Ref. Ares(2020)159389 - 10/01/2020

VETBIONET

Veterinary Biocontained facility Network for excellence in animal infectiology research and experimentation

Deliverable D9.2

Adaptation of light sheet confocal microscopy to the imaging of living pathogens and host cells interactions (cell cultures and biopsies)

Due date of deliverable: M24

Actual submission date: M35

Start date of the project: March 1st, 2017 **Duration:** 60 months

Organisation name of lead contractor: LEICA

Revision: V1

Dissemination level	
Public	X
Confidential, only for members of the consortium (including Commission Services)	
Classified, as referred to in Commission Decision 2001/844/EC	

Table of contents

1. Summary 3
2. Introduction 4
3. Results 5
4. Conclusions 9
5. Annexes 10

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

1. Summary

For this project we have upgraded a confocal microscope with Light sheet functionality in a P2 lab of INRA in Nouzilly. Light sheet microscopy is a highly suitable way of imaging sensitive samples or fast biological processes by illuminating the specimen only in a single plane. Since there is no out-of-focus excitation, phototoxic effects can be reduced to the focal plane. It also means that you automatically have optical sectioning and you can image specimens in 3D by moving the sample through the light sheet.

Objectives:

The goal of this deliverable 9.2 was to adapt an existing confocal microscope SP8 already in a P2 lab with Light sheet functionality. This adaptation should allow imaging of living pathogens and host cells interactions (cell cultures and biopsies) with a gentle single plane illumination. Another goal was also to keep all confocal functionalities after this adaptation.

Rationale:

Light sheet microscopy usually requires a dedicated optical setup on an independent system, where the illuminating and detecting objective are perpendicular to each other. For this upgrade we decided to install a TwinFlect mirror device, which deflects the illuminating light sheet at a 90° angle and allows the integration of the illumination and detection beam path into the vertical axis of an existing inverted Leica TCS SP8 without compromising confocal functionality. The transmitted arm of the microscope was modified to bring the detection path of the Lightsheet system (composed by a filter wheel and filters on objective holder for the detection objective, a 25x Water immersion) with 2,5 mm twinflex mirror and a sCmos camera for detection of Lightsheet signals. We also provided an active table and a compressor as this type of microscopy is more sensitive to vibration. Finally, we had to adapt an incubator specifically designed for this kind of set up in order to control the environmental conditions of the observed samples.

Teams involved:

Leica Microsystem

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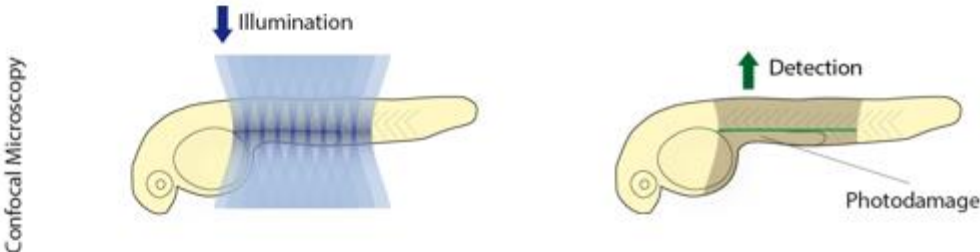
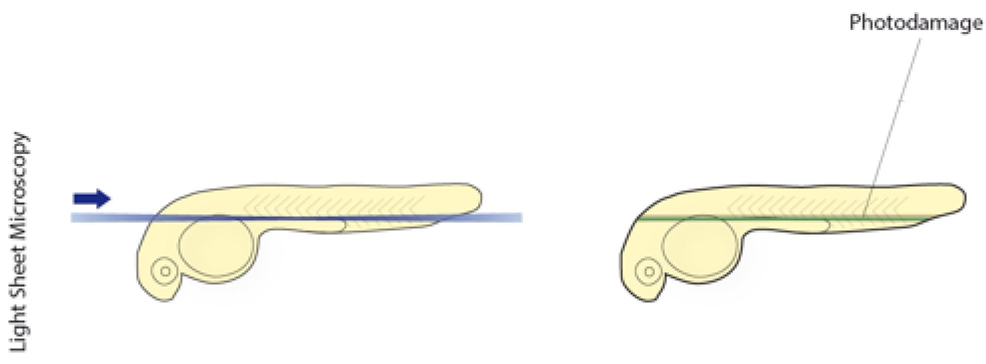
2. Introduction

Imaging interaction between live pathogens and host cells require long term experiments in a controlled and safe environment. Traditionally a confocal microscope (Laser scanning or spinning disk) are used for this kind of experiment as they are able to make optical sectioning that removed the out of focus blur and give to the user more precise pictures and 3D resolved acquisition.

