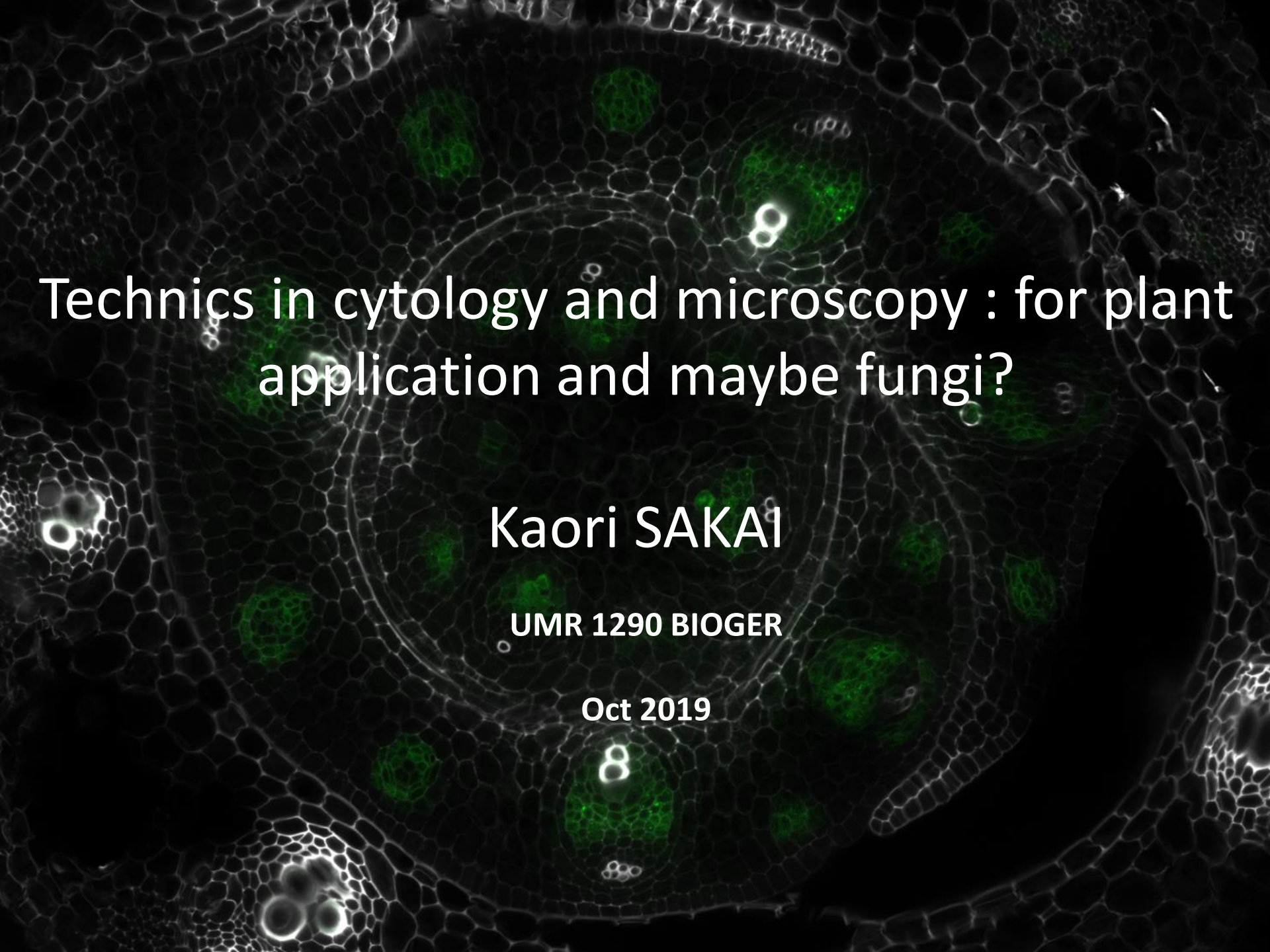


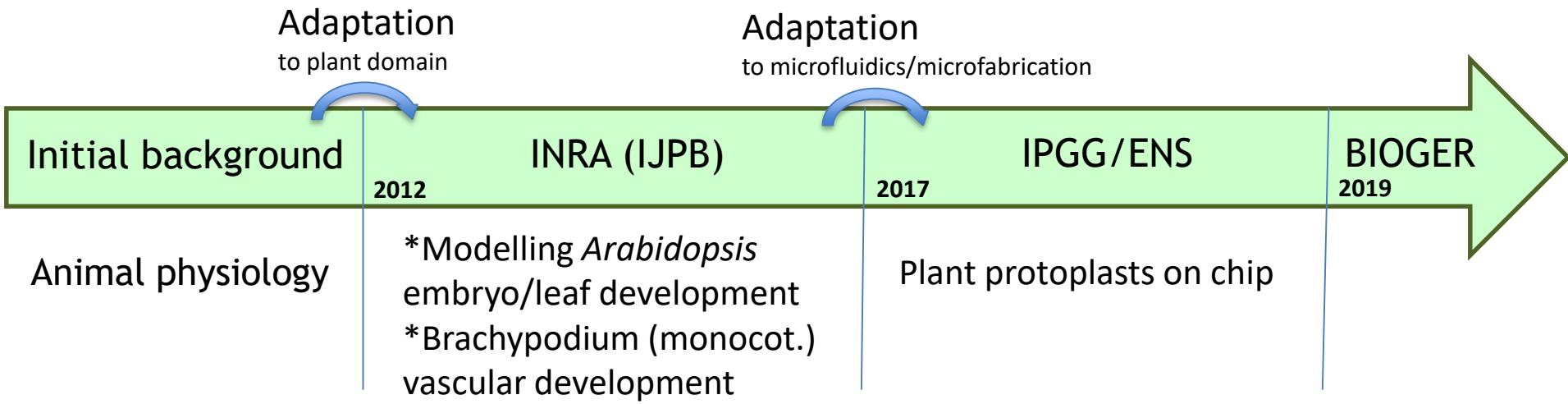
Technics in cytology and microscopy : for plant application and maybe fungi?

