Multiphoton Microscopy for Bacterial Imaging: A Label-free Solution Resting on Endogenous Two-photon Fluorescence

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Abstract: We demonstrate the interest of multiphoton microscopy (MPM) for imaging bacteria without any labelling process. Six families of bacteria are tested: *Escherichia coli*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Clostridium perfringens*. For each of these bacteria, the image of a cell is recorded through a multiphoton microscope thus revealing the 3D shape of these bacteria. For the first time, the images of such bacteria are recorded without any labelling solution. A protocol of controlling the image produced is led thanks to a standard staining protocol with carboxy fluorescein diacetate (CFDA) for *E. Coli* and *Staphylococcus epidermidis*. Similar object shapes with or without labelling are produced, thus validating the label-free images generated by MPM. Then, the two-photon excitation spectra are measured for each of these bacteriaand the emission spectra delivered by *E. Coli* and *Bacillus subtilis* are shown. The origin of the two-photon fluorescence (TPF) emission of the bacteria thanks to the nonlinear imaging solution is discussed regarding to the TPF excitation and emission spectra of metabolic indicators.

1 INTRODUCTION

Currently, no label free solution devoted to bacterial imaging exists and labelling bacteria remains a technical challenge. Several techniques have been used for bacterial detection and identification, such as conventional laboratory-based culture media, polymerase chain reaction (PCR), immunological techniques, and Raman spectroscopy (Yoon 2021). However, these methods are often expensive, time-consuming, they need complex procedures, or can lead to false positive/ negative results. With the recent technical advances of bacteria detection using fluorescence-based dyes, it is now possible to assess simultaneously the physiological states of cells with two or three different fluorescent dyes that target specific biomolecules and physiological processes (Yoon, 2021, Wilkinson 2018). The use of flow cytometry multiparameter analysis in conjunction with fluorescent dyes provides consistent information about physiological state of cells at a single level. However, these methods need complex protocol adaptation and complementary investigative techniques. Above all, further advances are required for “in situ” methods improvement in order to assess microbial viability without coloration steps. To this purpose, other more direct technical solutions still have to be developed. Therefore, the need in new solutions resting on microscopic tools for the optical characterization of bacteria is high.

In this publication, for the first time in the field of biomedical microscopy and in the field of microbiology bacteria, we propose to merge multiphoton microscopy (MPM) (Larson, 2011) and

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bacterial imaging, in order to reveal the image of such a biological object through a nonlinear optical process. Several set of experiments usually involved for biological, medical or vegetal samples such as cells, tissues, of whole organs (Gobel 2007, Plotnikov 2006, Hortholary 2021) are now newly presented for nonlinear characterisation of bacetria. We are testing six families of bacteria: *Escherichia coli* (*E. Coli*), *Staphylococcus epidermidis*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Clostridium perfringens*. For each of these bacteria, morphologic characteristics are gathered into Table 1, showing the diversity of the sample chosen: each GRAM is represented (GRAM + and GRAM –), bacilus and coccus shapes, sporulant or not and aeroby or anaeroby. For such large diversity of bacteria, a preliminary test is led: a standard protocol of fluorescence imaging through a nonlinear process of two-photon fluorescence is involved. Thus, two-photon fluorescence images of these bacteria are produced. The first interesting result obtained concerned the ability of this strategy to deliver an image for each of the 6 bacteria selected. By consequence, a second set of experiment was led. For each bacetria, a recording of the two-photon excitation spectra highlights large regions of absorptions, and similar spectral shapes and regions of emission are recorded, as illustrated by the emission spectra of *E. Coli* and *Bacillus subtilis*. A 3D image of *Clostridium Perfringens* is proposed thanks to the application of our instrumental and computational pipeline FAMOUS resting on the estimation of the point-spread-function (PSF) of the system and the deblurring/denoising of the image (Lefort 2021). Finally, we raise a discussion about the origin of the endogenous fluorescence observed and about the interest of MPM devoted to the optical characterization of bacteria. More precisely, the question raised by the presence of fluorescence emission in the same emission range and biphotonic excitation range for all of the bacteria presenting such different characteristics led to the investigation about the presence of similar substances for all these biological objects. Our conclusions are therefore oriented towards metabolic indicators, substances existing for all of the living bacteria.