Unfortunately, this kind of microscopy need to illuminate all the sample with a high light dose that can cause photodamage and reduce the capacity to observe some phenomenon during a long time.



Another solution now is to use a single plane illumination. Since illumination ONLY excites one plane at a time, light sheet imaging is extremely gentle. Photodamage is also reduced to the plane that is observed. No point scanning involved, therefore, the plane is illuminated for a short period of time. Subsequently, one can afford to have an excellent sampling rate in z and t. That’s why light sheet imaging is ideal tool for in vivo applications



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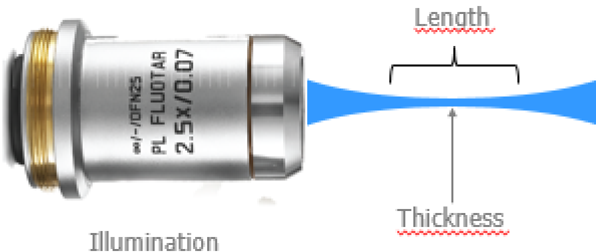
3. Results

For illumination, a rather low NA objective is used. The beam properties of this objectives determine the "length" and the "thickness" of the light sheet.

In the case we decided to use a 2.5x/0.07 illumination objective, the xy resolution (FWHM) is around 3.6µm. This value represents therefore the light sheet thickness.

The focal length of the 2.5x/0.07 is ~240 µm, therefore one can homogeneously illuminate a field of 240µm.



As we use a confocal laser scanning inverted microscope to generate the light sheet, we decided to generate a "scanned" light sheet (also called "virtual light sheet"), one such beam is scanned along a line to create a light sheet, hence many foci are put next to each other.

Alternatively, sometimes in light sheet microscopy, cylindrical lenses are used that create a "static" light sheet. There are several other parameters that determine a light sheet, *e.g.* profile of the illumination light. This could be either a Gaussian beam, a Bessel beam or Lattice illumination.

In our case our strategy uses a Gaussian beam profile that generate a light sheet by scanning the focus. This technic allows to use the existing lasers available on the confocal microscope (405, 488, 561 and 633 nm)

Basic principle of light sheet imaging is illumination from the side that will excite only one plane at a time. Fluorescence emitted by this one plane is captured by a detection objective that is oriented in a 90° angle relative to the illumination objective.

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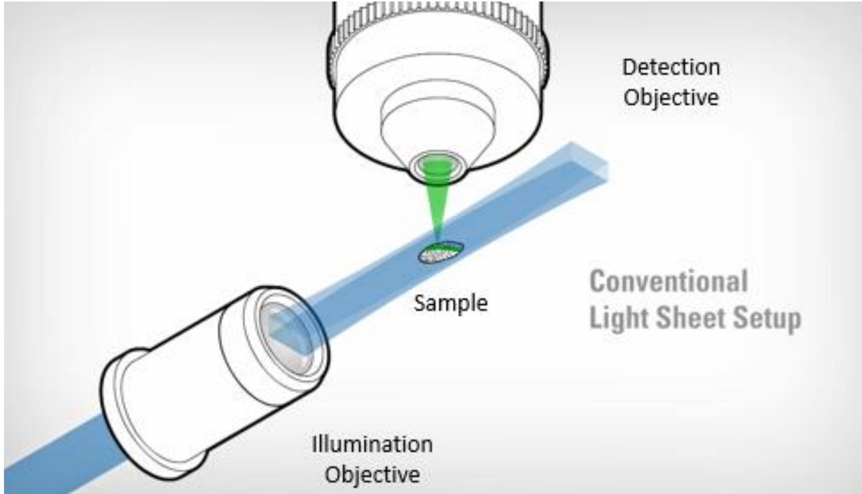
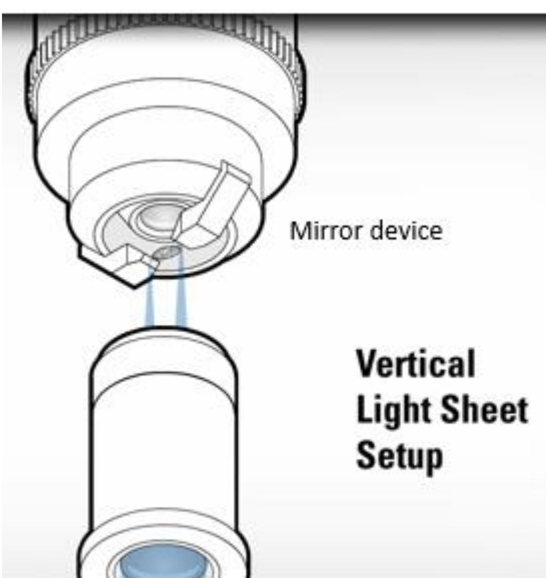


