iTheraMedical

Listening to Molecules

User Manual – Part 3

User Guide

inSight 64   inVision 128   inVision 256-TF

CONTENTS

1   MSOT overview

1.1   Organization of the MSOT User Manual

This manual is divided into 4 sections: MSOT Overview, MSOT Basics, MSOT Data Acquisition, and MSOT Data Analysis. Each section covers a variety of topics separated by themes. Frequently asked questions (FAQ) appear at the end of the individual section when applicable.

1.2   MSOT imaging features

Multispectral Optoacoustic Tomography (MSOT) imaging systems, including the MSOT inSight 64, inVision 128 and inVision 256-TF, are intended for anatomical, functional and molecular imaging of small animals. Data is acquired at 10Hz, allowing for deep tissue characterization of quickly changing dynamics. The custom made excitation laser is tunable in 1nm steps in the near infrared, and has a peak pulse energy maximum of at least 100mJ, enabling sensitive measurements through the entire depth of the imaging plane. Detection of ultrasound occurs through a custom-made array of piezocomposite, 5MHz ultrasound transducers, enabling 150µm resolution at depth.

1.3   Technical support

For any issues with system operation or software usage, please contact support@ithera-medical.com, or your local distributor.

2   MSOT basics

2.1   Photoacoustic effect

The photoacoustic effect occurs when light is absorbed by a molecule, and a sound is subsequently generated. Upon absorption, electrons within the molecule move to the excited state. Upon relaxation, energy is dissipated according to the quantum yield of the absorber either as fluorescence or as heat. When heat (in the low milli-Kelvin range) is generated, a thermoelastic expansion surrounding the molecule generates a pressure wave, which can be detected as an ultrasound signal.

2.2   Tomographic v. linear detection

Tomographic detection is characterized by the acquisition of signals from multiple angles around an object, while linear detection captures signals from a single side. Tomography enables more faithful imaging of complex shapes by capturing 2-dimensional signals in the imaging plane. Linear detection, predominantly capturing 1-dimensional signals, typically undersamples boundaries perpendicular to the axis of the detection, and suffers from shadowing of deep structures from those that are superficial.

The inSight 64 MSOT system has a 172 degree ultrasound transducer tomographic array, while the inVision 128 and inVision 256-TF have a 270 degree tomographic array.

FAQ: What is the coverage angle of the ultrasound detector?
The MSOT systems come with detectors with coverage angles of either 172 degrees (inSight 64) or 270 degrees (inVision 128 and 256-TF).

This is sufficient to image the entire animal in cross-sections. With the 270 degree detector design we get nearly a full tomographic view without loss of image quality in the upper half of the image (which is the case with 172 degrees).

The missing 90 degrees on top are compensated for by other detector elements (especially those on the 180 degree opposite side of the detector array) in our tomographic reconstruction approach.

FAQ: What are the limitations in the field of view in MSOT imaging?
The lateral resolution drops with the distance to the transducer center due to the focus of the detectors. The 256-element system has detectors that focus off-center and therefore provides a slightly extended focal field.

Depending on the absorbance of the object being imaged, light will be fully attenuated after a few millimeters to centimeters.

FAQ: What is the center frequency of the transducer?
The ultrasound transducers used in the MSOT inSight and inVision imaging systems have a center frequency of 5 MHz. The center frequency of the transducer defines the trade-off between resolution and imaging depth. Resolution increases with increasing frequencies. At the same time imaging depth decreases with increasing frequency due to increasing acoustic attenuation at higher frequencies. Five megahertz is a center frequency well suitable for imaging at depth required for an entire mouse cross-section. High-frequency approaches fail to penetrate deeper than a few millimeters.

FAQ: What impact do the different geometries have on imaging performance?

The MSOT inVision 128 and inVision 256-TF have a 270 degree angular coverage, while the MSOT inSight 64 has a 172 degree coverage. While the 270 degree array has sufficient angular coverage to generate complete cross-sectional images through an entire mouse, this is not always the case for the 172 degree coverage. In the case of the MSOT inSight 64, it may be necessary to reposition an animal to image a desired region of the body. The desired imaging region should be positioned towards the detector. For example, when using the MSOT inSight 64 the animal should be placed on its back to image the brain and kidneys. However, for heart imaging the animal may need to be positioned on its belly at a 10 degree angle (with its heart facing the detector).

In the case of the MSOT inVision 128 and inVision 256-TF, both with 270 degree angular detector coverage, cross-sectional imaging is possible throughout the animal regardless of positioning. It is therefore typically not necessary to reposition an animal. However, imaging performance is maximized when the region of interest is nearest the bottom of the transducer, as these regions will have access to more of the detectors in the 270 degree array.

2.3   Cross-sectional imaging

The inSight and inVision MSOT systems utilize a tomographic array of ultrasound transducers to acquire images. The transducer array is perpendicular to the axis of the animal; therefore, the images acquired are cross-sectional. A motorized stage is used to translate the animal through the imaging plane if desired. Offline, a stack of 2D images can be used to render a 3D volume.

FAQ: What is the minimum thickness of an acquired image?
The physical slice thickness is determined by the focus of the detector elements. In the focal field of the detectors this is approximately 800µm.

FAQ: What is the MSOT field of view?
The cross-sectional in-plane field of view of MSOT (x/y) is a square of up to 40x40mm (usually 20x20mm for mice). The field of view in z-direction (via translation) is 120mm (standard, for mice; extendable to 180mm for small rats).

2.4   Object illumination

A fiber bundle transmits the light from the laser to the imaging chamber. This fiber bundle divides the laser beam into multiple slit-shaped fiber light outlets (5 on each side of the ultrasound detector) in order to illuminate the imaging plane from 360 degrees.The light from the fiber covers an area of approximately $4cm^2$. With a maximum incident pulse energy of approximately 70mJ (100mJ, 70% fiber coupling efficiency), this generates a surface fluence of close to $20mJ/cm^2$, thereby within the ANSI limits of safe exposures (for humans).

Body hair can produce irregularities in the transmission of incident light and the emitted sound waves; or hair can produce complete opacity. In contrast to planar fluorescence imaging, where hair is sometimes only removed from the animal surface closest to the camera, hair must be completely removed for MSOT imaging. Since incident light and ultrasound detection cover the entire animal circumference, it is absolutely necessary to remove hair from the entire circumference of the mouse in the desired imaging region.

FAQ: Laser pulse energy
Laser pulse energy is key to image quality because the photoacoustic signal is directly proportional to light fluence (i.e. light energy reaching the absorber). Therefore, double the pulse energy results in double SNR.

The pulse energy is wavelength dependent, but iThera lasers that operate in the near-infrared (NIR) range typically have up to 120mJ pulse energy. The maximal energy is achieved between 730nm-750nm. The laser self-diagnostic tool (see software manual) can be used to monitor the actual laser performance.