Kaori SAKAI

UMR 1290 BIOGER

Oct 2019

Overview of my experiences



Biological model
Animal
Plant
Bacteria

microfabrication

Microscopy
Confocal
Fluorescence microscopy
Home made microscopy

Laser Capture Microdissection

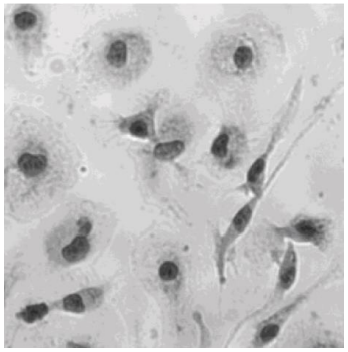
Biological sample preparation
Histology
Cell culture
Molecular biology

Image analysis
ImageJ / Metamorph
C language/ImageJ macro

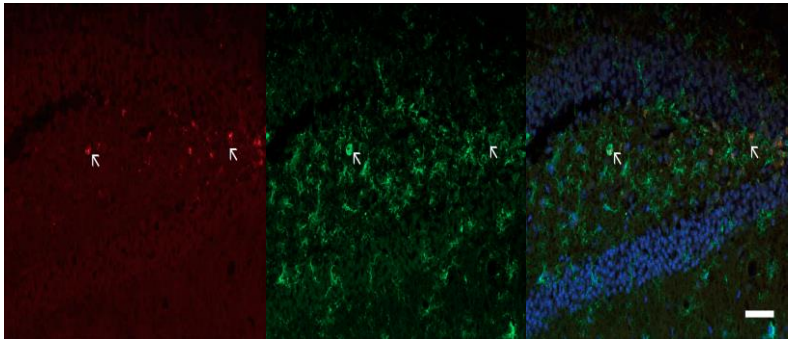
Intership during Master (Vyas S., Katz R., Mallat M.)

Master in Science and Technology- Integrative Biology and Physiology – Speciality Aging biology and longevity, Pierre et Marie Curie University (UPMC, Paris VI, Sorbonne University)

Primary cell culture

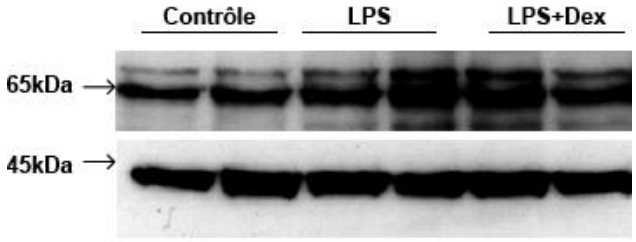


Fluorescence microscopy

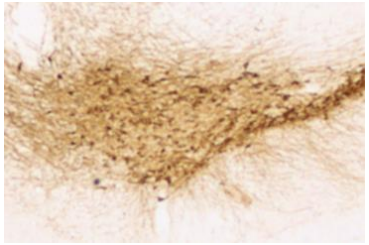


(Apotome: 1D-SIM)

Molecular biology



Immunohistochemistry



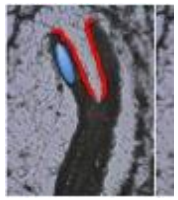
Research subject:

Amyloid-β 1-42 peptide effect on microglia –role of NADPH oxidase

Effect of aging on neuromuscular network

Role of Glucocorticoid receptor in immune response regulation during neurodegeneration

SPS Project : Modelling Developmental Mechanisms



Transcriptomic (URGV platform)

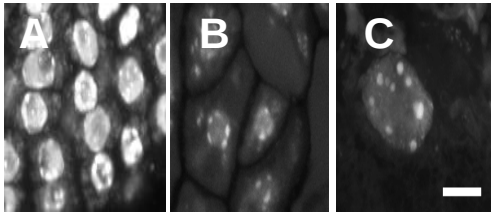
Transcriptional regulation networks during seed maturation

Laser Capture Microdissection (LCM)
RNAseq with small amount of RNA (down to 50pg)

Embryo development ⟷ Nuclear architecture

Dynamics of nuclear organisation during seed development

F.I.S.H set up



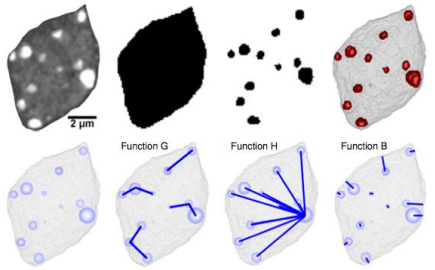
DAPI-stained nuclei of *A. thaliana* embryos.

Computational modelling

Modelling dynamics of nuclear organisation

Sample preparation : conserved 3D structure and able to segment

Set up of multi- sample/position and automatized z-scan process



modelisation of 3D spatial distribution of object (Arpon J.)

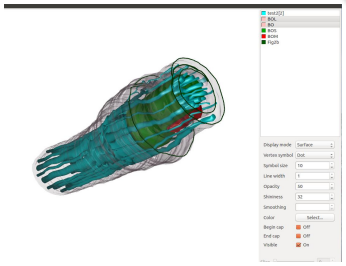
References:

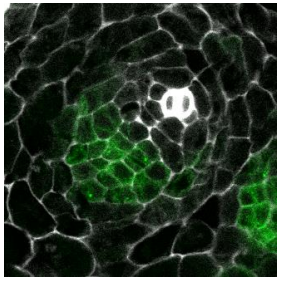
Sakai et al., Plant methods, 2018
Combining laser-assisted microdissection (LAM) and RNA-seq allows to perform a comprehensive transcriptomic analysis of epidermal cells of Arabidopsis embryo

Del Prete et al., Cytogenet Genome Res., 2014
Nuclear architecture and chromatin dynamics in interphase nuclei of Arabidopsis thaliana

BRAVO Project : Deciphering vascular development mechanism (ANR)

Computational modeling of vascular network





Vascular gene related transgenic lines

Detailed description of vascular:
-development
- patterning
-cell differentiation

Image analysis (segmentation)
Cytology / Histology
(microtome, vibratome, cryotome)
Microscopy
(confocal)

