Réseau meetochondrie

GDR 3159

dépasser les frontières

AMMi

ATELIER

Dynamique mitochondriale sur cellules humaines et végétales

Morphologie, fusion et nucléoïdes mitochondriaux

Dynamique mitochondriale en champ large

Utilisation de protéine fluorescente photoconvertible pour l'analyse de la fusion mitochondriale

Analyse d'image

université angers

SFR 4207 Angers Qualité et Santé du Végétal Quasav

IRHS Institut de Recherche en Horticulture et Semences

INRA SCIENCE & IMPACT

AGRO CAMPUS

université angers

CHU ANGERS CENTRE HOSPITALIER UNIVERSITAIRE

Programme

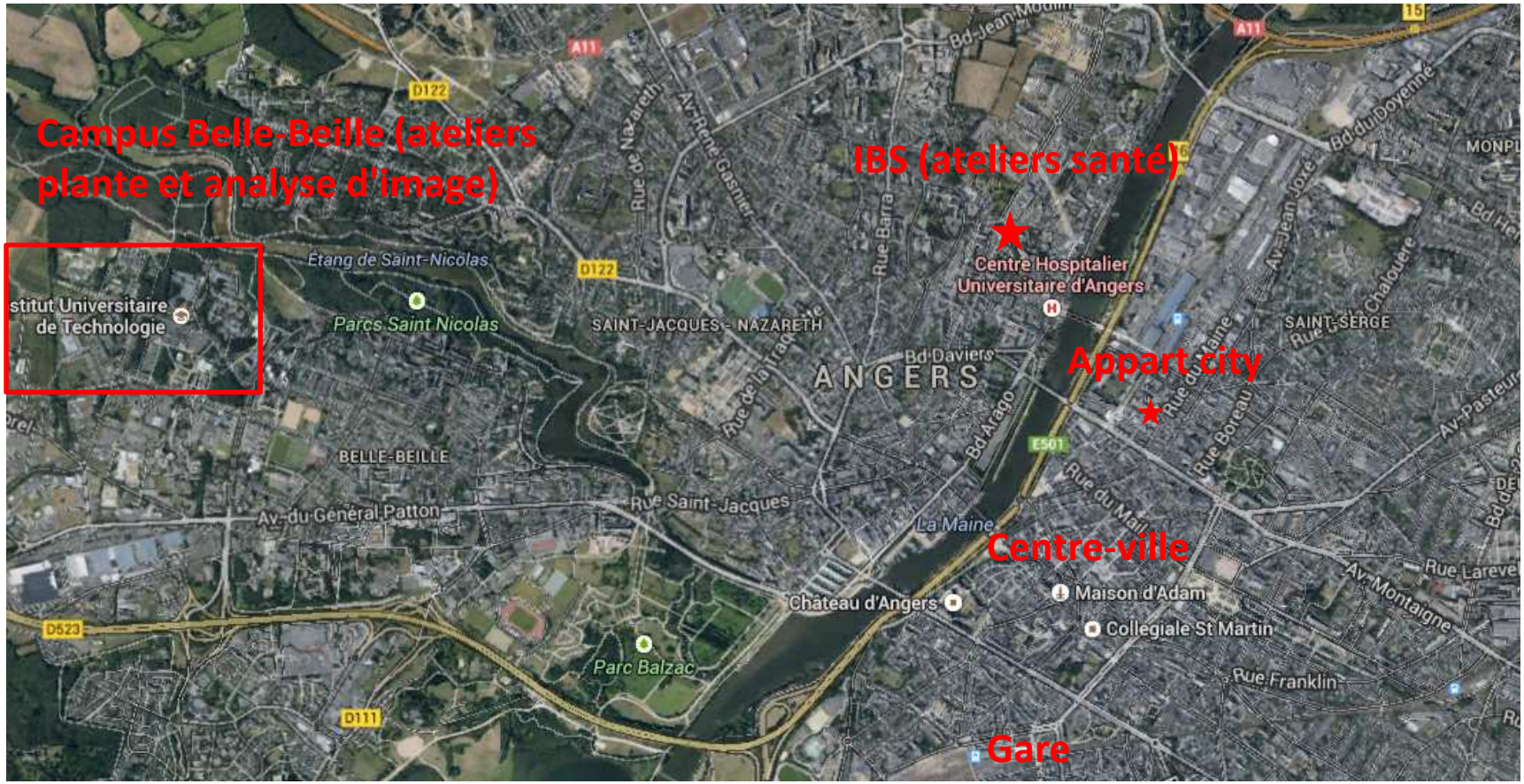
Mer 25 juin	13h00-14h00	Accueil, Café & snacks
Batiment IBS, CHU	14h00-14h20	Intro & présentation de l'atelier *(D Macherel/ P Reynier)*
	14h20-14h40	Biopics intervenants
	14h40-15h10	Biopics participants
	15h10-15h25	Importance de la dynamique mitochondriale *(P Reynier)*
	15h25-15h40	Dynamique fonctionnelle *(M Rojo)*
	15h40-15h55	Diversité et hétérogénéité de la dynamique
	16h00-16h30	Aspects techniques- matériel fixé *(M Rojo)*
	16h30-17h	Aspects techniques - matériel végétal *(D Logan)*
	17h-17h30	Aspects techniques - in vivo *(A Chevrollier)*
	17h30-17h50	Aspects techniques- Imagerie *(A Chevrollier, R Perrot, M Ferré)*
	17h50-18h20	Présentation ateliers de microscopie *(A Chevrollier / David Logan)*
	18h20-18h30	Formation Groupes
	18h30-19h30	Transfert à pied (via Appart-city si besoin)
Brasserie du Théatre	19h30-20h30	Conférence 30 min / discussion *(Stephan Wagner)*
	20h30-	Diner Brasserie du Théatre