FAQ: Should nude mice be used, or should mice be shaved prior to imaging studies?

Hair absorbs light, which results in a strong surface signal in MSOT. It also has a different speed of sound than tissue and captures air bubbles, thus resulting in ultrasound signal artifacts.

Nude mice can be used to avoid having to shave. Alternatively, mice can be shaved in combination with using a depilatory cream to completely remove hair prior to imaging.

Black mice (e.g. C57Bl) can also be used for MSOT imaging, but the hair must be removed. In a subset of black mice, concentrated deposits of melanin can be present on the skin surface. These patches will strongly absorb light and thereby reduce or possibly block light transmission to the interior of the animal. This is a more common issue in young mice (i.e. less 8 weeks old).

FAQ: Pump and OPO laser characteristics

iThera uses a pulsed OPO laser. The pump laser is a Nd:YAG solid state laser that delivers light at one wavelength (in the case of a NIR OPO: 532nm), and the OPO is used to tune the wavelength in single-nanometer steps. Therefore no filters or lamps are used.

The pulse energy differs at different wavelengths. In the range from 700-900nm it should be from 80-120mJ. At 680-700nm and 900-980nm, the laser intensity drops by 30 percent or more. Further, OPO lasers have a pulse-to-pulse energy fluctuation of 5-10 percent, particularly at the lower and higher ends of the wavelength range. Since the MSOT signal is proportional to the light fluence, it is necessary for quantitative measurements to measure the actual pulse energy of every pulse. The optional laser-integrated pulse energy meter in combination with a normalization function during image reconstruction achieves this desired correction for energy fluctuations.

FAQ: Is protection from the laser necessary during operation?

No laser protection devices (safety goggles) are required during operation of the system. The MSOT system is certified as a Class 1 laser product and eliminates the risk of harmful laser light exposure by a light-tight enclosure and safety interlocks.

Warning: do not disable any interlocks!

FAQ: What is the function of the laser-integrated pulse energy meter?

The functions of the laser-integrated pulse energy meter are two-fold:

1) Normalization of the optoacoustic signal for fluctuations in laser energy (all OPO lasers fluctuate significantly from pulse to pulse but also between different wavelengths)

2) control of laser stability and health (as a remote service diagnostic tool)

FAQ: What is the process for troubleshooting laser issues?

The first step of troubleshooting laser issues is to run a diagnostic self-test. The integrated OPO energy meter and spectrometer can be used by the user to monitor the laser performance.

If the diagnostic self-test indicates that the laser energy is significantly reduced, the next step would be to contact a Service Engineer at iThera or from your local distributor. When possible, remote access to the system can allow a technician to view a log file of laser performance over time, and identify or at least rule out the source of the issue. If necessary, a service intervention will be scheduled.

2.5   Ultrasound detection

The custom-made ultrasound arrays are defined by different number of detector elements, element size and element focus according to the model (inSight 64, inVision 128 or inVision 256-TF). In all cases the ultrasound detectors collect broadband signals and have a central frequency of 5MHz. This allows practical acquisition of signals between 50kHz and 7MHz and provides an in-plane spatial resolution of 150μm at depth. The broadband acoustic response based on the photoacoustic effect from large objects is weighted towards low frequencies, while small objects have a higher-frequency response.

2.6   Coupling

When an object is placed in the imaging plane (e.g. a mouse), there is a distance between the surface of the object and the surface of the detector. It is necessary for seamless ultrasound signal propagation to use a coupling medium to fill this distance. Since water has a similar speed of sound as tissue, it serves as an ideal coupling medium. The imaging chamber is therefore filled with water prior to imaging.

In order to separate the imaging specimen from the water, a holder is used in combination with a thin plastic membrane to create a water-tight compartment within the imaging chamber. When an animal is placed properly in the holder, it shall not come in direct contact with the water in the imaging chamber.

A user should take care to observe whether any air bubbles have accumulated between the surface of the animal's skin and the plastic membrane, as air bubbles create artifacts in signal propagation based on differences in the speed of sound in air versus tissue (wave reflections at phase boundaries). All air bubbles should be eliminated prior to imaging by either manual perturbation or repositioning the animal.

Ultrasound gel can be used to optimize the interface between the plastic membrane and the animal's skin. While ultrasound gel is a common coupling medium, the premise for its use in this case is to increase the adhesion of the foil to the animal's skin without producing bubbles. A thin layer of gel is sufficient for this purpose.

FAQ: What is the membrane used for small animal imaging in water?

The membrane used in the MSOT system to protect the animals from water is a 7 μm thin PE foil, a thin version of commonly used wrap foil in the kitchen. This foil has optimal properties with respect to flexibility (to avoid air gaps) and sturdiness (to avoid ruptures).

The PE foil has an optical transmission in the NIR wavelength range of >95% and a speed of sound that is within a few m/s of water. PE is therefore ideally suited as membrane material.

FAQ: What impact does animal positioning have on coupling and image performance?

The nose cone freely rotates, allowing the user to place the animal in the holder in variable positions. For imaging the brain, the coupling between the plastic and the animal head is ideal when the animal is placed in the holder on its back. On its belly, it is possible that due to the proximity of the nose cone, the foil does not fully close around the reducing imaging performance.

Imaging of the legs can be achieved with positioning on the back or belly; however, the user must take care to apply additional ultrasound gel between the animals' legs to avoid air bubbles and resulting image artifacts.

2.7   Intrinsic signals

The MSOT inSight 64, inVision 128, and inVision 256-TF imaging systems are standard-equipped with a laser that operates from 680-980nm. In this range, the predominant sources of contrast that are intrinsic to animals are deoxygenated hemoglobin, oxygenated hemoglobin, and melanin. Other absorbers, such as myoglobin, cytochrome oxidase, bilirubin and water may also contribute to measured intrinsic MSOT signals, depending on the laser illumination wavelength.

In addition, it is possible to use genetic reporters to enhance intrinsic signals from organs or sub-organ structures, or from tumors. Multiple genetic reporters have been documented in the literature. For example, melanin and iRFP (a fluorescent protein that absorbs and emits in the NIR) have been used as optoacoustic genetic reporters.

FAQ: How are anatomical images acquired with MSOT?
In photoacoustic imaging there is no image without laser illumination. An ultrasound detector measures the ultrasound signal generated by the photoacoustic effect – i.e. the acoustic wave generated when photons from a laser pulse are absorbed by intrinsic or extrinsic absorbers in tissue.

The 'anatomical image' is a representation of absorbance of all tissue-intrinsic absorbers at the laser wavelength chosen for illumination. Oxy- and deoxy-hemoglobin are the strongest intrinsic absorbers and therefore dominate the image in case of absence of extrinsic absorbers (fluorophores, dyes, nanoparticles absorbing in the NIR).