Screening mutants with aberrant vasculature

Transcriptomic data from vascular bundles at different development stage

Laser Capture Microdissection (LCM) RNAseq with small amount of RNA
RNA chip

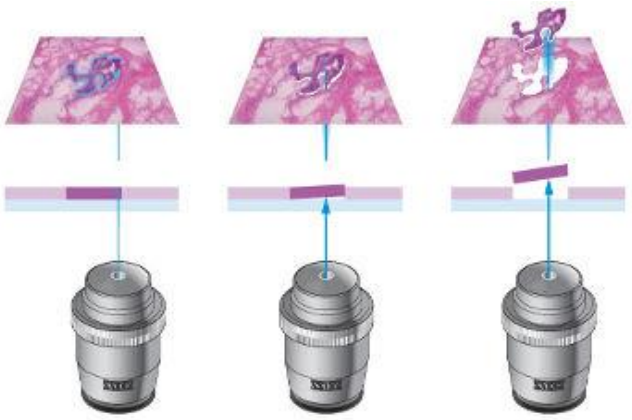
Transcriptomic (URGV-IPS2)

Laser Capture Microdissection

Laser Capture Microdissection

Aim: to concentrate sample content by cutting and isolating the region of interest

Each sample type has it own optimum cutting parameters



Questions you should ask:

1-**How to recognize the region of interest? :**

-determine if isolation is possible OR not

-important step to avoid any "tissue contamination"

-critical step to be efficient against sample degradation (RNA, chemicals)

2-Fixed or fresh tissue / thickness?

-possible RNA modification by fixation process (Acetic acid/Ethanol for RNA is better)

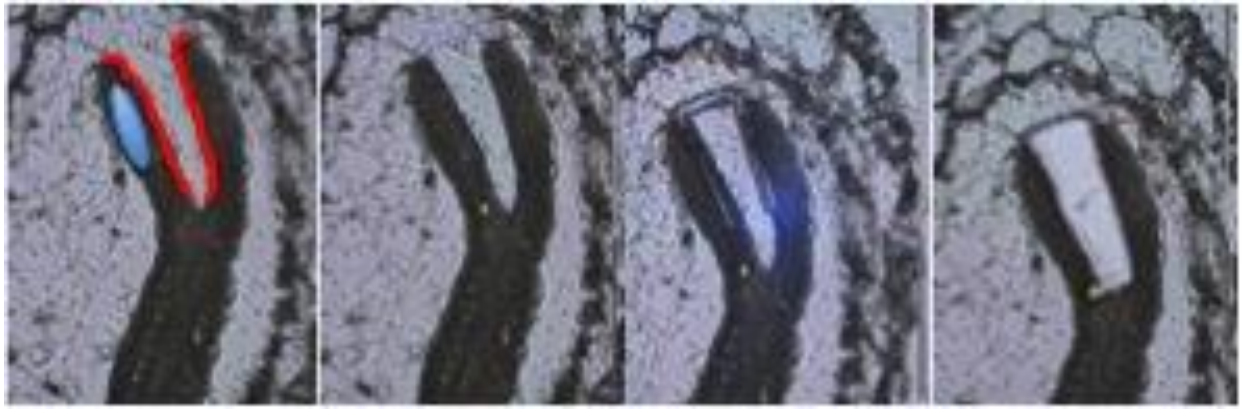
- too thick: could lead to tissue contamination, efficiency of sample harvest by laser microdissection (laser power, laser focus/diameter)

3-Enough quantity possible for analysis:

-minimum amout tested : RNA- (RNAseq, NGS-Illumina-IPS2, 2-3ng total_200-300pg/uL)

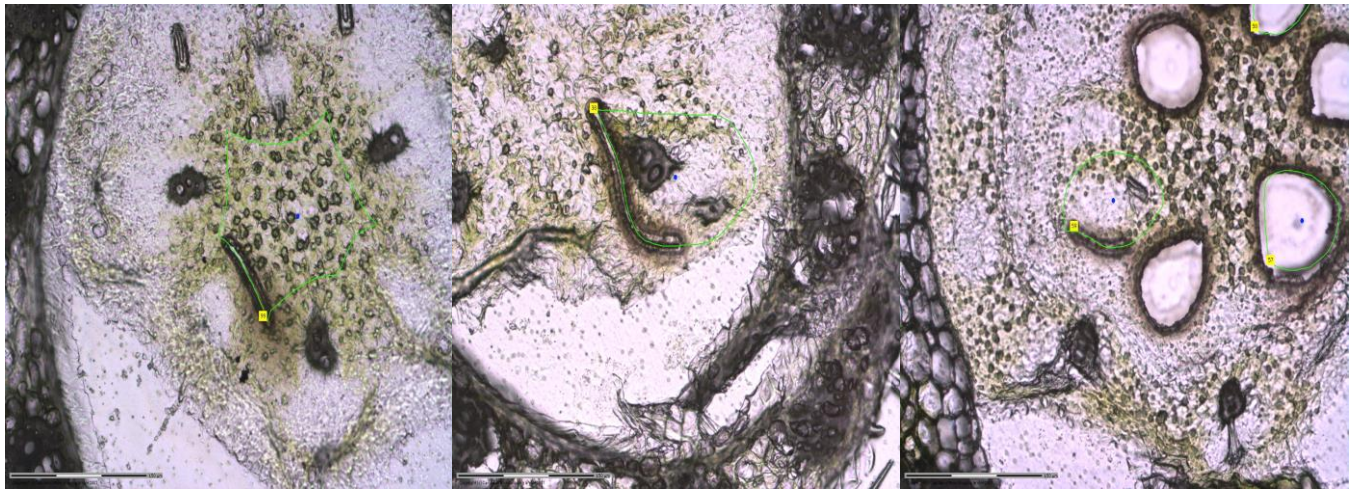
Sakai et. al
Combining laser-assisted microdissection (LAM) and RNA-seq allows to perform a comprehensive transcriptomic analysis of epidermal cells of Arabidopsis embryo, Plant Methods, (2018)

Some examples



Arabidopsis embryo
Acetic Ac./Ethanol fixation
Paraffine embedding
Microtome cutting (8um)

