Two-photon deep imaging through skin and skull of Zebra finches: Preliminary studies for in vivo brain metabolism monitoring

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ABSTRACT

Zebra Finches are songbirds which constitute a model for neuro-ethologists to study the neuro-mechanisms of vocal recognition. For this purpose, in vivo and non invasive monitoring of brain activity is required during acoustical stimulation. MRI (Magnetic Resonance Imaging) or NIRS (Near InfraRed Spectroscopy) are suitable methods for these measurements, even though MRI is difficult to link quantitatively with neural activity and NIRS suffers from a poor resolution. In the particular case of songbirds (whose skin is thin and quite transparent and whose skull structure is hollow), two-photon microscopy enables a quite deep penetration in tissues and could be an alternative. We present here preliminary studies on the feasibility of two-photon microscopy in these conditions. To do so, we chose to image hollow fibers, filled with Rhodamine B, through the skin of Zebra finches in order to evaluate the spatial resolution we may expect in future in vivo experiments. Moreover, we used the reflectance-mode confocal configuration to evaluate the exponential decrease of backreflected light in skin and in skull samples. Following this procedure recently proposed by S.L. Jacques and co-workers, we planned to determine the scattering coefficient \(\mu_s\) and the anisotropy \(g\) of these tissues and make a comparison between fixed and fresh skin and skull samples for future Monte Carlo simulations of the scattering in our particular multi-layered structure.

Keywords: two-photon fluorescence, microscopy, neuro-imaging, scattering media, songbird

1. INTRODUCTION

In order to understand vocal recognition and social behaviors of animals, it is quite obvious that in vivo experiments are crucial. Moreover, analyses of the neuro-mechanisms underlying vocal recognition created a need for reliable and non invasive tools to probe activations of brain area involved in vocal processes. Amongst all animals, songbirds, and particularly Zebra Finches (\(Taeniopygia guttata\)) are interesting to study because they happen to be specialists in vocal emission and recognition, which are deeply involved in their social interactions. In order to monitor neuro-activations, functional MRI and NIRS (Near Infra-Red Spectroscopy) have been used and have demonstrated their efficiency.\(^2\)\(^-\)\(^4\) Unfortunately, the BOLD (Blood Oxygen Level Dependent) signal measured in fMRI experiments remains difficult to link quantitatively with parameters expressing neural activity. On the contrary, NIRS gives quantitative information about cerebral blood volume (CBV) and hemoglobin oxygen saturation level which reflects cerebral activity, but it is not an imaging method and it suffers from a poor millimetric resolution. In other terms, we have to admit that the study of neuro-mechanisms requires the use of different and complementary monitoring methods.

For more than ten years now, two-photon microscopy has been widely adopted for in vivo imaging because of its ability to keep a good resolution (a few micrometers), even in the depth (1mm) of scattering biological samples.\(^5\)\(^-\)\(^7\) In more recent years, two-photon microscopy has been used for in vivo brain monitoring.\(^8\)\(^-\)\(^13\) Many of the studies have been performed on rats or mice and proved very efficient. Unfortunately, the thickness (1.6-2.2mm) and the highly scattering properties of rats and mice skull requires to remove a part of the skull and

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replace it with a glass window. Other approaches tend to reduce many problems associated with craniotomy by reducing the thickness of the skull to about 200-250µm. Miniature head-mounted elements and optical fibers have been successfully employed on freely moving rats, but still requires craniotomy. More recently, two-photon micro-endoscopy experiments have been tested with very thin gradient index lenses, allowing to keep micrometer resolutions, even 1.5mm deep inside the brain. In most of these various multiphoton in vivo brain studies, the monitoring relies on the study of the dynamics of red blood cells in brain capillaries. This gives a reliable information about neural activations in quite small brain areas. By staining the plasma with various fluorophores, contrast can be obtained between the fluorescent plasma and the absorbing blood cells. These studies, based on red blood cells counting seem to require quite a good resolution which necessitates craniotomy. However, the feasibility of such a measurement has been demonstrated through 250µm thinned skull. Another way to monitor brain activity can be the use of endogenous fluorescence of NAD(P)H or flavoproteins involved in the energy metabolism of neurons. Even if this endogenous fluorescence is quite weak, it seems to be an interesting way as it does not seem to require as much resolution as the counting of red blood cells.