2 EXPERIMENTAL SETUP

The experimental setup involved in this study rests on a two-side contribution including optical & computational engineering on the one hand and bacterial process engineering on the other hand. First the bacteria are chosen thanks to their specificities for a complete representation of the different kinds of bacteria existing. Then, the instrumental and computational solution resting on MPM is presented and our original solution FAMOUS devoted to the image restoration is presented. We note here that for all the images presented in this publication, false colours have been used. Indeed, the recording of the fluorescence signal emitted by the bacteria endogenously or after a fluorescence labelling process is led by a PMT which detects a signal intensity level, discretely coded.

2.1 Bacteria

2.1.1 Six Bacteria Chosen

The experimental characterization protocol for imaging bacteria is tested with six bacteria from different families. Table 1 gathers the properties of the related bacteria (Prescott 2018).

Table 1: Characteristics of the bacteria used.

	E. coli	Staphylococcus epidermidis	Proteus vulgaris	Pseudomonas fluorescens	Bacillus subtilis	Clostridium perfringens
GRAM	-	+	-	-	+	+
Shape	Bacillus	Coccus	Bacillus	Bacillus	Bacillus	Bacillus
Sporulation	No	No	No	No	Yes	Yes
Aerobic	Yes		Yes	Strict		
Anaerobic		Facultative			Facultative	

In order to cover the great complexity of bacterial structure and physiological features, a large variety of biological models such as gram-negative, gram-positive and spore forming bacteria were used in this study.

2.1.2 Protocol of Preparation

The six bacteria studied (E. *coli, Staphylococcus epidermidis, Pseudomonas fluorescens, Proteus vulgaris, Bacillus subtilis, Clostridium perfringens*) were cultured in a TSB liquid culture medium (Trypto-Casein-Soy BK046HA, Biokar). 1.2mL of bacteria culture in TSB liquid culture medium was removed and centrifuged at 5400rpm for 15 minutes at room temperature. The pellets were taken up in a volume of 4 mL of PBS and their optical density was measured for $\lambda = 600$ nm. Observations were made at optical densities ranging from 0.1 to 1.6. The bacteria were cultured for 2 to 3 days in an oven at 37°C and stored at 4 ° C after resuspension in PBS.

2.2 The Instrumental and Computational Pipeline FAMOUS

The raw images of bacteria are recorded thanks to a multiphoton microscope. The endogenous fluorescence recorded in this situation exposes MPM to nonnegligible levels of blur and noise especially in the case of endogenous fluorescence recordings, this method requires ad hoc computational processing strategy to produce 3D images with an adapted visual quality. In this part, we present the specificities of the pipeline FAMOUS, combining MPM and our computational strategy.

2.2.1 Experimental Setup

Multiphoton Microscope. The experimental setup involved in this set of experiments rests on a standard multiphoton microscope, associated with an original computational strategy of image recovery. Figure 1. presents the resulting experimental setup. The whole instrumental and computational chain has been presented in details in (Lefort 2021). A commercial multiphoton microscope from Olympus (reference BX61WI) was involved for the whole setup. The laser excitation had a tunable central wavelength between 650 and 1080 nm. For the imaging experiments, the central wavelength of the laser excitation, a mode-locked titanium-doped sapphire laser (Ti: Sa) was fixed at 740 and 840 nm. When the evaluation of the two-photon excitation spectra was led, the central wavelength of excitation was tuned between 680 and 1040 nm with scanning steps of 20 nm. During this specific procedure, the laser average power was followed and kept constant thanks to optical densities all along the recordings. The microscope stand was composed with a scanning device with two galvanometric mirrors. The detection system is composed by a set of two dichroic mirrors and two photomultipliers tubes (PMTs), each of them being coupled with a bandpass filter.