Jeu 26 juin	07h30-07h50	Transfert Appart-City=> ARES (Autocar) // IRIS (à pied)
IBS // ARES	08h00-10h00	Ateliers microscopie par demi-groupe
	10h00-10h30	Pause
	10h30-12h30	Ateliers microscopie par demi-groupe
	12h30-12h50	Transferts IRIS=> ARES (Autocar)
ARES	12h50-13h30	Plateaux-repas à l'ARES
	13h30-13h50	Transferts ARES=> IRIS (Autocar)
IRIS // ARES	14h00-16h00	Ateliers microscopie par demi-groupe
	16h00-16h30	Pause
	16h30-18h30	Ateliers microscopie par demi-groupe
	18h30-18h50	Transfert ARES => Appart-City (Autocar), IRIS-Appart-City (à pied)
Restaurant du Quai	20h-	Diner

Ven 27 juin	07h30-08h00	Transfert Appart-City=> Faculté des Sciences -Fac Sci (Autocar)
Faculté Sciences	08h00-10h00	Ateliers analyse image par demi-groupe (Bat A, A115 // Bat L L232)
	10h00-10h30	Pause café et gâteaux britanniques
	10h30-12h30	Ateliers analyse image par demi-groupe
	12h30-13h30	Plateaux-repas à la Faculté des Sciences
	13h30-15h00	TP sur data (Bat A, A115)
	15h00-16h00	Table ronde : bilan, questions (Bat A, A118)
	16h00-16h20	Transfert Faculté des Sciences => Gare SNCF (Autocar)

Atelier "Voir la dynamique mitochondriale"