In order to realise illumination from the side on an inverted microscope, the TwinFlect mirror was developed.



In Light sheet microscopy as Illumination is coming from the side, structures that are far away from where the light is coming from are getting blurry. By illuminating from the opposite side, these structures now become better illuminated. By applying a two-sided illumination, the penetration capabilities of light sheet are doubled. Finally, the images that are obtained by left and right illumination are fused to get a merged image of superior quality.

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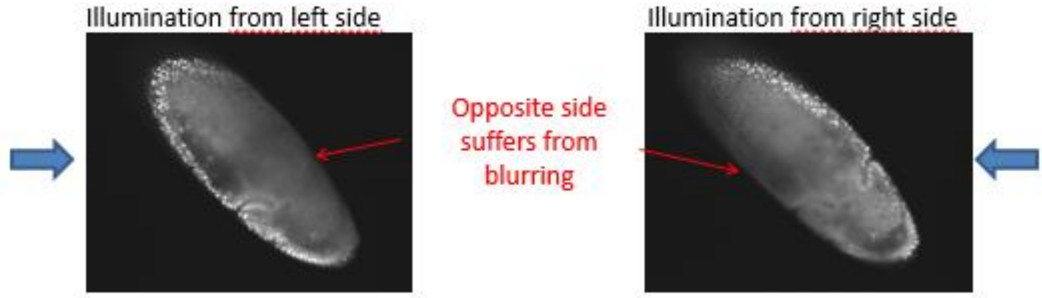
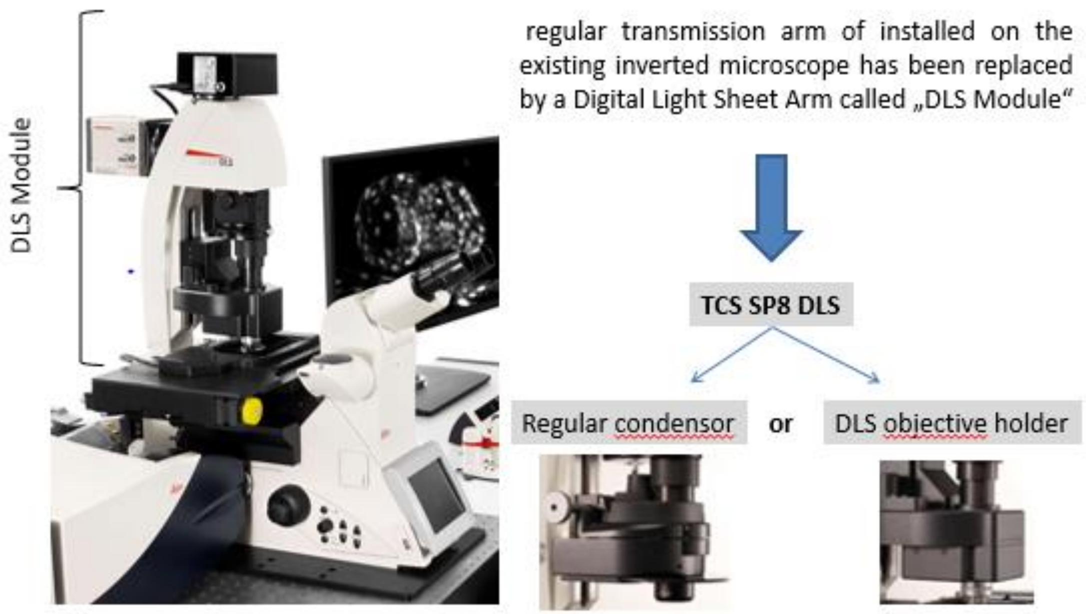