Recording of the Instrumental Response Function. The main experiments of bacterial imaging involved a single PMT, excepted when the instrumental contribution to the image has to be characterized (see below section 2.2.3 Computational solution). In that case, the second PMT is requisitioned. For this specific part of the experiment, the biological sample is mixed with standardized object, composed by fluorescent microsphere having a diameter of 200 nm. The fluorescence emission is detected between 575 and 630 nm by the second PMT. The emission efficiency of these objects is significantly higher than the endogenous fluorescence emitted by the bacteria. The sensitivity PMT is consequently adjusted. Starting from the recordings of the fluorescence emission of the microspheres, the estimation of the instrumental response function, also named the 3D Point-Spread-Function (PSF) is recorded. Two detection spectral ranges are specifically recorded: between 420 nm and 500 nm on the one hand, and between 575 nm and 630 nm on the other hand. The emission of fluorescence is characterized thanks to a punctual adaptation of the detection channel. Indeed, a homemade system of emission detection is positioned thanks to a fibered spectrometer. Thus, the emission spectra have been delivered for few bacteria.

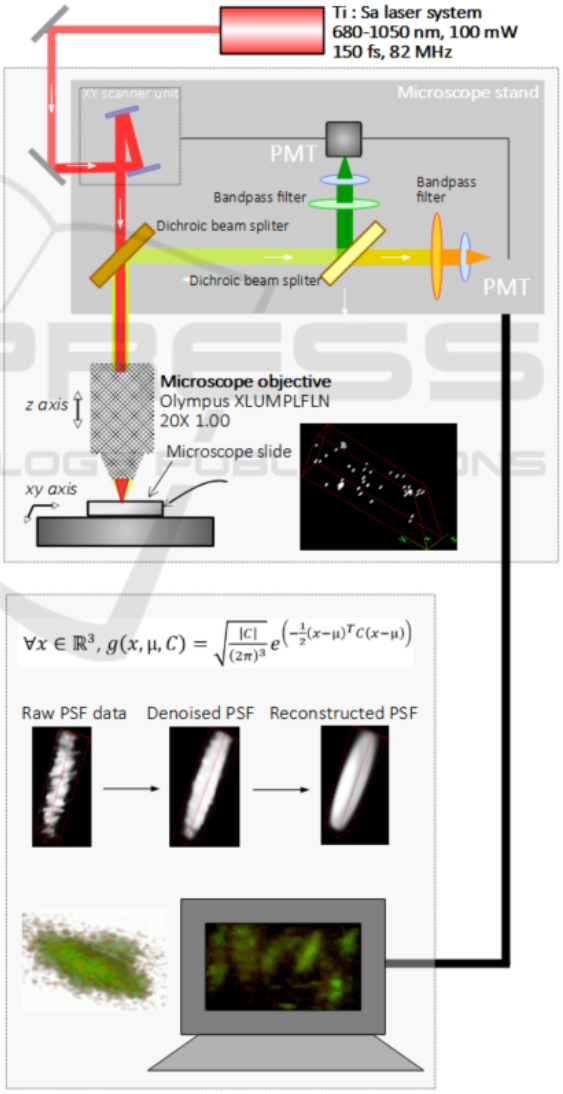

Figure 1: Experimental setup for recording the multiphoton images of bacteria, associated with a computational strategy for 3D image recovery.

2.2.2 Principle of MPM

MPM is an optical solution devoted to the production of images which rests on a principle of nonlinear optics. TPF, a nonlinear process which results from the third-order of susceptibility of the fluorophore $\chi^{(3)}$, is the main process involved in MPM for generating the detectable optical signal. Alternative sources of contrast exists, such as second harmonic generation (SHG) and three-photon fluorescence (ThPF) for example. SHG is an instantaneous phenomenon of nonlinear coherent light scattering which requires drastic conditions of phase-matching while combining the simultaneous interaction of two photons with the structure probed. In our case, the six bacteria tested are good candidates for two-photon excitation and do not seem to be at the origin of the generation of a second harmonic signal. The interest of MPM for bacteria rests on two facts. First, the multiphoton process is involved for wavelengths in the near-infrared range, a spectral range far from the one-photon absorption and emission ranges and lowly energetic compared to linear fluorescence processes. Thus, the bacterial integrity is preserved during the image recording and the spectral absorption ranges can be scanned without any risk of spectral superposition between the excitation and the emission windows. Thus, the endogenous fluorescence emitted from bacteria can be recorded. Then, the TPF solution offers an optical sectioning inherent to its principle resting on a mechanism weakly likely consequently occurring exclusively at the focal point of the system where the photon density is enough for producing a two-photon absorption process. Parasite light, usually emitted from the upper and lower optical plans are de facto highly reduced, contrary to one-photon processes producing photon in these plans sometime mechanically removed thanks to a more or less opened pinhole (confocal microscopy).