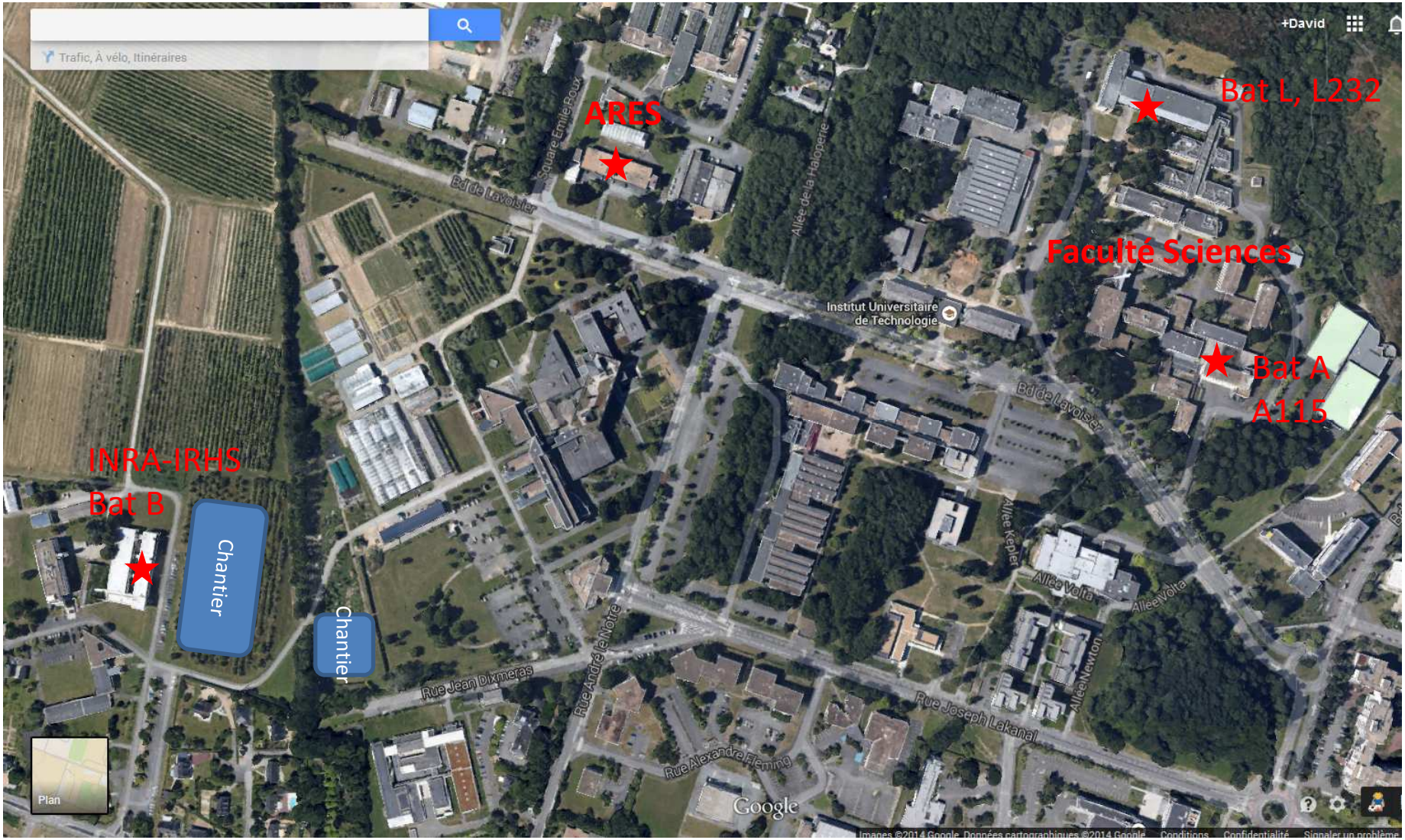
Aspects géographiques



Localisation ateliers Université d'Angers/INRA
Campus de Belle-Beille

ARES : microscopie à fluorescence
IRHS : confocal

Faculté des Sciences, analyse d'image (Bat A et bat L)



Liste des Participants

	Nom	Mail	Laboratoire	Tel	Ville
Dr	ARDAIL, Dominique	dominique.ardail@univ-lyon1.fr	Faculté de Médecine Lyon-Sud Charles Mérieux	661839537	Oullins Cedex
Dr	BADIA, Eric	eric.badia@univ-montp1.fr	Université Montpellier 1	411759892	Montpellier
Mme	CIRER, Bénédicte	benedicte.cirer@bayer.com	BAYER SAS	472852593	LYON
Mme	DARTIER, Julie	julie.dartier@gmail.com	UMR INSERM1069 - Nutrition Croissance et Cancer -	247366306	TOURS
Dr	DUCHENE, Anne-Marie	anne-marie.duchene@ibmp-cnrs.unistra.fr	Université de Strasbourg	367155369	Strasbourg
Mme	DUROUX-RICHARD, Isabelle	isabelle.richard@inserm.fr	INSERM U844	499606383	Montpellier
Mme	EL FISSI, Najla	najla.elfissi@gmail.com	Institue de Biologie et du Développement Marseille (IBDM)	651628830	Marseille
M	FABRE, Philippe	philippe.fabre@sanofi.com	SANOFI	534634113	Toulouse
Dr	FLEURY, Valérie	valerie.fleury@sanofi.com	Sanofi	160497819	CHILLY MAZARIN
Mme	JACQUET, Agnès	agnes.jacquet@sanofi.com	SANOFI	160496107	Chilly mazarin
Mme	JUBLANC, Elodie	agnes.jacquet@sanofi.com	INRA - UMR866 DMEM	499612823	Montpellier
Dr	JUGÉ, Romain	romain.juge@ens-lyon.fr	UMR5239 Lyon	(0)4 26 23 59 43	Lyon
	MONTAROU, Gérard	montarou@clermont.in2p3.fr	Lab Physique Corpusculaire de Clermont Université Blaise Pascal	607135755	Aubiére
Dr	PLANCHAIS, Séverine	severine.planchais@upmc.fr	UNIVERSITE PIERRE ET MARIE CURIE (PARIS 6)	144276232	PARIS Cedex 05
M	ROUSSIGNOL, Gautier	gautier.roussignol@sanofi.com	SANOFI-Aventis R&D	33499774182	Montpellier
Mme	SCHOLTES, Charlotte	charlotte.scholtes@etu.univ-lyon1.fr	Université Lyon 1	673955759	VILLEURBANNE