Brachypodium vascular bundle
Liquid N2 fixed
Cryoprotected embedding
Ethanol deshydrated
Cryocrotome cutting (20um)



Overview of Laser Capture Microdissection experiment

Adapt biological material preparation suitable for RNAseq/chemical analysis

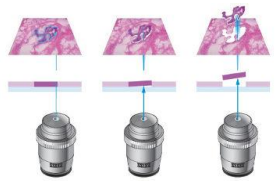
Fixed tissue (ex. Embryo project)

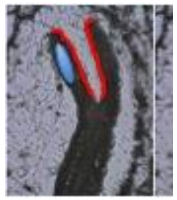
Adapt sample preparation

Fresh tissue (vascular project)

Set up sample preparation

LCM : optimum surface, thickness, time to preserve RNA quality



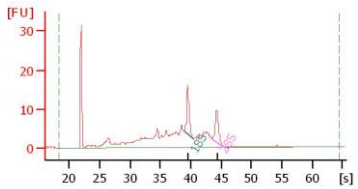


Samples fixation : 4% PFA, Ethanol/Acetic Acid, etc…
Sample conservation : Paraffine, cryoblocs, solution (ethanol)

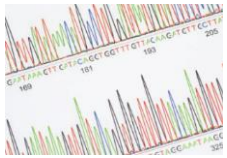
RNA extraction : Rneasy FFPE kit, picopure kit

Chemical extraction/analysis

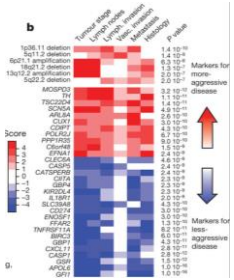
Check RNA quality: Agilent



NGS sequencing: Illumina



Transcriptomic platform



Transcriptomic data of the whole microdissected tissu :
For qualitative, semi quantitative analysis

Platform with LCM : IJPB (plant), I2BC (animals)
LCM working group (in French, Luc Legres)

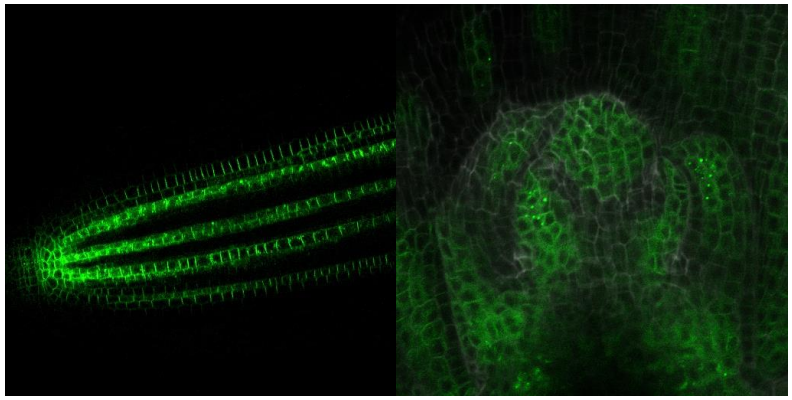
Cutting technics –sample preparation in histology

Sample for microscope observation, but before…

Living OR fixed → Clearing/Staining

Whole mount

Sections

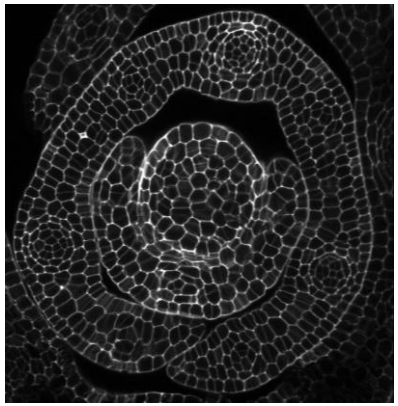


In vivo root

Fixed meristem

Frozen: cryostat
Vibration: vibratome
RT solid embedding: microtome, ultramicrotome

Mounting media (vectashield, citufluor, etc…)
Choice could be crucial for optimum observation



Cryostat cut, monocot leaf, autofluorescence, only in cityfluor

Comparison of cutting method

Did in the past (IJPB)

ROC

	ultramicrotome	microtome	cryotome	vibratome
Thickness range	0,05-10um	1-20um	10-200/500um	20-500um
cutting	Glass/diamond knife	Disposable microtome blade		Disposable blade
Sample fixation	fixed	fixed	Fresh/fixed	Fresh/fixed
Embedding method	resine	Parrafine, wax	sucrose	agarose
Cutting condition	RT	15°C	-20°C	RT
Conservation method	RT, decades	4°C, years	-80°C	4°C, humid
Some critical points	Ethanol used during sample preparation degrade/inactivate functional fluorescent protein as GFP, but some technics to conserve GFP (ROC)		Set up of cutting temperature is crucial for structure conservation	Tissue floating Cutting could be hard depending on tissue softness
Possible application			Fluorescent Transgenic lines GUS transgenic lines	

Ultramicrotome/microtome

fixed samples

BIOGER

For very thin sections

Dehydration/heating step included in sample preparation could affect protein conformation

Sample preparation process is long (5 days)