2.2.3 Computational Solution

3D image recovery rests on the estimation of the PSF model thanks to the algorithm FIGARO (Chouzenoux 2019). A multivariable Gaussian fitting process delivers a mathematical model of the transfer function of the instrument thanks to the recording of the signal coming from the fluorescent microspheres. The instrumental contribution at the image is thus characterized. Then, the 3D image recovery can take the contribution of the instrument to the image in consideration and specifically remove its role. In our case, for the 3D image recovery, the algorithm from (Chouzenoux 2013) in its accelerated version, is involved. The block distributed majorize-minimize memory gradient (BD3MG) minimizes a least-squares criterion, regularized with a smoothed total-variation term reducing noisy artefacts and a quadratic penalty to constrain the range of the pixel intensities.

3 LABEL-FREE IMAGES OF BACTERIA

3.1 3D Endogenous Images of Bacteria

The illustration of bacterial imaging is led with *E. Coli*. No labelling process is involved in this part. Figure 2 illustrates the image recording of *E. Coli*. Figure 2A presents the large field of view including regions of interest where bacteria look isolated; in the white square, at least two bacteria are identified. Figure 2B shows the 3D point of view of the bacteria present into the white square, revealing a potential

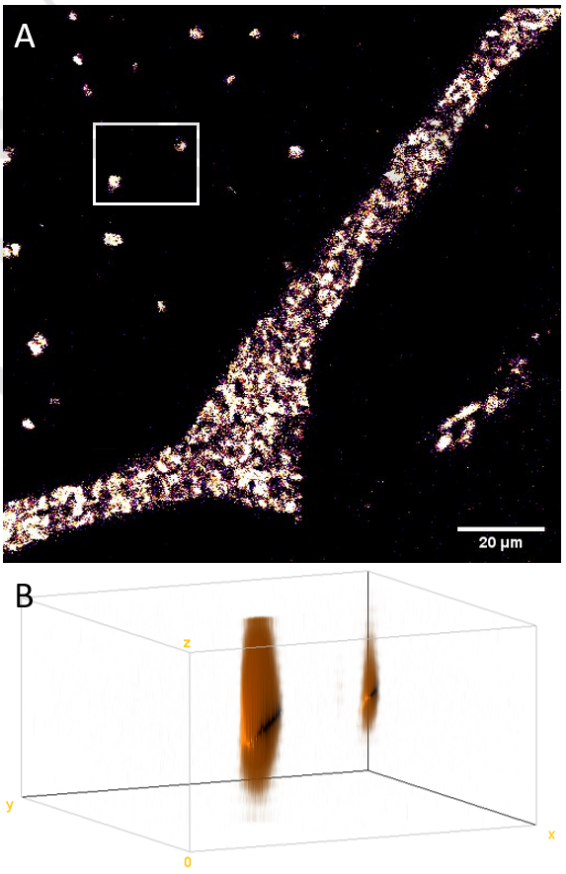

Figure 2: Endogenous fluorescence of *E. Coli*. A. A 2D record of multiphoton images of *E. Coli* label free. B. A 3D reconstruction of the image of *E. Coli*.

superimposition of two bacteria on each other. The *Bacillus* shape expected for E. Coli is observed.