If one images at a single wavelength (anywhere in the 680-980nm laser range), the photoacoustic signal represents the absorbance of all absorbers present in the tissue, both intrinsic and extrinsic. By using multiple wavelengths it is then possible to separate the contribution of each individual absorber to the composite image and thereby visualize their individual distribution and concentration. The single wavelength 'anatomical' image can be used to show tissue background, and the multispectral information can be overlaid on top of this background image. Anatomical images at approximately 850nm offer a compromise between light penetration into tissue, laser performance and low absorption from water, which increases significantly above 900nm. However, this is a user-defined setting that can be optimized empirically depending on the experimental goals.

2.8   Extrinsic signals

Extrinsic signals are those not normally present in the animal and may be cells or reagents introduced by the researcher. Any agent that absorbs in the NIR can increase the optoacoustic signal. For a separation from intrinsic tissue background signals through spectral unmixing, an absorber that has a distinct absorption spectrum must be used. As blood is the predominant source of intrinsic contrast, a sufficient concentration must be injected in order to see an increase in signal in the blood. Typically, an optical density greater than 0.5 OD (after dilution in the blood volume of the animal) is required to see an increase in contrast in a blood vessel. Since some injected agents will accumulate in an organ or biological locus (e.g. a tumor), some contrast agents that are not visible in the blood after injection will nevertheless accumulate to a sufficient extent to be visualized at a particular site and time point.

FAQ: Is it possible to get continuous images from pre- to post-injection?

Yes, to image a mouse continuously before, during, and after injection, one should use a tail vein catheter that is long enough to reach through the port in the chamber flap and can thus be accessed with the imaging chamber closed. One should note the dead volume in the catheter to deliver the desired dose.

2.9 Phantoms

Phantoms are sometimes used to characterize injectable contrast agents in a controlled setting. Through the use of phantoms, the sensitivity and linearity of an optoacoustic system can be determined in regards to the agent of interest.

Phantoms have variable properties, including size, shape, composition, and their resulting absorption and scattering properties. Based on the illumination and detection geometry of the MSOT inSight and inVision imaging systems, an ideal phantom has a diameter of 2cm. Phantoms shall scatter incident light in order to homogenously illuminate the imaging specimen. Phantoms can be absorbing or non-absorbing, which affects the comparison of phantom results to *in vivo* studies. Absorbing phantoms with a μ_a (absorption coefficient) of 0.2 cm^{-1} are considered 'tissue mimicking.'

An appropriate phantom should be made in consideration of light and sound propagation. A material should therefore be chosen for MSOT phantoms where optical and acoustic attenuation is minimal and where the speed of sound is close to that of water (1480 and 1520 m/s at 21° and 34°C, respectively).

Most phantoms that are created by users must be made shortly before use, typically using an agar recipe. Alternatively, iThera Medical offers a durable phantom that can be used repeatedly. The iThera Medical phantom is scattering, but does not mimic tissue with respect to absorption. Therefore, phantom lower limits of quantification are generally 5-10-fold lower than quantification limits in a blood vessels or tissue, depending on the particular absorber being used and the local environment surrounding the vessel.

FAQ: Can a phantom be used for calibration of the MSOT system?

Using a phantom for calibration of *in vivo* signals is not completely accurate as it does not take into account actual light fluence in the region of interest. This is related to the heterogeneous presence of intrinsic tissue absorbers.

The durable phantom provided by iThera Medical includes insertions which are used to calibrate the MSOT signal prior to shipment. It also includes two cavities that can be filled with probes to measure respective optoacoustic absorbance spectra and determine their lower limit of quantification. Customer-made agar phantoms with straw insertions can equally well be used for this purpose. However, agar has the disadvantage that it is not durable and needs to be replaced after a couple of days. It is also not sturdy and disassembles in water.

FAQ: How much difference in intensity between probe and control is considered significant?

One way of measuring signal with a phantom is to measure multiple locations within the phantom (i.e. measure 2mm of phantoms in 0.5mm steps, with one cavity filled with probe, the other with the non-absorbing buffer) and calculate an average and standard deviation of probe and buffer. Significance can then be tested by T-test or ANOVA. One can subtract or divide the sample signal from the buffer signal.

It is important to carefully select the ROI within the well in order to avoid measuring signal from the edge of the cavity (contamination or artifacts from phase boundary between phantom material and

probe/buffer). It is also important to use the probe vehicle as a control, unless this solvent is very absorbent in the wavelength region of interest (at wavelengths >900nm water absorbance does become significant).

2.10 Animal physiology

Animal physiology impacts intrinsic sources of MSOT signal. And, intrinsic signals affect measurement of extrinsic signals. It is therefore optimal to have an animal with stable physiology for measurements of MSOT signals, whether one measures oxygenation or the signal from an administered contrast agent.

Core body temperature is one source of variability of intrinsic MSOT signals. If, during anesthesia induction or during animal handling, the core body temperature of the animal is reduced, this will cause a hemodynamic response (i.e. vasoconstriction) in the animal that is observable later by MSOT. When the animal is placed in the imaging chamber, the core body temperature will return to normal due to the heating of the water in the chamber, and a second hemodynamic response (i.e. vasodilation) will occur. During this acclimation period, it is not advisable to perform measurements wherein one will make a comparison of signal values, since the absolute signal values will change according to the concentration of blood within a given region of interest. It is also advisable to use a heated injection pad to minimize the effect from changes in core temperature.

In addition, if one desires to measure oxygenation, one should consider the impact of inspired air on global oxygenation levels within an animal. Inspiration of 100% oxygen, for example, can mask hypoxic areas. For such measurements, one should consider using an anesthesia system that is tunable with respect to oxygen concentration to reach a compromise between anesthesia stability and imaging performance.

FAQ: What is the source of images from the mouse atlas?
The reference anatomy images provided in the mouse atlas are cryoslice images (i.e., photographs from animals sectioned with a cryostat).

2.11 Multispectral data acquisition

2.11.1   Online v. offline reconstruction

Multiple reconstruction algorithms are available for the generation of images from the acquired acoustic data. The 'backprojection' algorithm is based on the well-established related methods for tomographic imaging with MRI and x-Ray CT. It is computationally very efficient and therefore used for reconstruction of images in real time during acquisition. However, this algorithm has limitations, for example the amplification of high over low frequencies, thus emphasizing organ boundaries versus accurately representing the entire volume. This algorithm is also available offline for comparison to images seen during acquisition. However, for post-processing, the 'model-based' algorithm is generally performed for image reconstruction. Considered more quantitatively accurate, this model takes into account the geometry of the transducer to produce a more accurate image. This accuracy comes at a cost, however, with respect to reconstruction time.

2.11.2   Temporal resolution: wavelengths, averages, positions, step size

The temporal resolution of the MSOT systems is determined by the acquisition settings. In the simplest setting, a maximum temporal resolution is achieved by acquiring data at a single wavelength. In this case, the temporal resolution is limited by the firing rate of the laser: 10Hz.