Sample conservation: years

Cryo-microtome - Cryostat

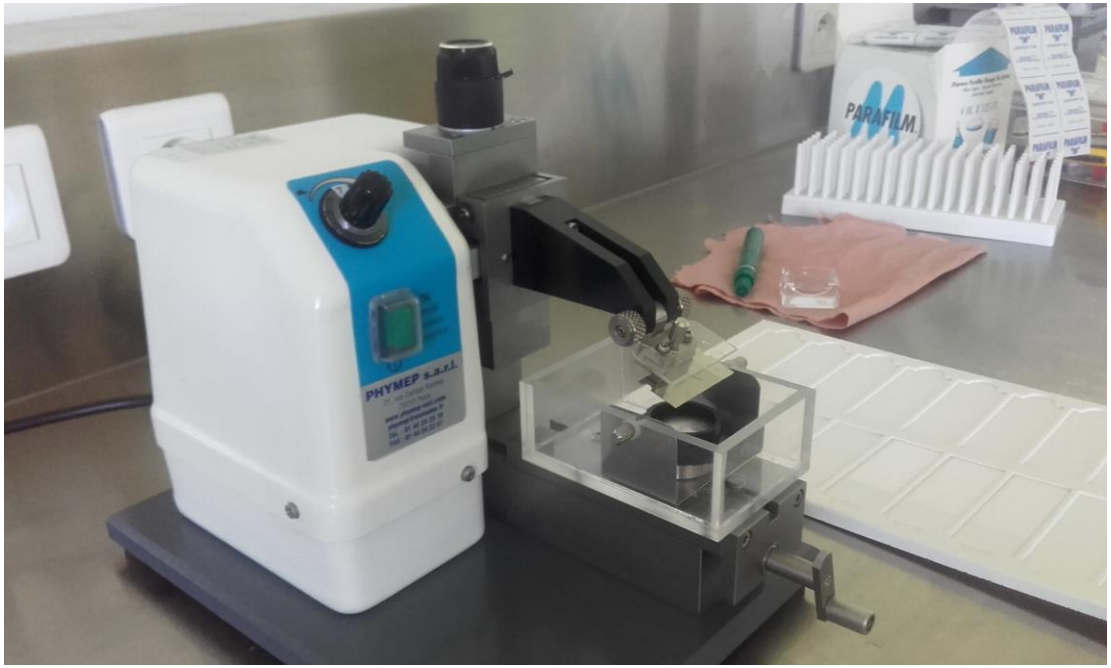
Cryo microtome : on fresh or fixed samples

Faster sample preparation than parrafine or wax
Better conservation of RNA integrity than fixed samples (no crosstalk)
No lost of GFP signal or other ethanol sensitive construction
Thickness between vibratome and microtome (10 to 200um)
Sample conservation : at -80°C for long term storage

Vibratome : microtome with vibrating blade

on fresh or fixed samples

BIOGER



Fast**est** sample preparation

No lost of GFP signal or other ethanol sensitive construction

Without any freezing or embedding process (is possible)

Adapted more for thicker slicing

Staining

Labeling method

Did in the past (IJPB-ENS)

	Autofluorescence Chemicals dyes	Immuno localization	In situ hybridization		Transgenic lines
target	Chemical substrate	protein	DNA	RNA	Proteins/promoter activity
detection	Chemicals: CMAC: vacuole Lignin: auto ex405 Syto62: nucleic acid-red	Antibodies, Direct Indirect	Probe, signal amplification		GFP, GUS construction Direct Indirect

PLANNED TO DO:

Make a list of staining chemicals available and share it
(google sheet, share point,
a system which doesn't need a file upload- and could be updated online)

Microscopy/Fluorescence microscopy

Microscope slide scanner : BF/epi-fluorescence

Trial but not used (IJPB)

Hight throwput image acquisitions

Conditions needed for the use of slide scanner:

-same size/region of slide to scan

-sample preparation well established

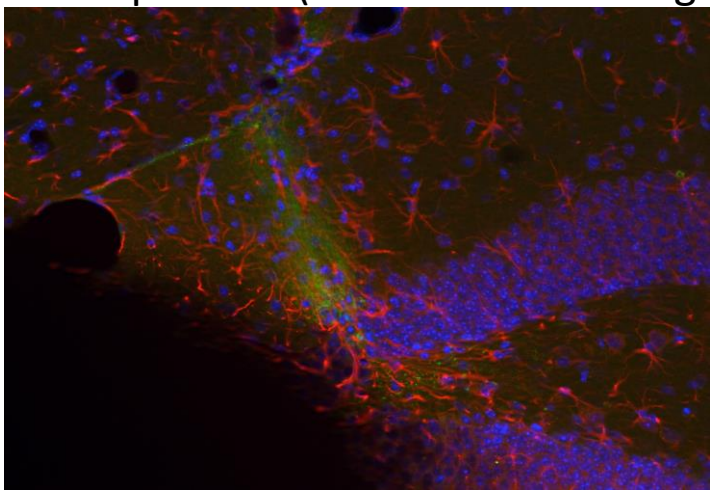
REFERENCES

Legland D. et al., 2017. Histological quantification of maize stem sections from FASGA-stained images. Plant Methods, 13: 1-11

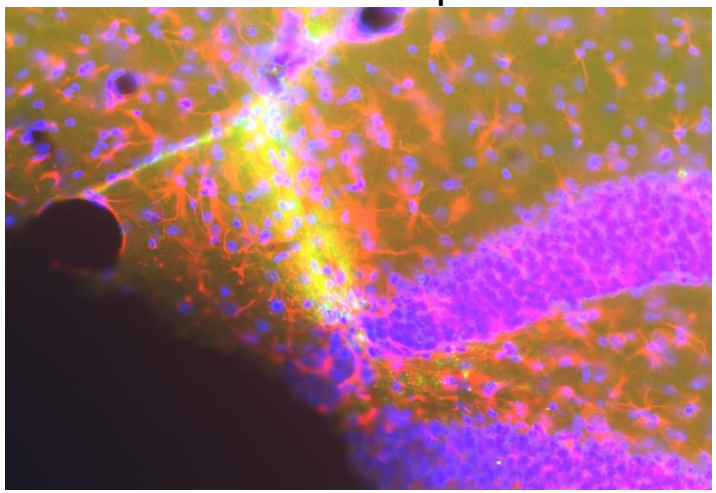
Girard, C. et al, 2015. AAA-ATPase FIDGETIN-LIKE 1 and Helicase FANCM Antagonize Meiotic Crossovers by Distinct Mechanisms M. Lichten, ed. PLOS Genet., 11, e1005369

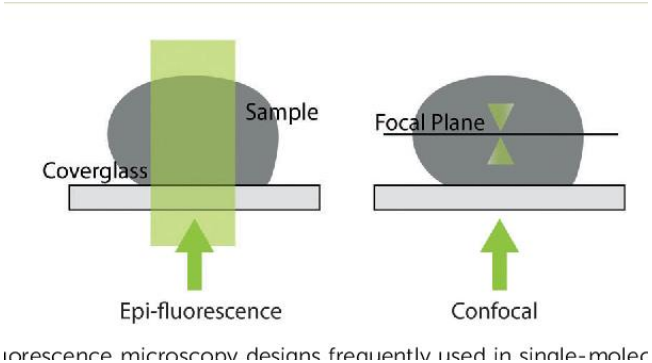
Apotome (ZEISS, « 1D-SIM »)