3.2 Control Process of the Object Shape Detected at the Image

A control set of experiment were led thanks to the observations resting on labeled bacteria. The objective was to confirm that the endogenous fluorescence and the resulting image shape of objects were emitted from bacteria. Two bacteria were tested with this protocol. We labeled the bacteria *E. coli* and *Staphylococcus epidermidis* with CFDA (carboxy fluorescein diacetate), an enzymatic activity indicator most often used in flow cytometry[7]. CFDA, a non-fluorescent molecule, is lysed by the esterase of bacteria and converted to CF (a fluorescent form) once in the cytoplasm of the cell, allowing living cells to fluoresce with enzymatic activity. 10 µL of CFDA were added to 1 ml of bacterial solutions in PBS. The solution was placed in an oven at 37°C for 30 minutes and then rinsed with PBS. The purpose of these markings is to find bacteria more easily under the microscope and thus confirm their shape and distribution on the slide in order to then compare the results with the absence of marking. Figure 3. illustrates the images of *E. coli* recorded with the labelling with CFDA.

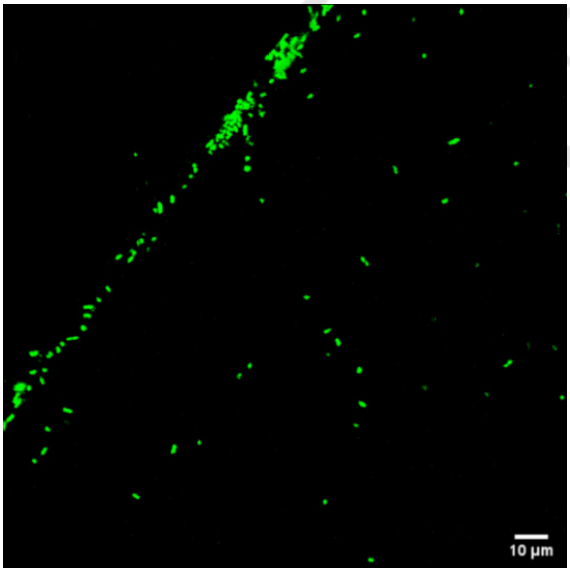

Figure 3: Image of a *E. coli* cells recorded with the labelling with CFDA. Excitation wavelength: 740 nm and detection wavelength fixed between 420 and 500 nm.

Similar information at the image are resulting from this set of experiment, thus validating the endogenous images of bacteria presented from the endogenous fluorescence (Figure 2A). As expected, the level of contrast is greater with the labelling process involving a substance efficiently fluorescent. Moreover, the protocol of labelling bacteria does not produce a labelling of all cells, since CFDA only stain enzymatically active bacteria. The quantity of bacteria revealed at the image looks thus smaller than without labelling (Figure 2A).

3.3 3D Representation of Bacteria through the Pipeline FAMOUS

The *Clostridium perfringens* where imaged in experimental conditions, fitting with the technical recommendation required for the correct application of the instrumental and computational pipeline FAMOUS: fluorescent microspheres with a fixed diameter of 200 nm were mixed with the culture of *Clostridium perfringens*.

Stacks of images were recorded with an imaging step of 0.1 µm all along the imaging depth of 8 µm. Figure 4 shows the superposition of two images: the raw acquisition recorded in red resulting in more or less condensed dots and the restored image revealed in green at the image. Figure 4A represents an extraction from the 80 2D stacks of the recorded image of *Clostridium Perfringens*. Figure 4B illustrates a 3D reconstruction of the *Clostridium perfringens* gathering raw and reconstructed signals. In the present situation, the stack of the raw images was recorded with a reduced average power compared to standard 2D recordings. Indeed, the duration of a 3D image recording is 80 times longer. Thus, for a too high average power level deposited on bacteria, the resulting energy on the sample produces a movement of the bacteria at the image under the local temperature increase (Lefort 2015).

4 SPECTRAL ANALYSIS OF BACTERIA

The origin of two-photon emission fluorescence from bacteria is investigated through the study of the two-photon excitation spectra and the spectral range of emission.