For multispectral measurements which can increase the specificity of the acquired signals, the temporal resolution is determined by three factors: the number of wavelengths, the number of averages, the number of stage positions, and the step size between adjacent stage positions.

Increasing the number of wavelengths can increase the specificity of a signal, but only to a certain extent. Adding wavelengths indefinitely does not further increase specificity. Rather, it compromises temporal resolution. Five to seven wavelengths are typically used for a multispectral measurement in vivo, as a compromise between speed and specificity. Depending on the number and spectra of intrinsic and extrinsic chromophores of interest, above typically ten to fifteen wavelengths there is only marginal improvement of spectral resolution.

Averaging is also a determinant of temporal resolution. Averaging pertains to the number of sequential laser pulses that are 'averaged' into a single data point before the laser continues to the next defined wavelength. In principle, averaging increases the contrast to noise ratio for a given acquisition. Averaging is also a method by which a user can cope with motion. For most *in vivo* applications, using 10 averages is a good compromise between increasing contrast to noise as well as accounting for motion related to breathing (typically 1 Hz) on the one side, and good temporal on the other side.

The scanning position is also determined by the user. For a given selection of wavelengths and averages, the scanning region is the next determinant of temporal resolution. One achieves the highest temporal resolution by continuously scanning a single cross section. This mode is recommended for preliminary studies where the behavior of a compound is not yet known, or when the kinetics of an injected agent are known to be fast. Alternatively, one can scan multiple stage positions. Increasing the number of stage positions decreases the temporal resolution. This mode is recommended when one has *a priori* knowledge of the probe pharmacokinetics.

The step size between adjacent stage positions also has significant influence on the acquisition time. Physical slice thickness is approximately 800μm according to the focal zone of the ultrasound detector. By oversampling the slice thickness, a better image quality for 3D data representation can be achieved. In practical terms 0.5 mm is a good standard step size for large regions, and 0.2-0.3 mm for small regions.

2.12 Multispectral data analysis

Multispectral data analysis relies on a pixel-by-pixel analysis of the change in signal amplitude as a function of measured wavelengths. For a stack of images (defined by a single cycle of multispectral measurements), each pixel in the image will have a spectrum. This spectrum is a composite of underlying spectra from absorbers present at that location. For example, in an animal, deoxy-hemoglobin, oxy-hemoglobin and an injected agent may all be present in different concentrations at the same pixel. The relative concentrations of each absorber will define the composite spectrum. Multispectral data analysis extracts the relative contributions of individual absorbers to the composite spectrum. The output is an image that depicts the distribution and relative concentration of each individual absorber.

2.13 Validation of MSOT data

MSOT data can be confirmed by analyzing the concentration of fluorophores with other modalities following an MSOT experiment. For example, blood samples can be used to measure absorbance or fluorescence with a spectrophotometer. Alternatively, planar fluorescence approaches can be used on whole animals or excised organs.

FAQ: How can tissue samples best be imaged?

Tissue samples can either be embedded in agar put directly onto the foil which is part of the animal holder. Embedding in agar is preferred for control of position, orientation, and homogeneous diffusion of light throughout the sample. If necessary, a water or ultrasound gel can be added in the foil to improve coupling.

2.14 Computer performance

The MSOT systems only come with a pre-installed Windows 7 operating system and the ViewMSOT software as well as the required hardware drivers. No additional software is installed to avoid conflicts with the high performance requirements of real-time image acquisition. Intermittent updates and other computational resource consumption from 3rd party programs, including virus scanners, can interfere with data acquisition, and result in the loss of data.

3 MSOT data acquisition

3.1 Data structure: Study concept

Within ViewMSOT, data is organized into studies and experiments. A study is a collection of experiments that have common experimental goals.

3.2 Data format

3.2.1 Scans v. exported images

A scan file contains the raw data, including study names, annotations, and MSOT signals collected according to the user settings (wavelengths, times and positions). One can also export some or all of the scan data as images. However, the images cannot be loaded into an offline system for analysis. The entire scan must be exported for offline analysis.

3.2.2 ViewMSOT v. MATLAB

ViewMSOT provides functionality for MSOT system control, data acquisition, reconstruction, multispectral analysis, visualization of the data, and quantitative analysis of the data. However, users may also load the scan files into MATLAB and perform reconstructions and/or analyses according to his/her needs.

3.3 User presets

3.3.1 Creating new presets

The organization of related experiments into studies allows the user to save 'study presets', which define acquisition, reconstruction, and multispectral analysis parameters, so that the user can apply the same experimental settings to related experiments. If a user alters a setting from one scan to the next— for example, the speed of sound, number or choice of wavelengths, or reconstruction algorithm—there can be a significant difference in the resulting data, making a comparison impossible. Therefore, it is imperative to maintain study settings for comparisons between scans.
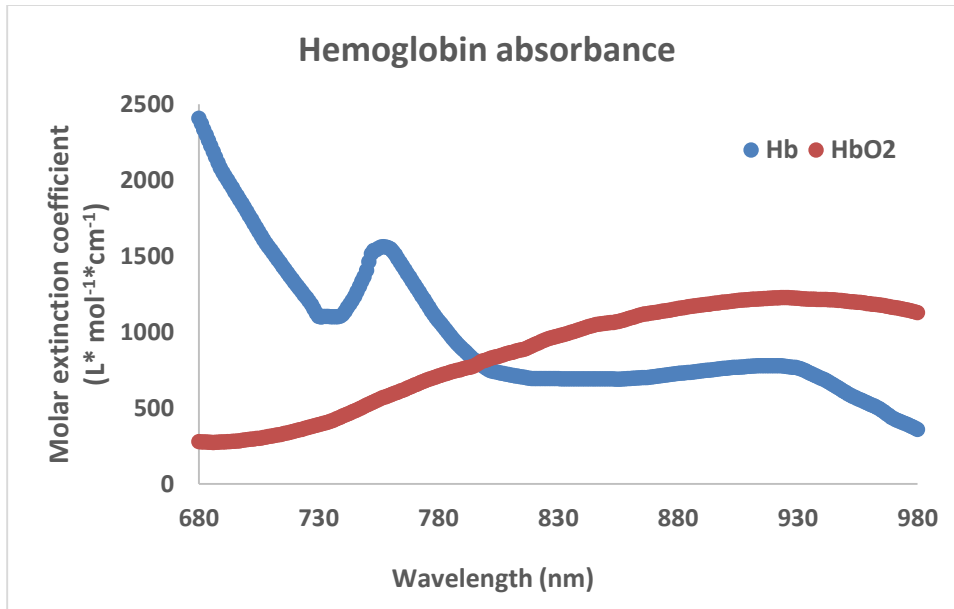
3.3.1.1 Wavelengths

3.3.1.1.1 Core wavelengths

When choosing the wavelengths for a particular study, a user must consider which absorbers will be present in the imaging plane. For a phantom, this is determined by the user. For an animal, the relevant absorbers are a combination of those that are intrinsic (i.e. deoxy-hemoglobin, oxy-hemoglobin, and possibly melanin) and those that are extrinsically administered (i.e. something that the user has injected into the animal). Because *in vivo* experiments always have blood contrast, some wavelengths are essential to use during image acquisition ('core wavelengths').