Apotome (confocal “like” images)



without apotome

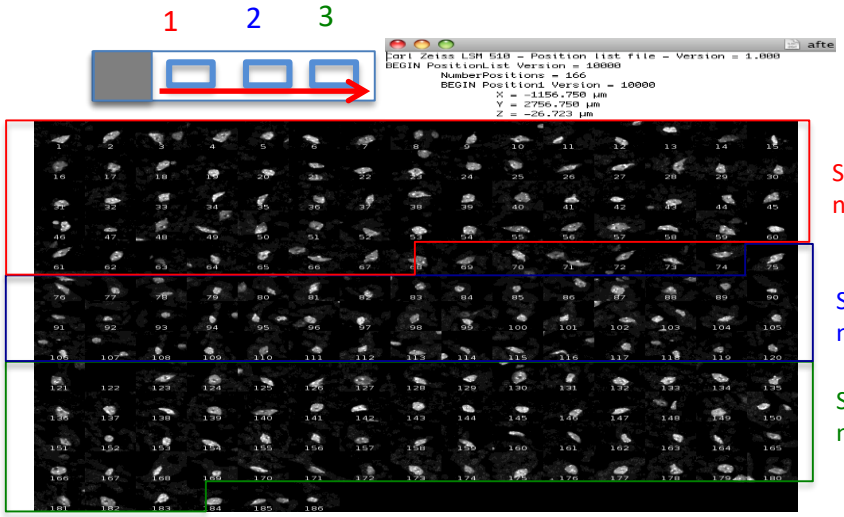




Confocal:
Limit the illuminated region and acquisition of signal to the focal plane

Confocal microscopy

Multi- sample/position and automatized z-scan process made available for confocal microscope users



