$, SNR: $F(2, 44) = 936.42$, $p < 0.001$, and CNR: $F(2, 44) = 1279.14$, $p < 0.001$. All Bonferroni-corrected Post Hoc group differences were significant at the $p < 0.001$ level for normalized PA intensity, SNR, and CNR. To calculate normalized PA signal, SNR, and CNR values, we averaged the PA image intensity within the yellow dotted-line circles indicated in Fig. 7A–C (Contrast was calculated from an equivalent area on the contralateral noninjected side). These pre-tests demonstrated that only 0.1 % (50 picomoles) of the planned amount of intracranially injected X-gal must be converted to X-gal product to produce a detectable PA signal.

### 3.3. PA imaging of X-gal product in stimulated rats ex vivo

We imaged perfused and fresh-frozen brains extracted from Fos-LacZ rats following acute cocaine injection, shock-tone pairing, or control (naive) conditions (see Section 2.6 for more details). The PA probe was placed 10 mm above the sample as shown in Fig. 5. The overlaid US and PA images are shown in Fig. 8. The obtained PA images showed higher PA signal intensity in the high Fos-expressing mPFC (PrL and IL) region on the X-gal-injected (Right, R) side in all 6 imaged animals (Fig. 8 and Videos 1–6). However, US image quality suffered from the tissue damage induced by the freeze/thaw process used (Fig. 5 3rd row). In addition, repetitive image quantification using 15 replicated PA volumes of the mPFC yielded statistically significant signal differences between non-stimulated (naive) and stimulated groups (both cocaine and shock-tone paired) independent of brain preparation method (i.e., perfusion or fresh-frozen) (one-way ANOVA, perfused: $F(5, 1) = 7527.98$, $p < 0.001$, fresh-frozen: $F(5, 1) = 762.73$, $p < 0.001$) (Fig. 9). In this study we confirmed X-gal product formation in the mPFC through light and immunofluorescence microscopy using an Olympus BX-51 microscope with Microsuite software using a DAPI filter set (see Fig. 10).

PA intensities on the X-gal injected side (right, R) and vehicle-injected side of the mPFC were quantified and the results presented in Fig. 9. All Bonferroni-corrected Post Hoc group differences were significant at the $p < 0.001$ level for the perfused groups. All group comparisons were significantly different ($p < 0.001$) in the fresh-frozen group except for the following: naive vehicle vs shock-tone vehicle, and shock-tone X-gal injection vs cocaine X-gal injected. Importantly, all vehicle vs. X-gal injected and all stimulus vs. naive group comparisons were significant ($p < 0.001$) in both perfused and fresh-frozen brains. The smaller signal differences and greater background observed in the fresh frozen results may be indicative of a future in vivo limitation. Future experiments may need to employ additional methods to better separate and quantify both X-gal product and hemoglobin derived PA signals.

### 4. Discussion

The main outcomes in this proof-of-concept study are as follows: we quantified the lower limit of sensitivity for PA imaging to detect X-gal product at depths up to 15 mm in a intralipid brain-like phantom (see Fig. 6); we showed a detectable PA signal from X-gal product with the concentration of as low as 5 µM in an ex vivo rat brain experiment (see Fig. 7); we evaluated the capability to detect Fos-expressing neuronal ensembles in cocaine and shock-tone stimulated transgenic rats, showing statistically significant differences in PA signal in the mPFC between stimulated and naive rats and signal differences in all rats between X-gal injected and vehicle sides (see Figs. 8 and 9).

Fos is an immediate early gene product that has long been considered a marker for neuronal activation [46,47]. Use of the Fos promoter to induce expression of LacZ produces β-gal that cleaves exogenously administered X-gal to form a PA contrast agent selectively, within activated Fos-expressing cells. It has been shown previously that cocaine administration and shock-tone pairing cause activation in the mPFC [48–55].

We used X-gal as a PA contrast agent because: (i) X-gal is currently the only known contrast agent used with the Fos-LacZ gene reporter system for neuroactivity reporting [13,27]; (ii) the absorption peak of X-gal product is at 635 nm, which is sufficiently far from that of oxy-hemoglobin (at 556 nm) and deoxy-hemoglobin (at 576 nm) (see Fig. 6) [44]; (iii) X-Gal absorbs light most effectively within a biological pH range (the maximum absorbance was between pH 7.0–7.5 with no more than a 20 % drop in absorbance throughout the pH range 6.0–8.0); (iv) there is an inherent signal amplification associated with X-gal through the β-gal enzymatic system that improves the detectability of X-gal product and hemoglobin derived PA signals.
this contrast agent; (v) X-gal product is a more stable contrast agent when compared to voltage sensitive dyes, calcium sensitive dyes, and blood oxygenation changes allowing the staining and imaging of Fos-expressing ensembles to happen at different time points.

The stimulated animals we used may have experienced neuro-inflammation and hypoxic glial activation during intracranial injection of X-gal; however, we controlled for this using the stimulus-free naive control group. We showed significant differences in PA signal detected