If one considers the absorption spectra of oxygenated (HbO_2) and deoxygenated hemoglobin (Hb) in the NIR (see Figure 1), then the selection of core wavelengths can be understood. The first, 700nm, is a wavelength where the absorption from HbO_2 is low and the absorption from Hb is high. The next two wavelengths, 730nm and 760nm, are chosen since they reflect local minima and maxima, respectively, in the Hb absorption spectrum. 800nm is chosen as it is very close to the isobestic point of HbO_2 and Hb.

Finally, 850nm is chosen because the absorption from HbO_2 is relatively high and the absorption from Hb is low. If one is scanning a pigmented mouse, the use of these five wavelengths is also sufficient to characterize the change in optoacoustic signal amplitude from melanin, which shows a gradual declining slope in the NIR. Choosing these core wavelengths provides optimal results for the un-mixing algorithms.

(Figure 1. Absorption of hemoglobin in the NIR)

3.3.1.2 Extrinsic absorber wavelengths

In addition to the core wavelengths that are used to characterize blood and skin signals, one might also need to measure or cope with signals from administered probes. In this case, it is important to select additional wavelengths that reflect unique aspects of the relevant absorption spectrum or spectra. For single-peak spectra, it is generally sufficient to add one or two wavelengths (e.g. the absorption maximum, and possibly a wavelength that would characterize decreasing signal if the core wavelengths are insufficient to do so). For more complex spectra, it may be necessary to choose additional wavelengths.

It is important to select sufficient wavelengths to characterize all of the major absorbers that may be present in the imaging plane, even if the user may not be interested in all of them. Otherwise, the un-mixing algorithms may not adequately separate the contribution of individual absorbers to the composite image.

Further, it is also important to select extrinsic probes that have unique spectra. If a probe spectrum has a similarity to another absorber in the experiment (whether intrinsic or extrinsic), then the ability to separate signals from one another will be compromised.

3.3.1.3 Tradeoff between temporal resolution and un-mixing efficiency

Increasing the number of wavelengths can increase the specificity of a signal, but only to a certain extent. Adding wavelengths indefinitely does not further increase specificity. Rather, it compromises temporal resolution. Five to seven wavelengths are typically used for a multispectral measurement in vivo, as a compromise between speed and specificity.

3.3.2 Averaging

Averaging is also a determinant of temporal resolution. Averaging pertains to the number of sequential laser pulses that are 'averaged' into a single data point before the laser continues with the next defined wavelength. In principle, averaging increases the contrast to noise ratio for a given acquisition. Averaging is also a method by which a user can cope with regular motion, such as the heart beat or respiratory motion, by averaging images from all phases of that cycle of motion.

Since the spectral analysis is performed on a per-pixel basis following the sequential acquisition of multiple wavelengths, the analysis can become corrupted if the animal is in different positions from one wavelength to another. For example, when an animal is under anesthesia, it may breathe at approximately 1 Hz. If ten wavelengths are used for a multispectral acquisition, and no averaging is used, then the acquisition of some wavelengths will occur during a breath, and some will occur in between breaths. This will lead to poor spectral un-mixing results. Alternatively, if one chooses ten averages per wavelengths, then 1 wavelength will be acquired per breathing cycle, and the averaged image will display the animal in the same location from one wavelength to the next.

For most in vivo applications, using ten averages is a good compromise between increasing contrast to noise and accounting for motion related to heart beat (10 Hz) and breathing (1 Hz) on the one side, and maintaining a high acquisition rate on the other side.

3.3.3 Spectra

For live multispectral analysis, it is favorable that the absorption spectrum of the probes of interest have been imported into the ViewMSOT software. The live multispectral analysis uses a linear regression algorithm, where given spectra are fit to the pixel intensities across different wavelengths, which requires the input of the expected absorption spectra. Not every offline spectral analysis algorithm requires an input absorption spectrum; however, as discussed in section 4.2, the linear regression algorithm is the most suitable for comparing one scan to another.

For measuring absorption spectra, a user typically uses a third-party commercial spectrophotometer. It is important to measure wavelengths within the working range of the iThera MSOT imaging systems: 680-980nm, ideally in 1nm steps. Spectra not covering this entire range may not be used with MSOT experiments in the case that spectral information for a measured MSOT wavelength is unavailable from the spectrophotometer data.

FAQ: Is it necessary to measure the spectrum of a new probe with a spectrophotometer prior to MSOT imaging?
There are several methods for spectral unmixing in the ViewMSOT software, some which require predefined spectra (fitting of known spectra to measured pixel values) and some which do not (blind unmixing).

For 'linear regression' and 'Guided ICA', one must input the spectra of interest and the algorithm fits the known spectra to the pixels.

There are also statistical blind unmixing methods where the unmixing algorithm looks for characteristic absorbers and generates the spectrum (e.g. PCA/ICA). The rationale for using different unmixing algorithms is discussed in section 4.2 of this manual.

3.4   Using prior presets

Within a study, it is possible to load multiple study presets. A user may have multiple kinds of experiments in mind for a particular study. For example, one may desire a high temporal resolution

during and directly after injection of a probe, but different settings to conduct a volumetric scan (i.e. a scan covering many stage positions) when the probe concentration has stabilized. The study presets are designed to enable the user to easily toggle between experimental conditions, and to maintain similar acquisition settings for related experiments.

3.4.1 Saving modifications

If one desires to modify a study preset, then changes must be made before entering acquisition mode. During acquisition mode, changes made 'on the fly' are not saved.

Because comparability of scans is dependent on identical acquisition settings, it is not recommended to change acquisition settings frequently. At the beginning of a study, a user should identify empirically the ideal imaging settings, and apply these settings to the rest of the study.

3.5 Preview / acquisition mode

3.5.1 Speed of sound

The speed of sound is an important parameter to focus an image. It defines the translation of run time into distance, from the signal source inside the object of interest to the detector. The appropriate speed of sound is determined by the physical properties of the tissue or object being measured, as well as its temperature and that of the water in the imaging chamber.

To optimize the speed of sound, find a small object (such as a blood vessel in a mouse, or an insertion in a phantom), and adjust the slider until the object comes into focus.

As with other settings, the same speed of sound should be used to compare one scan to another, as altering the speed of sound can alter the pixel values in the reconstructed image.