1-Select position

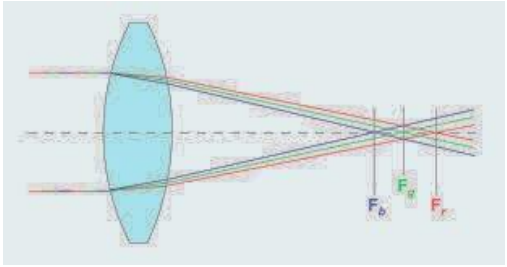
2-O/N Scan
(12h, 186 samples

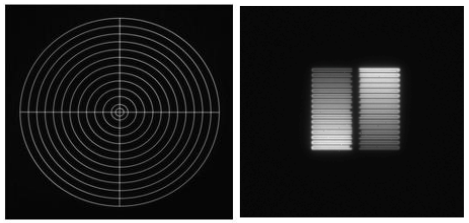
3-Save and analyse

Indroduction of Argolight, calibration slide to the microscopy platform

Metrology for microscopy

Chromatic aberration

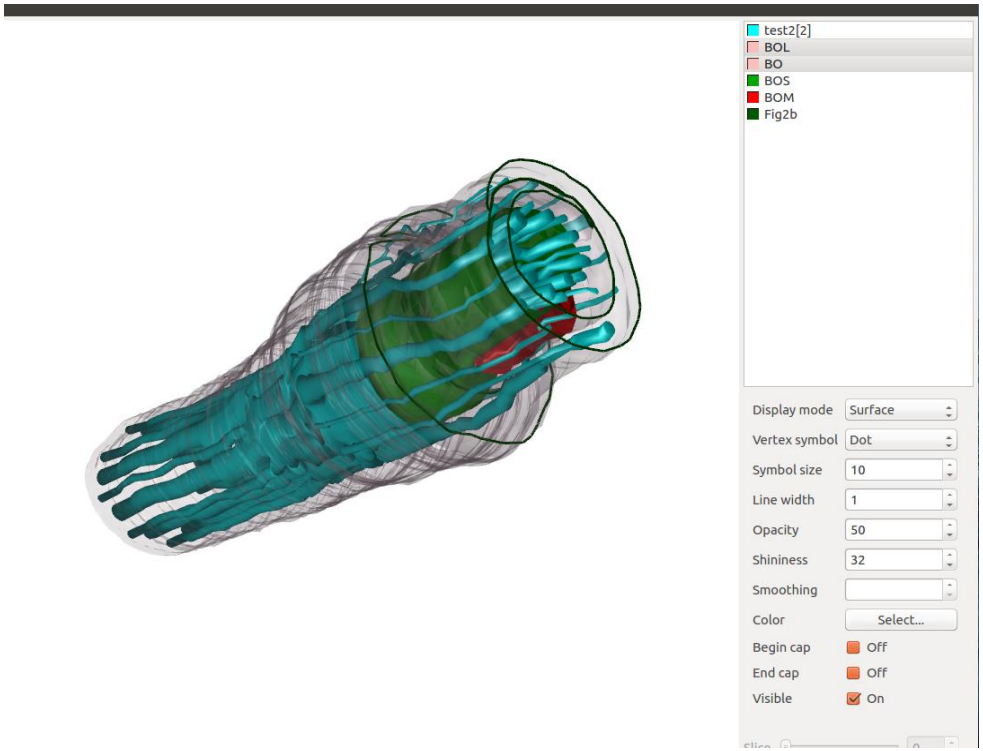




Geometric aberration

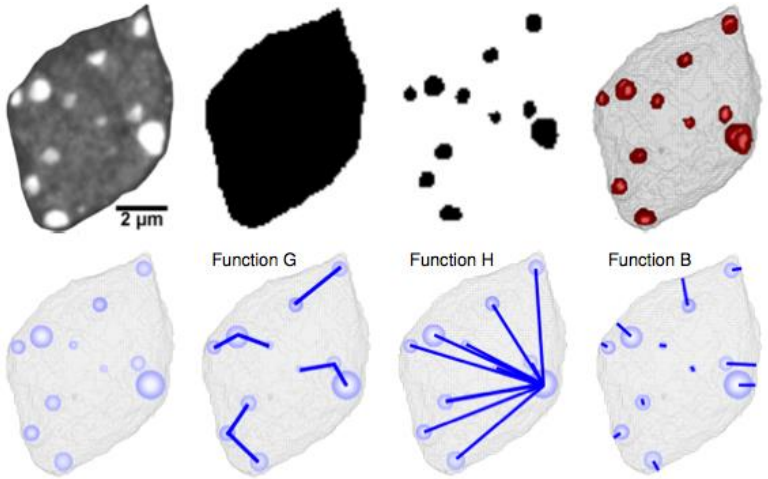
Image analysis - modeling

Image acquisition/3D reconstruction



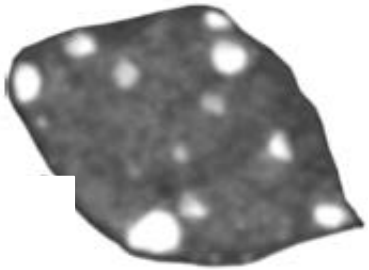
ImageJ, FreeD

Modeling



Collaboration, IJPB
P. Andrey's team

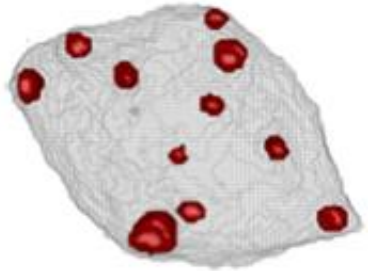
Image analysis – modeling : process



1-Image acquisition:
-acquisition parameters
-data storage (file name)

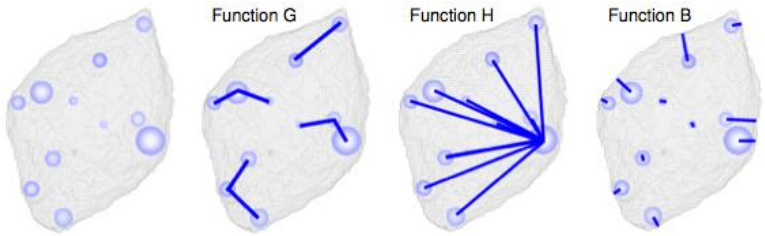
Critical step need to set up by biologist
NEVER USE jpeg!!
Always **keep raw data**

2-Image segmentation:
-isolate the objet od interest
-threshold parameters depending on objects



3-Image reconstruction

4-Modeling

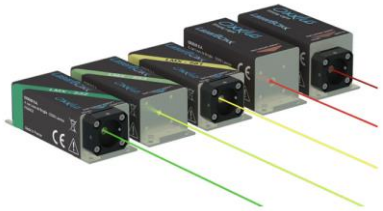


Microscope, let's take care!

Excitation sources

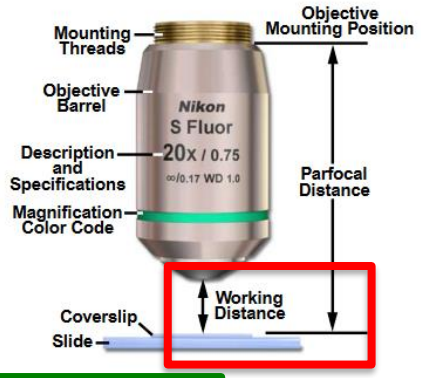
Vapor lamp (sometime toxic as mercure
2 000hours of use
~6 000 euros

Diode
10 000 to 100 000 hours of use
~10 000 euros



Laser
> 1000 hours of use
~20 000 to 150 000euros

Figure 1 - Objective Working and Parfocal Distance



Objectives

100 to 12 000 euros
(long working distance)

Cover slip

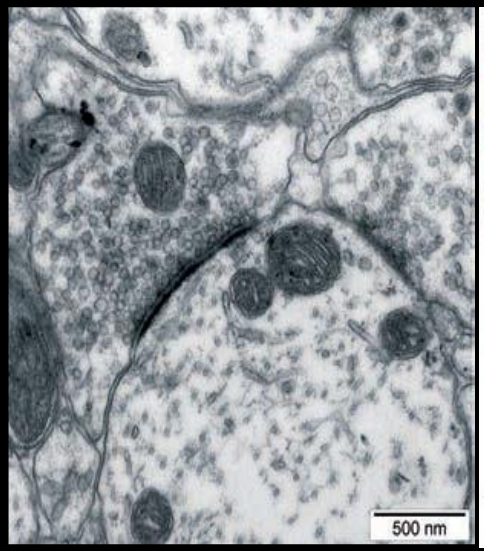
1,5# is for precise fluorescence acquisition
~5times more expensive than classical cover slip

Thank you
for your attention

Electron Microscopy

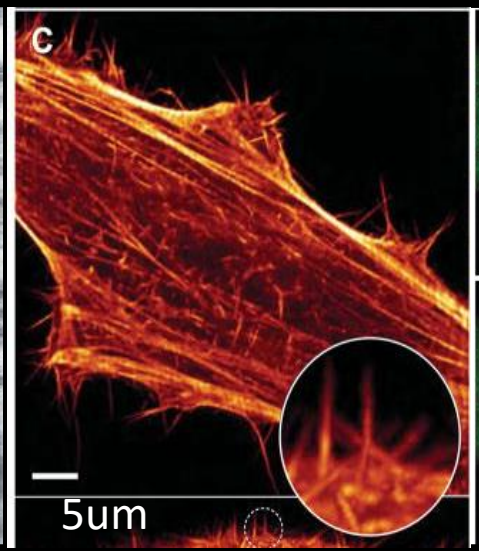
Optical Microscopy

Transmission Electron Microscopy
Ultrastructure



Resolution: electron size
but :
No fluorescence
No dynamic
Limit of staining tools
(no fusion protein)