In the case of songbirds like Zebra Finches, craniotomy is problematic as the birds are quite fragile. Moreover, Zebra Finches have a pneumatic skull structure composed of 25µm-thick internal and external cortical bones linked by approximately 250µm-high trabeculae. Indeed, the skull of Zebra Finches is much thinner than the skull or rats or mice, and mainly composed of air. Our goal is to evaluate the possibility to perform two-photon imaging in vivo through the skull, and maybe the skin, of Zebra Finches. Particularly, the brain area we would like to investigate is the NCM (caudomedial neostriatum) which is highly involved in song processing. This brain area is located between the caudal and the dorsal part of the bird’s head and 1.2mm to 2.7mm below the external surface of the head. The hippocampus could also be an area of interest and is located above the NCM, between 0.6 and 1mm below the surface of the head.

At first sight, this particular skull structure is more transparent than the skull structure of small mammalians, however, as the refractive index and scattering properties are very heterogeneous in all directions, it is important to evaluate the effect of this structure on the image quality and the axial and lateral resolutions of multiphoton microscopy. This heterogeneous structure is likely to distort the image. As a first step before setting up in vivo procedures and experiments we decided to work on a simplified structure (i.e. only on fixed skull samples) and to image a clearly identified object. The object we chose to image is a 10µm-hollow core fiber filled with Rhodamine B, which possesses a high two-photon absorption cross section. In this work, we will present various images and processed XYZ stacks performed on fixed skull samples, and particularly we will try to quantify the distortion and loss of resolution induced by the scattering of the incident beam while passing through the skull sample.

Besides, it could be interesting to use simulations of light scattering inside the skin and skull of Zebra Finches in order to better evaluate the feasibility of in vivo monitoring of the neuro-activations. This requires accurate knowledge of the scattering coefficients of fresh skin and skull samples. We used the recent experimental procedure described by S. L. Jacques et al. based on reflectance-mode confocal measurements of the exponential decay of reflectivity as the focus is moved along the optical axis inside the scattering sample. This measurement allows the determination of both the scattering coefficient $\mu_s$ and the anisotropy $g$ from the parameters $\rho$ and $\mu$ describing the exponential decay of reflectivity. This determination requires the performing of Monte Carlo simulations that we have not performed yet. However, comparisons between the exponential decay coefficients $\rho$ and $\mu$ of fresh and fixed samples are presented, allowing us to evaluate the effect of fixing tissues to perform more convenient experiments.

2. MATERIALS AND METHODS

In order to perform two-photon imaging as well as reflectance mode confocal imaging of skin or skull samples, we used a TCS-SP2 confocal microscope from LEICA Microsystems. The laser system was a Ti:Sapphire MIRA 900 oscillator from COHERENT pumped with a 5W VERDI Nd:YAG laser at 532nm. The laser system delivers typically 800mW, 200fs pulses with a 76MHz repetition rate. The wavelength can be adjusted between 710 and 900nm with a maximum power around 800nm. The experimental setup is represented on Figure 1. Only the elements that were relevant during our experiments are represented.
2.1. Two-photon imaging experiments

In the first set of experiments, i.e. for two-photon imaging, two-photon fluorescence is collected just after the microscope objective via a dichroic beam splitter which is transparent to wavelengths greater than 715nm. The collected visible radiation spectrum is split by another dichroic beamsplitter in two channels (non-descanned photomultipliers), one collecting blue/green radiations up to 550nm and the other one collecting yellow/orange/red radiations starting from 550nm. As the confocal pinhole is useless for two-photon microscopy, the use of such non-descanned detectors enables to collect the maximum fraction of fluorescence from the focus. Microscope objectives that were used are long working distance water-immersion LEICA objectives (HCX APO L U-V-I 20× NA-0.5 and HCX APO L U-V-I 40× NA-0.8). An electro-optical modulator (EOM) was used to adjust the laser power at the entrance of the confocal system. This was particularly useful to automatically compensate for energy losses due to scattering as the focus moves inside the samples.