4.1 Two-Photon Excitation Spectra

We characterized the two-photon excitation spectra of each strain of bacteria. In that aim, the central wavelength of the laser excitation is tuned each 20 nm, between 680 and 1080 nm and the laser average

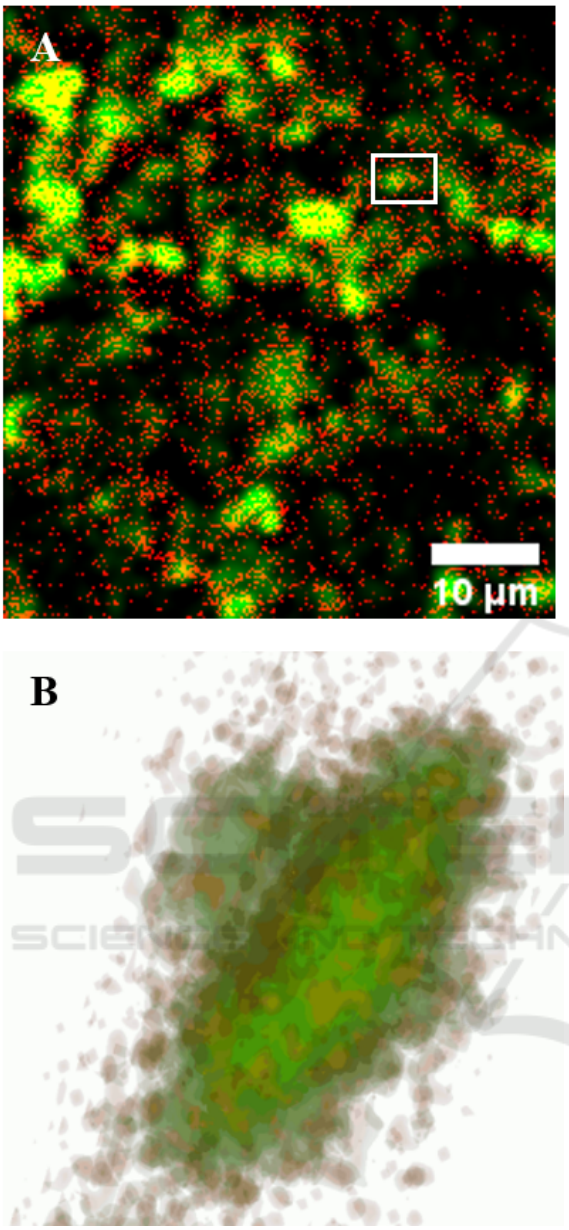

Figure 4: Image of *Clostridium perfringens* label free cells. Red dots: the endogenous emission of fluorescence from the bacteria. In yellow appears the superimposition of raw and reconstructed signals. Green: reconstructed signal of image through the pipeline FAMOUS with a contribution of red decreased compared to green.

power is kept constant all along the recordings. A ten of images were recorded for each of the central wavelengths, each of them containing many individual bacteria. A ten of bacteria were selected for each image and their fluorescence intensity were then recorded in function with the central wavelength of excitation. Figure 5 gathers the normalized intensity of the two-photon excitation spectra of each of the six bacteria.

The emission range of detection is between 420 and 500 nm. The protocol presented at the previous part is involved. The observation of the fluorescence excitation ranges of each of the six strain tested highlights a similar shape, all of them being contained between 700 and 800 nm.

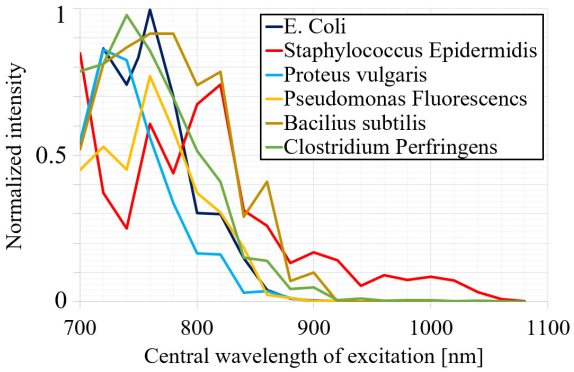

Figure 5: Two-photon excitation spectra of each of the six bacteria recorded between 680 and 1080 nm each 20 nm for a constant average power of 20 mW upon the bacteria.

4.2 Emission Spectra

For two bacteria, we had recorded the two-photon emission spectra. Figure 6 highlights the emission spectra for *E. Coli* and *Bacillus Subtilis*.