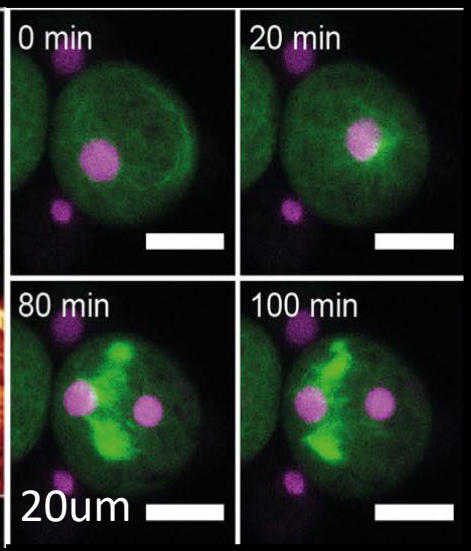
Super Resolution
Between confocal and TEM
(actin, tubulin)



Resolution : 50nm in x, y, z axis
Fluorescence, Ph, DIC
z-stack, numerical zoom
But:
Dynamic is more difficult
Technics for probes/sample/set up preparation is recquired

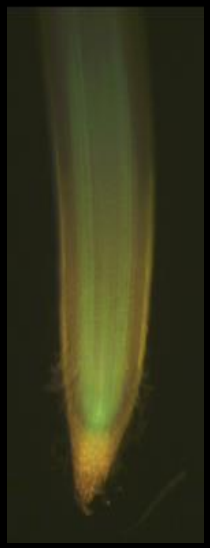
Hot topic, more and more commercial devices

Confocal
Tiny structure in 3D



Resolution : 0,2um in xy axis
0,4um in z axis, spectral (lambda)
Fluorescence, Ph, DIC
Dynamic
z-stack, numerical zoom

Wide Field / Fluorescence
(video microscope)



Resolution : xy 0,2um but in z is the thichkness of sample
Fluorescence, Ph, DIC
Dynamic
but :
No z-stack (need deconvolution)
Single frame (no zoom)

More and more « home made » set up : cameras (micromanager, metamorph)

Free-D Homepage

Synopsis Features Download Help Plug-ins Tools

SYNOPSIS

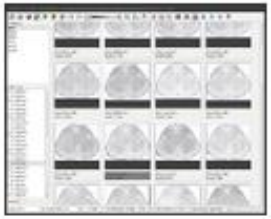
Free-D is a three-dimensional (3D) reconstruction and modeling software. It allows to generate, process and analyze 3D point and surface models from stacks of 2D images.

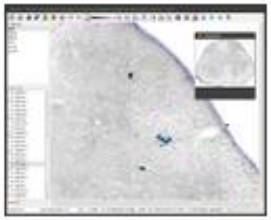
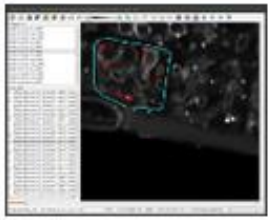
Free-D is an integrated software, offering in a single graphical user interface all the functionalities required for 3D modeling. It runs on Linux, Windows, and MacOS.

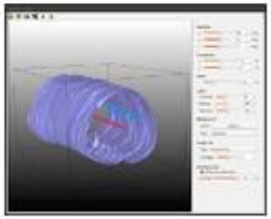
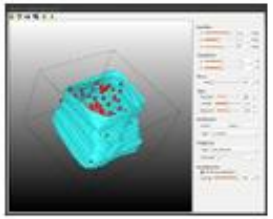
Free-D is developed by the **Modeling and Digital Imaging** team of the **Institut Jean-Pierre Bourgin**, INRA Versailles, France.

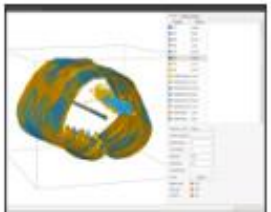
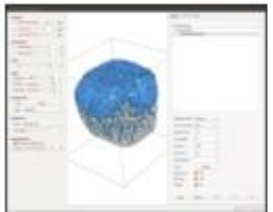
LATEST RELEASE: Free-D 1.13 (2016.07.28). See the **major changes** here.

FEATURES



Stack browsing

> can handle multiple stacks of several hundreds of Gb
> can handle section images of several hundreds of Mb
> supports variable inter-slice spacing and pixel calibration

Image segmentation

> manual delineation and pointing on 2D images
> automated 2D and 3D segmentation (contours, spots)
> segmentation editing, resampling, and smoothing

Image registration

> interactive rigid transformations (translation and rotation)
> several display modes for registration visualization
> registration based on images and/or segmentations

Model reconstruction

> instant rendering of segmentation and registration actions
> easy navigation through series of stack reconstructions
> permanent storage of poses of interest

Quantitative analysis

> quantitative measures (counts, lengths, areas, volumes, etc.)
> analysis per slice, per 2D item, per 3D reconstructed model
> export as spreadsheet for further statistical analysis

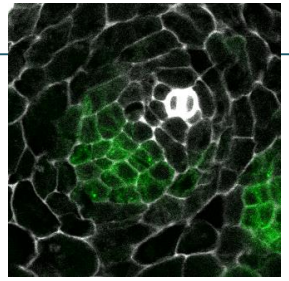
Spatial normalization and atlasing

> groupwise 3D shape registration and averaging
> linear and non-linear spatial normalization
> statistical density estimation and visualization

BRAVO Project : Deciphering vascular development mechanism (ANR)

Computational modeling of vascular network

Image analysis (segmentation)
Cytology / Histology
(microtome, vibratome, cryotome)
Microscopy
(confocal)



Laser Capture Microdissection (LCM)
RNAseq with small amount of RNA

RNA chip

Task-4
Screening mutants with aberrant vasculature / gene cloning

2nd Cell Wall team (IJPB)
(R.Sibout, A.Lemaire, P. Lebris, S.Anltem)

Task-1
Detailled description of vascular development, patterning and cell differentiation

Task-3
Functional genomics
Identification and characterisation of T-DNA mutants or whole genome sequenced mutants

Promoter cloning compatible with INTACT method

Task-2
Transcriptomic data from vascular bundles

Sub-task-1
microdissection

Verification of gene expression (qPCR, *in situ*)

Comparison with late expressed gene (CELLWALL- KBBE project)

RNA extraction of Microdissected samples

Gene differentially expressed

Sub-task-2
RNA-seq and data analysis

Data analysis

RNA seq

Transcriptomic platform (URGV-IPS2)