![Diagram](image)

**Figure 1.** Schematic representation of the microscopy configurations used in this work. (a) Main elements of the LEICA TCS-SP2 microscopy system used in this work for confocal reflectance mode measurements of the diffusion coefficients or two-photon imaging of the hollow fiber filled with Rhodamine B. For two-photon imaging: BS: dichroic mirror transparent to visible radiations, DBS1: dichroic beam splitter transparent to NIR radiations starting from 715nm, DBS2: dichroic beam splitter separating the visible radiation reflected by DBS1 into two channels (λ > 550nm and λ < 550nm), NDD1 and NDD2: non-descanned detectors (photomultipliers). (b) Details of the sample used in two-photon imaging: IM: immersion medium, Sk: skull, HF: hollow fiber, RhB: Rhodamine B, St: stand. For confocal reflectance mode: BS: 30/70 beam splitter, Sc: XY-scanner, MO: microscope objective, S: sample, TS: translation stage, P: confocal pinhole, PMT1: photomultiplier.

The hollow fibre we used had a 120µm total diameter and a 10µm hollow core, which simulates a single rectilinear blood capillary. We used roughly 2cm-long fibers, the core of which was filled with Rhodamine by capillarity and sealed with glue at both extremities. In order to have a maximum fluorescence, without any quenching problems, various tests were performed with Rhodamine 6G and Rhodamine B in ethanol with concentration varying from $10^{-5}$ to $10^{-1}$ mol/l. The highest fluorescence was recorded with Rhodamine B for $10^{-2}$ mol/l and for a wavelength equal to 850nm. This concentration and wavelength were kept for all the two-photon experiments presented in this work.

A couple of Zebra Finches with an average weight of 12g were sacrificed with a lethal intra-muscular injection of pentobarbital. Their head were plucked a couple of days days before the death. The blood was removed from the vascular system by cutting aorta just after the death of the animal in order to avoid staining of the bone or skin tissues with blood during the dissection. Approximately 4mm×4mm pieces of skull were taken between
the dorsal and the caudal parts of the bird’s head and along the sagittal suture. These skull samples were fixed with a 4% paraformaldehyde solution.

As described on figure 1-(b), the fiber was set on a 100µm deep rectilinear furrow carved on a plexiglas half-cylinder whose curvature radius is equal to 7mm, which is the average curvature of the Zebra Finches head. In order to prevent the filling of the skull’s pneumatic cavity with water, the immersion medium we used for the microscope objective was a transparent gel, mainly composed of water (echography gel). This gel also held the skull sample and prevented it from sliding during the recording of the image stacks.

2.2. Reflectance mode confocal experiments

In the second set of experiments, i.e. for reflectance mode confocal measurements, the dichroic beam splitter DBS1 is removed and the BS beam splitter is replaced with a 30/70 beam splitter (30% of reflection and 70% of transmission). The microscope objective that was used is a long working distance water-immersion LEICA objective (HCX APO L U-V-I 40× NA-0.8). The pinhole was set to 21.27µm which corresponds to the 40× magnified size of the inner Airy pattern at the focus. In these conditions, the light collected by the PMT1 photomultiplier is proportionnal to the light backreflected by the sample. For safety reasons, our LEICA confocal system is designed to prevent NIR radiations from entering the photomultipliers of the confocal head. Thus, these experiments were performed with a wavelength of 710nm in order to be as close as possible to the two-photon experimental conditions described in part 2.1.

In reflectance mode confocal configuration, the amount of reflected light collected by the microscope objective mainly comes from the focus and is linked to the backscattering properties of a thin slice in the sample. This reflectivity decreases exponentially as the focus penetrates inside a scattering samples.\(^\text{15}\) The reflectivity decrease as a function of depth inside the sample can be represented by the following equation:

\[ R(z) = \rho \exp(-\mu z) \]