FAQ: What are the optimal speed of sound settings?
In general, the speed of sound is an important parameter for image reconstruction. It should be optimized by the user during preview for accurate visualization of the imaging sample.

However, the setting during acquisition has no impact on your collected (and saved) data, since, after acquisition, one can reconstruct with the correct speed of sound and thereby generate images with optimal focus.

3.5.2 Image display

Image display settings can alter the visible contrast, but such changes do not change the acquired data. It is possible to change the dynamic range of the color scale, adjusting pixel minimum and maximum colors, brightness, contrast, color, and transparency. For spectral analysis, it is also possible to show or hide layers.

3.5.3 Selecting regions

3.5.3.1 Temporal resolution vs. region size

The scanning position is determined by the user. For a given selection of wavelengths and averages, the scanning region (number of slices, defined by scan region and step size) is the determinant of temporal resolution. One achieves the highest temporal resolution by continuously scanning a single cross-section. This mode is recommended for preliminary studies where the behavior of a compound is not

yet known, or when the kinetics of an injected agent are known to be fast. Alternatively, one can scan multiple stage positions. Increasing the number of stage positions decreases the temporal resolution. This mode is recommended when one has *a priori* knowledge of the probe pharmacokinetics and wants to look at the biodistribution and other probe properties in larger regions of interest (e.g. an entire tumor or organ).

3.5.3.2 Single position(s) vs. continuous regions

With *a priori* knowledge, a user can judge whether scanning a region is sensible, or whether scanning a single cross-section is preferred. If, for example, a probe has fast kinetics, then it may be possible that during the time it takes to scan a region of the mouse, the probe concentration could change significantly from the beginning of the scan to the end. Offline 3D reconstructions of the tissue volume and quantification of probe properties may then be compromised by inadequate temporal resolution.

3.5.4 Real-time MSP

3.5.4.1 Online v. offline

Multispectral data analysis occurs in two regimes: online (during acquisition) and offline (post-processing). The online, real-time multispectral processing offers a fast preview. Online and offline analyses can differ since they can use different image reconstruction and spectral unmixing methods. The online analysis depends on the real-time backprojection reconstruction algorithm. Further, it uses the linear regression algorithm for spectral unmixing. In offline post-processing, the user has the option to use the quantitatively more accurate (but also less time-efficient) model-based reconstruction, as well as other multispectral un-mixing algorithms (see section 4 for more details regarding multispectral analysis).

3.5.4.2 Update frequency

In the online preview, the multispectrally processed image will update after each cycle of multispectral measurements (i.e. after completing the full range of wavelengths specified in the study preset).

3.5.4.3 Autoscaling

By default, all layers in a multispectral image are autoscaled. One can adjust the image display settings by disabling the autoscale and using the image threshold tool to, for example, eliminate the visualization of low signals.

If one is conducting a kinetic study where the concentration of a probe will be observed over time, it is useful to establish a baseline before injection. The user can disable the autoscale so that changes in signal intensity after the injection can be observed relative to the baseline signals.

3.5.4.4 Snapshots

One can use the snapshot tool to save an image during a preview or scan. The images appear in the acquisition window, can be annotated, and are automatically available offline.

Snapshots are images of a scan. However, the scan itself is not saved with the snapshot. Therefore, it is not possible to alter image display settings, or reconstruction or multispectral processing settings of a snapshot. Snapshots are intended to be visual aides to gauge change over the course of an experiment, or to save a preview image quickly without the need for additional processing steps.

4 MSOT data analysis

4.1 Reconstruction

4.1.1 Backprojection

The 'backprojection' algorithm is used to quickly reconstruct images for live preview. While it can also be used offline, this algorithm has limitations, including, for example, the emphasis of high-frequency acoustic responses. However, the computational performance of this algorithm is often very useful in establishing a rough estimate of the experimental outcome within a short timespan.

4.1.2 Model based

The 'model-based' algorithm is the preferred offline reconstruction algorithm. This model takes into account the geometry of the transducer to produce a more accurate image. This accuracy comes at a cost, however, with respect to reconstruction time.

4.1.2.1 Matrix size considerations

The 'model-based' reconstruction algorithm requires more computational power than a 'backprojection' reconstruction. A matrix is used to model the geometry of the transducer during reconstruction. This matrix has a large impact on reconstruction time, as the file size of the matrix can be anywhere from 100MB to 10GB or more, and this file must be loaded into RAM before the reconstruction can begin. The first time a particular matrix is used (individual matrices being defined by number of pixels, field of view and speed of sound), it must first be calculated, leading to computational overhead a delay in the inception of image reconstruction. Once a matrix is calculated, it is saved for future use. When model-based reconstructions are executed, there is an initial delay while the matrix is loaded into RAM, and then the reconstruction of images proceeds.

The size of the model-based reconstruction matrix, and the requisite amount of associated RAM, is often a limiting factor in offline reconstruction on a particular computer.

4.2 Multispectral analysis

4.2.1 Basic methods

4.2.1.1 Subtraction

This algorithm subtracts an image acquired at one wavelength from an image acquired at another wavelength. This approach is the most commonly used in photoacoustic multispectral imaging and suitable if one has *a priori* knowledge of the spectra of interest. The user would then subtract a wavelength reflecting maximum absorption from the absorber of interest from a wavelength of low absorption. Use of this algorithm is limited to cases where the change in pixel intensity is only defined by the change in concentration of one absorber.

4.2.1.2 Ratio

Similar to the subtraction algorithm, this method divides the signal acquired at one wavelength from the signal acquired at another, according to the formula [(a-b)/a]. The same conditions and limitations described above for the subtraction algorithm apply to this algorithm.

4.2.2 Spectral fitting: Linear regression

Linear regression is a procedure using known spectra that allows for reproducible results across studies. A spectrum for each absorber of interest must be preloaded into the ViewMSOT software. The spectra of the known absorbers are then fit to the pixel intensities across different wavelengths to create component images for each selected absorber. Based on the mathematics involved, the situation may change with the number and kind of spectra selected. It is necessary to always select more than one spectrum and never more than the total number of wavelengths acquired in a particular scan. As a recommendation, in *in vivo* experiments background absorbers such as Hb and HbO2 should always be selected along with desired agents of interest.

4.2.3 Statistical approach: PCA/ICA

PCA/ICA is a blind method that does not require prior spectral knowledge. It can retrieve spectra based on the statistical properties of the dataset. Principle component analysis (PCA) is performed initially, which identifies the most dominant absorber in the image. This dominant absorber is the basis for the subsequent algorithm, independent component analysis (ICA), which iteratively searches for other relevant absorbers. The number of components, specified by the user, are then randomized during the ICA procedure such that the first component is no longer the principle component.

This algorithm is often useful for verifying the localization of a signal, as an image is produced with an associated spectrum. The similarity of the spectrum to the known spectrum of interest is a determinant of confidence in the localization. However, since this is a statistical algorithm, there will be a variation from one analysis to the next of the same data set. Furthermore, pixel values are normalized, and the intensity is therefore not comparable between scans.