To combine the illumination with a conjugated detection objective and twinflex mirror we had to redesign the existing arm of the inverted microscope. Our goal for this adaptation was to keep all existing confocal functionalities



Go through the DLS detection path. The Regular condenser has been replaced by a DLS objective holder.

Here we placed a 25X Water immersion objective as detection objective. This objective got an XY resolution of 340 nm and 0,96 µm in Z with a working distance of 2,5 mm. This is the best resolution we are able to provide compatible for individual cell imaging. Distance between the 2 mirrors of the twinflex device is 5 mm.

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A rotation Plate allows to freely rotate detection objective and attached TwinFlect mirror, so that the mirror surfaces are aligned with the line scan (xt scan) which generates the light sheet.

Fluorescence that gets emitted by the illuminated plane gets collected by the detection objective and is transmitted onto the camera chip. On its way, the fluorescence has to pass fluorescence filters that are located in the DLS Objective Holder (= modified condenser). For this adaptation, we decided to use 2 specific emission bandpass filters for good spectral separation (504-545 nm for green Fluorescent proteins or dyes and 575-615 nm for red fluorescent proteins or dyes.) We also provided a notch 405/488/561/633 filters for fast multicolour acquisition.

Optical path contains a 1.8x lens which makes sure that ~2-fold oversampling is achieved on the 2048x2048 px camera chip.

For detection we choose our sCMOS camera (Leica DF9000). This camera got a pixel format of 2048 x 2048 with a pixel size of 6.5 µm. With a quantum efficiency of 82 % and a frame rate of 64 frame per second, it was a good choice for us for fast and gentle acquisitions.

Due to the frame rate and the bit depth of such cameras, Light sheet microscopy provide huge amount of data. The computer installed on this confocal microscope was not powerful enough to transfer and stock such amount of data at this speed. That's why we had to change the exiting computer with a more powerful computer

We selected this configuration: HPZ840 2x Quad Core with 128 GB RAM and 1.85 TB SSD RAID (Temp for Data Container) and 11TB HDD RAID to stock data. K4200 4GB Graphics Card to play 3D movies. Computer has been installed with restoration of all previous confocal data and configuration tools.

We also realized that such speed (64 fps in 2048 x 2048) compare to a classical confocal microscope who provide a frame rate around 1 fps in this condition make the system more sensitive to vibration. Therefore, we had to provide an active table and a compressor that give an Air flow of 1 bar to isolate the updated system from vibration. This allow us to solve vibration issue caused by other device in the room.

After upgrading the existing confocal microscope with all these optical device, finally we had to design an incubator compatible with our digital Light sheet solution.

For this we asked to Okolab to design a chamber compatible with our system.

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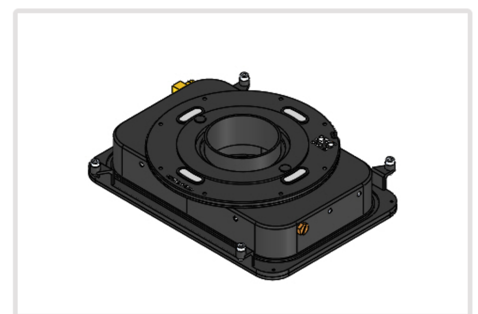
H301-LG-DLS

Compatible with Leica DLS module, it allows usage of illumination and detection lenses. Fits in the Super Z galvo stage, by replacing the Leica specimen holder



The chamber can fit:

- 35/60 mm Petri dishes

Features:

- Compatible with Leica DLS module, it allows usage of illumination and detection lenses
- Includes an objective collar for detection lenses Leica HC APO L 10x/0.3 W and Leica HC FLUOTAR L 25x/0.95 W
- Uniformly heated by means of embedded electric resistances and covered with a temperature controlled lid
- Spring locks hold Petri dishes, in correct position inside the chamber
- Perfusion holes: 12 channels for 2.0 mm O.D. tubings are available
- The chamber screws onto Super Z galvo stage
- Chamber weight, 130 g.
- To control the humidity into the chamber humidity module is recommended: HM-ACTIVE

This chamber allows the control of humidity level to prevent medium evaporation, temperature to simulate pathogen/host living condition and CO_2 level to keep a pH compatible with living cell. This is essential for in vivo long term experiment.