where \( R(z) \) represents the reflectivity of the sample at a distance \( z \) from the sample surface. \( \rho \) represents then the reflectivity on the surface of the sample and \( \mu \) the attenuation coefficient. These coefficients are linked to the scattering coefficient \( \mu_s \) and the anisotropy \( g \), which would then lead to the reduced scattering coefficient \( \mu'_s = \mu_s(1-g) \). The coefficients \( \rho \) and \( \mu \) are not independently linked to the scattering coefficients \( \mu_s \) and \( g \). Indeed, for example, when the anisotropy \( g \) decreases, there is less light that is backscattered, which reduces \( \rho \), as well as the attenuation coefficient \( \mu \). Monte Carlo simulations of the scattering of light inside the sample are needed to find the couple \( (\mu_s, g) \) corresponding to a couple \( (\rho, \mu) \). We have not performed such simulations for the moment. However, as a first step, our goal here is to compare the reflectivity and attenuation coefficient of fresh tissues with the paraformaldehyde-fixed tissues we used in the previous experiments with Rhodamine B filled hollow fiber.

We realized 512×512×100 image stacks. The total transversal field was 750×750µm-wide and the height of a stack varied between 100 and 400µm depending on the sample roughness. We then plotted as a function of \( z \) (depth in the sample) the average reflectivity in a 20×20µm region of interest (ROI), which corresponds roughly to an average on 14×14 pixels. The typical z-profiles that were obtained are represented on Figure 2 for various ROI sizes. This figure shows the profile we obtained on two different parts of the sample (A and B) and with ROI sizes varying from 10×10µm to 40×40µm. As it can be seen on B, the decrease can be dependent on the ROI size. This was observed when the sample is locally not perpendicular to the optical axis. The reflectivity variations are then smoothed and the maximum reflectivity is not representative of the theoretical exponential decay. For this reason, the ROI were selected on part of the stacks where the sample was quite perpendicular to the optical axis. According to these measurements, the 20×20µm size of the ROI was chosen as it seemed to be the best compromise to have a representative position of the reflectivity maximum as well as enough pixels to make a significant averaging that prevents the experimental curves from oscillating to much.

These measurements and exploitations were performed on skull and skin samples. A first set of measurement were carried out on samples freshly taken just after the death of the animal (see procedure in 2.1) and kept in a petri dish filled with a Phosphate Buffer saline (PBS) solution, which was then used as the immersion medium for the microscope objective. In these conditions, five image stacks were recorded on various parts of one sample.
Figure 2. Reflectivity variations as a function of depth $z$ in the sample. A and B sets represent the exponential decay on two different parts of the sample. Each curve represents an average on squared parts of the image. Various sizes of these parts have been tested in order to determine the best compromise between averaging and smoothing of the maximum (see text for details).

(skin or skull) and 10 different ROI were considered. Immediately after that, each sample was rinsed with PBS and kept overnight in a 4% paraformaldehyde solution. Five images stacks were then recorded on the fixed samples and 10 ROI were considered on each stack. Hence, we worked on one fresh (and then fixed) skull sample and one fresh (and then fixed) skin sample. On each sample, 5 different stacks were performed and 10 ROI were considered on each stack, which represents 50 exponential decay measurements per sample.

Finally, it was necessary to calibrate the system to provide a true value of the reflectivity. In order to do that, all the settings of the microscope system were kept equal during all the experiments and a calibration stack was performed on a glass plate of known refractive index. Only the power of the laser were changed from one stack to another and taken into account in the calibration of the curves.

3. RESULTS AND DISCUSSION

3.1. Two-photon imaging experiments

Firstly, several stacks were recorded on skull samples, using the endogenous two-photon fluorescence of the samples. This was carried out to characterize the skull structure and is represented on Figure 3. Thanks to these images, we found that the average radius of trabeculae is 50 $\mu$m and there is about 12 trabeculae per squared millimeter. Seen from above, this means that 10% of the skull area is occupied by trabeculae. Previous histology studies performed in ENES laboratory indicates that the overall thickness of the skull lies between 250 and 350 $\mu$m in this part of the skull, and the thickness of the internal and external cortical bones lies between 25 $\mu$m and 50 $\mu$m.

Secondly, stacks were recorded with the hollow core fiber filled with Rhodamine B. An image sequence taken from a stack is represented on Figure 4. On this sequence the endogenous fluorescence of the external cortical bone and trabeculae is first observed, then, a distorted and enlarged image of the Rhodamine B filled core can be observed as the focus is moved inside the sample. Thanks to the electro-optical modulator, intensity compensation was used as the focus penetrates the medium in order to compensate for the intensity decrease of the excitation beam due to scattering.