Note: an artifact of this iterative algorithm is the mathematical inversion of spectra. The user must therefore take note of the spectra of the components. If a component is an inversion of a commonly known spectrum (i.e. the injected probe, Hb, or HbO_2), then the user should manually flip the spectrum (and the associated image) by using the 'Select Layers' icon.

4.2.4 Combination: Guided ICA

Guided ICA is an adaptive algorithm that uses a-priori knowledge of the absorption spectra in the sample in order to best adapt to the individual situation. It makes use of the statistical properties of the dataset, and thus does not allow for comparisons between different MSP data sets. As with linear regression, it is necessary to select more than one spectrum for analysis, and to not choose more components than the number of acquired wavelengths.

FAQ: Which wavelength should be used as the 'background' image for layered multispectral images?
This depends on the intended visualization of the user. If one wants to show an anatomical background image based on both states of hemoglobin, one can set 800nm (close to the isobestic point). Using wavelengths lower than 800nm emphasizes signal from deoxy-Hb and will reduce signal at depth due to light attenuation. One can change the background image used in the MSP at any time offline, so the ideal background image can be determined empirically.

FAQ: Which method (linear regression, PCA/ICA, guided ICA, etc.) typically gives the best MSP results?
This needs to be determined on a case-by-case basis and it also depends on the research questions being asked. If one is studying a certain mouse over time, the statistical methods (e.g. PCA/ICA) are not a good choice because the results will vary between scans (i.e. PCA/ICA spectrum on day 1 vs. day 2 could be different); and, PCA/ICA normalizes signal values. With linear regression this is not the case; therefore, it is useful for comparing animals over time or comparing one animal to another. However,

PCA/ICA can be a great tool to confirm that the observed signal is associated to the expected spectrum; and, it can be used to evaluate if the *in vivo* environment has an impact on the probe spectrum.

One case where linear regression is not optimal is performing linear regression analysis on animals / cross-sections with no probe injected or very low levels of probe present, while providing the spectrum of such probe for spectral unmixing. The algorithm will force-fit signals into each component and thus show false-positive signal from the probe. Linear regression should therefore only be used with spectra that represent absorbers found at MSOT-detectable concentrations in the imaging plane.

FAQ: Does including the melanin spectrum in linear regression or guided ICA improve the accuracy of spectral unmixing?

If melanin is present, yes. For nude mice melanin should be excluded. When a C57BL/6 is imaged, melanin should be included (due to force-fitting, see above).

4.3 Quantification

The region of interest tool can be used to quantify MSOT signals in a single cross-section, in volumes, or over time. Quantification is used to analyze changes in MSOT signal from one position (and/or time point) to the next.

Absolute quantification is influenced by heterogeneity of light fluence in the imaging object. One can model light fluence in an animal, but there will always be a margin of error. Heterogeneous light attenuation will lead to different light fluence in different regions of the animal and therefore to differing MSOT signal and calculated probe concentrations. While MSOT software is under continuous development to better account for heterogeneous light fluence, an *ex vivo* validation and calibration of the actual compound concentration should be conducted to more accurately approximate absolute quantification with MSOT measurements.

FAQ: How is oxygenation quantified?

MSOT signals can be spectrally unmixed to represent the contribution of oxy- and deoxy-hemoglobin components to the image. Without calibration these are arbitrary units, used to measure relative changes. The ratio of both components can be used for assessing the oxygen saturation in tissue and vessels. The total of both signals can be scaled to be 100.

FAQ: What other modalities can validate MSOT oxygenation measurements?

There are few methods to measure oxygen saturation in deep tissue and none is 100% quantitative. An alternative imaging method is the BOLD sequence in MRI.

Besides imaging methods, one can use pulse oxymeters and interventional needles for oxygenation measurement. The former have the disadvantage of only measuring very superficially (and unclear, where/what exactly), the latter can also probe deep tissue, but destroys the model due to the needle intervention and only samples a small spot in the region of interest.

Oxygenation measurement is one of the unique capabilities of MSOT imaging. A frequently requested application for oxygenation measurement by MSOT is the analysis of hypoxia in tumor models.

FAQ: How does one quantify concentration vs. MSOT signal?

There are two ways to accomplish this. First, if the injected concentration is known, and one would like to quantify compound concentration inside a blood vessel, one can assume that immediately after injection the probe has diluted in a volume of mouse blood volume plus volume of the injection. In this

way one can estimate how the MSOT signals compares to the actual concentration in a blood vessel following injection. Alternatively, one can bleed the animal and measure the absorbance of the probe in blood at different time points to calibrate the data. One could compare this to a standard curve of different concentrations of probe added to blood or BSA. This calibration, however, would only calibrate the MSOT signal for compound concentration in a specific region of interest, since other regions of interest will show other absolute MSOT values, influenced by local light fluence.

FAQ: What is the general protocol to measure the pharmacokinetics of injected agents?

The usual protocol is to perform an initial experiment to determine the blood dynamics / half-life of the probe. In the absence of *a priori* knowledge, a user can monitor a single cross-section multispectrally (or with a single wavelength in the case of compounds with very fast kinetics).

Once the blood half-life is known, a second experiment can be performed to determine biodistribution. For such studies, single cross-sections covering representative regions for the liver, spleen, and kidneys can be selected. By scanning individual cross sections as opposed to entire organ regions, one can maximize the temporal resolution.

To identify the accuracy of the pharmacokinetic result, one can confirm probe clearance from the bloodstream by taking blood samples at different time points. Confirmation of organ clearance rates would need to be done by *ex vivo* tissue sampling at different time points.

FAQ: How does one cope with motion when quantifying MSOT data?

Addressing regular motion from breathing and heart beat, the user has the following options: By using multiple averages (i.e. a number of sequential laser pulses at the same wavelength that are averaged into a single data point), the user can acquire an average image of the cross-section including all positions of the breathing and heart beat cycle. This will however lead to a loss of resolution due to motion blurring. Depending on the nature and timing of regular motion, one can use fewer or no averages, and then only use the data of same time positions in the motion cycle. This 'retrospective gating' has the disadvantage of requiring a manual selection of data after the experiment. Another option is to use a larger region of interest where the movement does not influence what tissue is inside the region of interest. This approach can also be used in case of irregular motion (e.g. sudden movement of the animal due to light anesthesia). And, finally, in some cases, thresholding the data (i.e. quantifying the top x percent of the data) can sometimes reduce variability within a certain region of interest.

FAQ: What is the unit of signal measured by MSOT?

The signal is a representation of pixel intensity due to ultrasound signal amplitude, and expressed as an arbitrary value. The definition of more meaningful and calibrated MSOT units is under development.