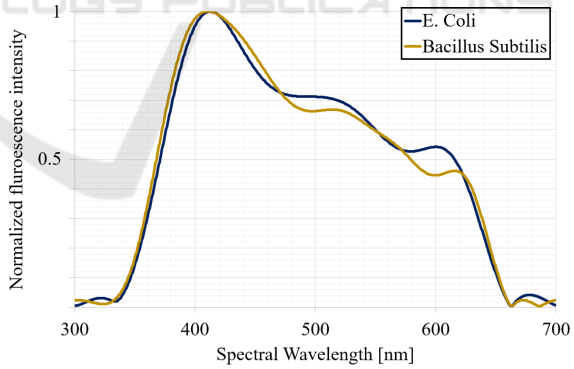

Figure 6: Fluorescence emission spectra for *E. Coli* and *Bacillus Subtilis*.

The emission range of these two bacteria covers the visible range with a maximum value at 400 nm and with a full width at the half maximum of 120 nm. We suspect the fluorescence spectra to be larger in the red range. But our system, resting on a multiphoton system, has a dichroic mirror involved for the separation of the beam between excitation starting at 680 nm and emission fixed with a central wavelength

of 650 nm. The decreasing slope of the fluorescence emission spectra around 650 nm is due to the dichroic mirror.

5 HYPOTHESIS ABOUT THE ORIGIN OF THE ENDOGENOUS EMISSION OF FLUORESCENCE

The main question raised by this work concerns the question of optical characterization of bacteria. Bacteria contain many fluorescent cellular components as protein tryptophan, a few other aromatic amino acids, nucleic acids, and some coenzymes (Surre 2018, Torno 2013). The spectra of these autofluorescence cover most of the spectral range because different endogenous fluorophores emit at different wavelengths of the electromagnetic spectrum (Surre 2018). Among the various endogenous fluorescent molecules, the influence of flavin adenine dinucleotide (FAD) and nicotinamide-adenine dinucleotide (NAD) are considered to be major. Indeed, the fluorescence of reduced nicotinamide-adenine dinucleotide (NADH) is commonly used as metabolic biomarker of cell due to its key role in the conversion of energy. Moreover, numerous optical studies that exist in literature present the two-photon excitation and emission spectra of fluorescence of NADH (Qin 2021, Hortholary 2021, Georgakoudi 2012, Lakowicz 2006). All of these works highlight a two-photon excitation range for NADH located between 700 and 800 nm and an emission range, cantered at 450 nm covers the range between 400 and 600 nm. Moreover, no distinct shape of bacteria can be visually identified at the image. Knowing that all of the bacteria contain metabolic indicators, this cluster of clues leads us to reasonably think that the fluorescence emission we have detected is originated from these chemical substances instead of membrane proteins. Therefore, for living bacteria the presence of fluorescence must be produced for all the bacteria presenting a metabolic activity.

6 CONCLUSIONS

These series of experiments bring new elements of answer to the question raised in introduction concerning the difficulties to visualize the bacteria, their presence, to see their shape, their composition. resting on two scientific facts, initially known but usually treated independently until the present works. Indeed, the endogenous fluorescence of metabolic indicators is not a new scientific result, whether it be by a one-photon or a two-photon fluorescence process. The presence of metabolic indicators in bacteria is also known. In the present set of experiments, the interest of a two-photon excitation strategy lies in the separation of excitation and emission ranges, with several hundreds of nanometers (450 nm for emission and 750 nm for the maximum of excitation). Thus, the endogenous fluorescence initially known as “optical noise” at the image for a one-photon fluorescence process is now easily separable from the excitation beam. Furthermore, our computational solution, adapted for the low intensity levels of detected signal resulting in a highly corrupted image allows to reveal for the first time the 3D optical image of a bacteria in its current environment without any labelling process. The construction of the image of the bacteria label free is thus accessible and gives a visual representation of the target.

For future works, one question emerges from all these experiments. The image of bacteria can be produced through endogenous fluorescence, which is an important information, especially when considering the characterization of bacteria density often led with flow-cytometry through a one-photon process involving labelling procedure. But by evidence, the human eye cannot recognize or differentiate two bacteria from one another when they originate from two different strains. In the case of a need concerning the identification of the bacteria, an assistance of the most recent computational strategies resting on artificial intelligence has to be deployed.

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