Four different skull samples were used to carry out two-photon imaging of the hollow-core fiber through the skull. The average $z$-projections of the stacks are represented on Figure 5. For each sample, both the red/orange/yellow fluorescence image ($\lambda > 550\text{nm}$) and the blue/green fluorescence image ($\lambda < 550\text{nm}$) are presented. As it can be seen, the fluorescence of Rhodamine B can be easily distinguished from the endogenous fluorescence of the bone because the fluorescence of Rhodamine B is located in the yellow/orange/red part of the spectrum, with a maximum around 575nm, while the endogenous fluorescence of the bone seems to be extending uniformly from blue to red parts of the spectrum. (a) and (b) experiments were performed with skull samples
as described previously while (c) and (d) experiments were performed with thinner skull samples with only one cortical layer, roughly 100\(\mu\)m-thick, that were taken from younger birds. In (a), (b) and (c), the \(\times 20\) objective was used, while the \(\times 40\) objective was used in (d).

In order to evaluate the lateral and axial extent of the fiber image, x-projections of the previous stacks are presented on Figure 6 (x represents then the axial direction of the fiber). Each of these stacks was obtained by selecting only 200\(\mu\)m-thick slices along the x axis. These slices were selected in particular parts of the image where the fiber image was not too distorted by the scattering in trabeculae.

Lateral and axial profiles of the fluorescence radiated from the Rhodamine B filled hollow core of the fiber are represented on Figure 7. As it can be seen, the image of the fiber core is enlarged by the scattering. The axial and lateral full width at half maximum (FWHM) of the fluorescence spatial repartition is summed up in table 1, as well as the position of the maximum of fluorescence from the skull external cortical part.

As a conclusion, these two-photon imaging studies indicate that it is possible to collect fluorescence at depths close to 1\(\text{mm}\) through the skull of Zebra Finches. However, from experiments (a) and (b), it is clear that this fluorescence was collected mainly between trabeculae. As these trabeculae cover only 10% of the skull area (seen from above), it remains possible to collect an important part of the fluorescence. The distortion and the enlargement of the image of the fiber remains problematic, especially in the axial direction of the beam, with
Figure 5. Two-photon images of the hollow core fiber through various skull samples. Each image is an average z-projection of the stack. The first line contains the images obtained with red/orange/yellow fluorescence (λ > 550nm) while the second line represents the images obtained with blue/green fluorescence (λ < 550nm). As it can be seen, (c) and (d) stacks were performed with skull samples having very few or no trabeculae. These samples were taken from younger birds and were approximately 100µm-thick and only composed of one single cortical bone.

Figure 6. x-projected stacks of the previously presented stacks (where x is the axial direction of the fiber).

an axial FWHM of the fluorescence repartition greater than 1mm in case of experiment (a). As it can be seen from experiment (c) and (d), a more moderate enlargement of 50-70µm in the lateral direction and 400µm in the axial direction can be achieved at depths greater than 700µm if working with younger Zebra Finches.
Figure 7. Lateral (left) and axial (right) profiles of the fluorescence radiated by the Rhodamine B filled hollow core fiber.

Table 1. Summary of the data extracted from the x-projection represented on Figure 6 (The position column is the position of the maximum from the skull surface).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lateral FWHM</th>
<th>Axial FWHM</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>141 µm</td>
<td>1.2 mm</td>
<td>0.71 mm</td>
</tr>
<tr>
<td>(b)</td>
<td>205 µm</td>
<td>0.98 mm</td>
<td>0.36 mm</td>
</tr>
<tr>
<td>(c)</td>
<td>53 µm</td>
<td>0.41 mm</td>
<td>0.74 mm</td>
</tr>
<tr>
<td>(d)</td>
<td>76 µm</td>
<td>0.36 mm</td>
<td>0.78 mm</td>
</tr>
</tbody>
</table>

3.2. Reflectance mode confocal measurements

As described in section 2.2, reflectance mode confocal measurements were performed on fresh and fixed skull and skin samples. Each reflectivity curve has been fitted with the theoretical expression \( R(z) = \rho \exp(-\mu z) \). The least squared fitting method was used to find the values of \( \rho \) and \( \mu \). Figure 8 represents the experimental curves and the theoretical fits for ten ROI taken from one stack recorded on a fixed skin sample. All the points were taken into account for the fit, starting from the maximum of the reflectivity curves.