5 MSOT data visualization

5.1 Image visualization

ViewMSOT uses 'Visualizations' to enable the user to analyze reconstructions and multispectral analyses. A Visualization must be given a name, and a scan or scans must be loaded to populate it. All image display and region of interest analyses are saved within the visualization for future reference. Analyzed images and region of interest quantification can be exported from within the Visualization.

When a Visualization is opened with a loaded scan, a default selection of images is displayed. The user can then choose which images—defined by wavelength, position and time point—are displayed in the Dashboard.

The main image on the left side of the screen shows a larger view of the selected image from the Dashboard. When autoscaling is disabled, any image display manipulations (image thresholds, contrast, brightness, color, or transparency) are applied to all images in the Dashboard.

5.2 Region of interest analysis

Region of interest (ROI) analysis can be performed by drawing a region of interest on the main image and selecting this region for the graph plot. The MSOT signal in the ROI can be depicted as change over positions, wavelengths or time. Toggling from one mode to another requires the user to re-select which data (i.e. positions or wavelengths) should be displayed.

In ROI signal quantification, it is possible to apply thresholds to the data. This is one mechanism by which users can cope with negative pixel values, an artifact of some reconstruction algorithms. The application of a threshold is determined by a drop down menu. Thresholds can be applied per image or per stack of images; or, a user can set numerical limits to quantified values (i.e. only quantify positive pixels).

5.3 Image and data export

Only the images shown in the Dashboard will be exported when the export icon is used.

FAQ: What hardware is used for data export?
For data export, one needs to use either the two external USB ports at the front or the LAN network connection. The system has no user-accessible DVD drive.

FAQ: How is MSOT data exported?
Region of interest and spectral data export is in xml format (for MSOT data) and csv format (for spectra). Both xml and csv can be imported in Excel or printed as PDF. As discussed elsewhere, the MSOT imaging system does not have Excel or PDF reading capabilities due to possible interference of required 3rd party software programs with real-time system operation.

Export of images and videos can be selected in jpg, tif, and mpeg-4 formats.

5.4   3D visualization

A stack of cross-sectional images can be used to render a 3D volume. A step size of 0.3mm, along with interpolation between cross-sections, can provide optimal 3D reconstructions (as compared to larger step size commonly used when not pursuing a 3D visualization).

FAQ: What programs are used for 3D visualization of MSOT data?

3D visualization can be performed by using a stack of 2D images and rendering a 3D volume. Basic 3D visualization tools are integrated into the ViewMSOT software. For more sophisticated visualization options, MSOT images can be exported and processed with 3rd party software such as ImageJ and Amira.

6 Appendix

6.1 Animal handling guide

Animal Handling Guide
1. **Choice of gas with isoflurane:** 100% oxygen normally 20% oxygen to optimize hypoxia measurements
2. **Place catheter if necessary; fix catheter in place with glue**
3. **Apply thin layer of clear ultrasound gel to increase adhesion of foil to skin**
4. **Deploy plastic foil**
5. **Place front magnet**
6. **Cut foil**
7. **Position animal holder**
8. **Position rear magnet**
9. **Confirm anesthesia port is connected to holder, as well as to the scanner**
10. **Place animal in holder (nose cone should be rotated according to animal position)** Positioning on belly: optimal for carotid arteries 　　　　　　　　　　 suitable for liver, bladder Positioning on back: optimal for kidney, spleen, liver, bladder 　　　　　　　　　　 required for brain Positioning at 10 degrees (heart at 6 o'clock position): optimal for heart
11. **Reapply ultrasound gel if necessary:** A thin layer should be around the circumference of the desired imaging region If imaging the legs, the space between the legs should be filled with gel
12. **Apply paw cuffs to regulate limb positioning (optional)**
13. **Close clamps around the holder** Ensure safe positioning of tail Ensure that the holder is flatly placed in the frame before closing. **<u>Incorrect positioning prior to closure will damage the clamp.</u>**
14. **Disconnect anesthesia temporarily, transfer animal + holder to chamber, reconnect anesthesia** Lowering the holder too quickly into the chamber can prevent flat closure of the foil above the animal, leaving space for a gap of air

6.2 Imaging guide for study presets

MSOT Imaging Guide for Study Presets	
Experiment settings:	
Wavelengths	Standard (for blood): 715, 730, 760, 800, 850 Additional: peak absorption for injected probe, inflection points, etc…
MSP Preview Spectra	Standard (for an animal): Hb, HbO_2 Standard (for a phantom): Uniform absorber Additional: absorption spectrum of interest (i.e. injected probe)
Background	For live imaging, wavelengths in the mid-800 range provide optimal animal contrast
Frames per wavelength	Phantom: 3 Animal: 10-20 (optimal to acquire 1 wavelength per breathing cycle) e.g. Laser fires at 10Hz, animal breathes at 1Hz; 10 averages = 1 wl / breath
Average frames	Allows user to manually average frames after acquisition, increases file size but gives flexibility to optimize averaging offline. Recommendation: average frames.
Repetitions per slice	Allows user to acquire sequential frames before moving to next designated stage position. In normal use cases, set to '1'.
Preview mode	Full sequence: allows multispectral preview during image 'preview' Single WL: allows user a static image during 'preview', contrast can be optimized Multi WL, 1 frame: fast multispectral preview; can be compromised by motion
Acquisition time schedule	User can schedule imaging time points to proceed automatically
Time interval for runs	Allows one to schedule intervals before repeating a defined scan region/set of wavelengths. Clicking 'infinite' allows scan to proceed until the scan is manually terminated.
Processing settings:	
Recon method	Live preview: backprojection Offline recommendation: model-based linear
Size/resolution	Reconstruction speed proportional to FOV
High/low pass filters	Not recommended, but the user can eliminate some of the acquired frequencies prior to reconstruction. Not possible for model-based linear.
MSP method	Standard: Linear regression Optional: see software manual for recommendations/use cases for other methods
Background	For offline analysis, wavelengths in the mid-800 range provide optimal animal contrast
Spectra	See 'experiment settings', above

6.3 MSOT imaging modes

MSOT Imaging Modes
Single wavelength, single stage position, no averaging **Application:** Ideal for achieving maximal temporal resolution of 10Hz. For example, when imaging the heart or perfusion when concentrations may change very quickly.
Multiple wavelengths, single stage position, with 10 averages **Application:** Ideal for measuring a change in concentration of a specific absorber, independent of background signals from hemoglobin. This mode can be used for imaging organ function, drug delivery or clearance. Ten averages are used to cope with motion related to animal breathing. A single stage position is used to maximize temporal resolution while imaging multispectrally.
Multiple wavelengths, multiple stage positions, with 10 averages **Application:** Ideal when the concentration of an injected agent is not expected to change during the duration of the imaging session. This mode can be used to image contrast agent distribution in 3D, drug delivery, and drug clearance.