For Lightsheet microscopy we need to isolate the sample from parasite light (noise reduction during acquisitions) that's why the designed incubator is black coloured.

4. Conclusions

This adaptation has been successfully installed and allows an existing confocal scanning microscope to perform light sheet experiment with a gentle single plane illumination from right and left side of the sample in a P2 environment. Transmitted arm and condenser of the existing inverted microscope has been modified to allow the installation of a light-sheet detection path. In order to provide a horizontal scanned light sheet, a device mirror has been installed next to the detection objective. Regarding the performance of the light sheet system, illumination Light sheet thickness is 3.6 µm and the illuminated field of view is 240 µm. Conjugated with a 25x Water immersion objective with a numerical aperture of 0,95 and a working distance of 2,5 mm, the system is able to acquire light sheet data with a XY resolution of 340 nm at 64 frame per second. Software and computer has been upgraded regarding these new functionalities (3 days of service engineer on site for installation and 3 days of an application specialist on site for testing and trainings). An incubator has been designed specifically for this kind of adaptation to control environmental condition for long term in vivo imaging.

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5. Annexes

List of provided items for this upgrade:

Upgrade of Leica TCS SP8 Flexible Supply Unit AOBS with a DLS module

Upgrade SuperZ

100	Okolab DLS Stage Incubation **No : 158006099**	1	9,150.00	9,150.00
200	Okolab Temperature Control (DLS) **No : 158006100**	1	6,876.00	6,876.00
300	Système Okolab contrôle CO² HUmidité **No : 158006094**	1	9,270.00	9,270.00
400	Insert Fix Inverse **No : 158004117**	1	1,110.00	1,110.00
500	Insert Petri 36mm SuperZ Type H pr DMI **No : 158004136**	1	515.00	515.00

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DLS Upgrade

600	TCS SP8 Digital Lightsheet (DLS) DMI6000 **No : 158007002**	1	95,131.00	95,131.00
700	Table optique active 900 x 900 pr STED **No : 158005600**	1	10,618.00	10,618.00
800	Compresseur pour table optique active IR **No : 158002540**	1	2,767.00	2,767.00
900	Base plate DMI 6000 **No : 158000630**	1	1,545.00	1,545.00
1000	Surplatine SuperZ Galvo type H pr DMI **No : 158004116**	1	13,944.00	13,944.00
1100	Obj. HC PL FLUOTAR 2.5x/0.07 **No : 15506523**	1	1,923.00	1,923.00
1200	Unité pour Trigger **No : 158004760**	1	5,843.00	5,843.00
1300	DFC9000 GTC DLS **No : 158005312**	1	17,393.00	17,393.00
1400	Obj. HC FLUOTAR L25x/0.95 W DLS **No : 15506399**	1	10,356.00	10,356.00
1500	Expert Workstation **No : 158003122**	1	16,126.00	16,126.00
1600	DLS TwinFlect 5mm **No : 158007011**	1	2,959.00	2,959.00
1700	Filter DLS 504-545 **No : 158007038**	1	1,332.00	1,332.00
1800	Filter DLS 575-615 **No : 158007039**	1	1,332.00	1,332.00
1900	Filter DLS 405/488/561/633 **No : 158007030**	1	1,332.00	1,332.00
2000	LAS AF SP8 Visualisation 3D **No : 158003203**	1	6,183.00	6,183.00
2100	Service Installation **No : 9I_DAY_INST_UPGR**	2	1,768.25	3,536.50
	Total H.T. :			**219,241.50**
	TVA 20%			+43,848.30
	Total :			**263,089.80**

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