Figure 8. Reflectivity curves measured as a function of the depth in a fixed skin sample. We present here 10 measurements carried out on 20×20 µm zones spread on various parts of one 512×512×100-stack. Experimental points (circles) are fitted with the theoretical reflectivity function \( R(z) = \rho \exp(-\mu z) \) (lines). The fitted values of \( \rho \) and \( \mu \) (in mm\(^{-1}\)) are given here as well as the correlation coefficient \( R^2 \).

All the fitted values of \( \rho \) and \( \mu \) were compiled and the result can be observed in Figure 9. This figure represents the box and whiskers plots for the four types of sample (fixed and fresh skull and skin samples). As it can be seen, the coefficients for fresh and fixed skull samples are obviously significantly different. As this is less obvious for the skin samples, a Mann-Whitney test was carried out, indicating that the data are significantly
different with a probability of equality inferior to \(10^{-6}\) and \(10^{-11}\) (see Figure 9). According to these data, no conclusion can be made concerning the value of the scattering coefficient \(\mu_s\) and the anisotropy \(g\), because this requires Monte Carlo simulations that are still in process. However, it is clear that the attenuation coefficients \(\mu\) and reflectivity on surface \(\rho\) are different for fixed and fresh samples. These results will be very useful as we are going to perform simulations of the diffusion in our situation and try to optimize our measurements for \textit{in vivo} studies.

![Figure 9. Box and whiskers plots of the coefficients \(\rho\) and \(\mu\) measured on skin and skull samples. The fresh samples were kept few minutes to several hours after the death of the bird in PBS(Phosphate Buffer Saline) solution, while the other ones were paraformaldehyde-fixed samples.](image)

4. CONCLUSION & PERSPECTIVES

Monitoring the brain activation on small songbirds is crucial to understand the neuro-mechanisms that underlie vocal recognition, emission and learning. The understanding of these complex mechanisms requires a multilevel approach as well as the use of various complementary monitoring methods. Two-photon imaging has been recently used for various \textit{in vivo} studies, demonstrating its potential for brain activation monitoring, unless craniotomy or thinning of the skull is required. In the particular case of fragile songbirds, craniotomy should be avoided. Fortunately, we have seen that the skull structure of birds is mostly composed of air and of two thin cortical bone layers that are quite transparent. The skin itself is quite thin (around \(100\mu m\)-thick). All these conditions can also be enhanced by the use of younger birds. In this work, we have showed that the fluorescence of a thin fiber filled with Rhodamine B can be clearly seen through the skull with a conventional multiphoton microscopy system. Unfortunately the enlargement of the incident beam is quite important, reaching \(200\mu m\) in lateral direction and roughly \(1\text{mm}\) in the axial direction. Moreover, the skin and other intracranial tissues were not present in our simple configuration. Solutions could be found with the use of younger birds, because in this case, we have seen that the resolution can be kept to a reasonable value of \(50\mu m\) in the lateral direction and \(400\mu m\) in the axial direction. New fluorophores optimized for two-photon absorption\(^{17}\) could be used, as their two-photon absorption cross section can reach \(2000\text{GM}\), which is ten times the cross section of Rhodamine B.\(^{18}\) As it has been previously demonstrated,\(^7,19\) the use of a regenerative amplifier is an interesting way to increase the imaging depth in biological samples. It remains obvious that a compromise has to be found between the resolution and the imaging depth we require. In our case, simulations of the scattering as well as further experiments are necessary to investigate if the quite peculiar structure of the bird’s skull will not affect too much our resolution and to find experimental conditions and approaches that would be likely to enhance this resolution, especially the axial one. That was the reason why complementary works were presented here as well, concerning the measurement of the scattering coefficients of the skull and skin, which will be useful for future simulations. Particularly, it has been showed that the exponential decay of backreflected light is different for fresh and for fixed sample, and this fact must be taken into account before attempting \textit{in vivo} experiments.
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