![Photoacoustic imaging of brain ex vivo in gelatin containing various concentrations of X-gal product (5 μM, 50 μM, or 500 μM). PA (monochromatic cyan) and US (greyscale) overlaid coronal images of rat brains injected ex vivo. Left to right: 3.0 mm anterior to injection site (anterior cortex and olfactory bulb), injection site (mPFC, dashed yellow circle), 6.0 mm posterior to injection site (dorsal hippocampus). A. 5 μM (12.5 pmol) X-gal product injected, B. 50 μM (125 pmol) X-gal product injected, C. 500 μM (1.25 nmol) X-gal product injected, D. comparison of normalized PA signal, SNR, and CNR at the injection site versus concentration of the injected X-gal product. Horizontal line artifacts were observed from the higher concentrations due to saturation of the PA signal. Ofb: olfactory Bulb, fmi: anterior forceps of corpus callosum, Hipp: hippocampus, SNR: signal-to-noise ratio, CNR: contrast-to-noise ratio, a.u.: Arbitrary units, AP: anterior/posterior. Error bars display standard deviation. All comparisons across concentrations within PA intensity, SNR, and CNR were significant at p < 0.001.](image-url)
by replicate imaging of Fos-expressing ensembles between acute cocaine or shock-tone pairing compared to naive controls in both perfused and fresh-frozen brains. This indicates that the stimulus paradigms are sufficiently activating Fos-expressing neuronal ensembles. Additionally, we used intracranial administration of X-gal to attempt to distinguish Fos expression in stimulus-activated neurons from Fos expression in the damaged-induced glial activation. Given that Fos protein expression (and hence β-gal expression) from glial activation would occur approximately 90 min from the time of injection, it is unlikely that Fos from this glial, non-ensemble cells would interfere with PA imaging of Fos from highly activated neurons that was timed to occur 90 min before PA imaging.

The combination of the LacZ gene reporter system with PA imaging offers a novel solution to the problem of imaging Fos-expressing neuronal ensembles. Fos-LacZ rats have previously been used as an ex vivo system to study neuronal ensembles \[13,19,56,57\] responsible for a variety of learned behaviors such as operant food seeking \[56,58\]. We have previously shown active neuronal ensembles in the mPFC after cocaine self-administration and extinction \[59\]. The application of X-gal enhanced-PA imaging in combination with LacZ gene reporter system has also previously been demonstrated in tumor demarcation \[22,60,61\]. The major advantages of X-gal enhanced F/M-PAT method we describe here, over fMRI \[16\] or modern optical techniques such as two photon microscopy \[62\], OCT \[5\], GRIN lens-based methods \[6\], and PA tomography of GCaMP6f \[17\], are the following: (i) our method uses direct measurement of Fos-expressing persistently active cells involved in neuronal ensembles during a stimulus or behavior \[13,19,27\] compared to more indirect blood oxygenation measurements or more
transient measurements of calcium or voltage changes, (ii) our PA probe has a large FOV, high spatial resolution that is currently marginally better or comparable to that of fMRI, and deep penetration depth adequate for imaging of the whole rat brain [4], (iii) the PA probe has a high frame rate allowing it to be used for simultaneous functional connectivity studies [4], and (iv) unlike other molecular techniques, the ability of F/M-PAT to measure Fos-expressing neurons can persist from a previous timepoint into an anesthetized imaging session. Although not shown in the current study, we intend to test our proposed methodology for non-invasive longitudinal in vivo studies and simultaneous vascular mapping and functional-hemodynamic imaging [4,17,22,24,63].

Label-free PA imaging of oxy- and deoxy-hemoglobin has been used in many studies to indirectly measure neuronal activity and to investigate the neurocircuitry that modulates, for example, reward-related...
behaviors [31,64]. To study the activity of a specific group of neurons, calcium-sensitive contrast agents and voltage-sensitive dyes have been used in combination with PA imaging [17,18]. However, changes in neuronal calcium or membrane voltage are more easily invoked, and therefore, are less specific measures of highly-active neuronal ensembles when compared to Fos-based methods. In addition, our technology allows imaging of Fos-expressing neuronal ensembles while under anesthesia because of the temporal separation afforded by the Fos-LacZ gene system. This is because Fos-LacZ is expressed in a window of time (90 min – 6 h post-stimulus) that is temporally separate from the stimulus or behavioral test [42,65,66]. This cannot be done using more immediate voltage or calcium imaging techniques because there is almost no delay between the behavior and activated calcium ensemble or voltage signal. Moreover, PA calcium imaging methods use GCaMP6f, which has its peak absorption at 488 nm; this short wavelength severely limits the penetration depth of this technique [37]. To make F/M-PAT more clinically useful, the method’s penetration depth could be further improved using a PA-sensitive nano-probe for Fos-protein with a higher absorptivity and peak absorption wavelength similar to advances made in PA imaging within oncology [38], or methods using deep learning [67]. For clinical translation, imaging should be performed though an acoustic window [68,69], or transcranial imaging should be performed with skull aberration compensation algorithms implemented. The most compelling reason to track Fos-expressing neuronal ensembles is that it provides researchers a tool that can focus on the sub-population of neurons most activated during a particular stimulus or behavior. This highly-active subpopulation of neurons have been previously shown to play key roles behind behavior and sustained neuronal activity is inherent to brain plasticity and learned behaviors [13].

5. Conclusion

In this proof-of-concept study we showed the capability of F/M-PAT imaging to detect Fos-expressing neuronal ensembles following acute cocaine injection or shock-tone pairing compared to naive control conditions in male Fos-LacZ transgenic rats. These findings demonstrate the potential for this method to be used in neuroscience studies underlying complex behaviors. It would do this by providing the means for a longitudinal in vivo method for visualizing and measuring neural activity in neuronal ensembles that appear to encode long-lasting memories following learning [70]. This novel method can be applied to rodent models aimed at understanding both normal and pathological neurological processes. The ability of F/M-PAT to selectively image Fos-expressing neuronal ensemble cells in Fos-LacZ rats across multiple stimuli makes it a broadly applicable technique to study the most persistently active components of neurocircuitry behind a range of behavioral processes such as fear learning or addiction, or neurophysiological processes that involve Fos expression such as seizure [71].

Author contribution

James Machynski: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Visualization, Validation, Writing - original draft, and Writing - review & editing. Shane Perrine: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing & review & editing. Alana Conti: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing & review & editing. Kamran Avanaki: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - Original draft, and Writing - review & editing.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.pacs.2021.100297.

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