Organisateurs et encadrants

David MACHEREL

Professeur de Physiologie végétale, Université d'Angers.
Equipe MITOSTRESS, Institut de Recherche en Horticulture et Semences, UMR 1345 INRA, Agrocampus-Ouest, Université d'Angers.

Site web équipe : http://www6.angers-nantes.inra.fr/irhs/Recherche/MitoStress
Google scholar : http://scholar.google.com/citations?user=VSfVAU8AAAAJ&hl=en
Contact: david.macherel@univ-angers.fr

Depuis le début de ma carrière, mes recherches ont essentiellement porté sur les propriétés des mitochondries végétales, et depuis une quinzaine d'années sur la tolérance au stress extrême (desiccation, température). Ayant toujours combiné différentes approches (physiologie, biologie moléculaire, biochimie) dans le cadre de l'étude des organites, j'ai naturellement intégré depuis une dizaine d'année la biologie cellulaire avec l'utilisation croissante des protéines fluorescentes, notamment pour la localisation subcellulaire. Nous avons ainsi récemment établi la distribution sub-cellulaire de l'ensemble de 51 protéines LEA (Late embryogenesis abundant) liées à la déshydratation chez Arabidopsis.

Recent publications:

- Candat A, Paszkiewicz G, Neveu M, Gautier R, Logan DC, Avelange-Macherel MH, **Macherel D** (2014) The ubiquitous distribution of LEA proteins across cell compartments offers tailored protection against abiotic stress. ***Plant Cell***, in press.
- Benamar A, Pierart A, Baecker V, Avelange-Macherel MH, Rolland A, Gaudichon S, di Gioia L, Macherel D (2013) A simple system using natural mineral water for high-throughput phenotyping of Arabidopsis thaliana seedlings in liquid culture. ***Int. J. H-T Screening.*** 4: 1-15.
- Candat A, Poupart P, Andrieu JP, Chevrollier A, Reynier P, Rogniaux H, Avelange-Macherel MH, **Macherel D** (2013) Experimental determination of organellar targeting-peptide cleavage sites using transient expression of green fluorescent protein translational fusions. ***Anal. Biochem.*** 434: 44-51.

Pascal REYNIER

PU-PH de Biochimie et Biologie moléculaire, Université et CHU d'Angers

Equipe mitolab http://mitolab.eu/
UMR CNRS 6214 – INSERM U1083, Biologie Neurovasculaire et Mitochondriale Intégrée, Bnmi.fr
Département de Biochimie et Génétique, CHU, 4, rue Larrey, 49933 Angers cedex 9
Contact : + 33 (0) 6 84 50 33 57 Pareynier@chu-angers.fr

Je m'intéresse principalement à la physiopathologie mitochondriale. Dans le cadre hospitalier nous réalisons le diagnostic biochimique et génétique des maladies mitochondriales. Sur le plan de la recherche nous travaillons plus spécifiquement sur les maladies mitochondriales neurogénérératives et les maladies de la dynamique mitochondriale. Notre approche intégrée (métabolisme énergétique, génétique mitochondriale, imagerie structurale et génomique) tente d'identifier de nouvelles pistes thérapeutiques.

Recent publications:

- Procaccio V, Bris C, Chao de la Barca JM, Oca F, Chevrollier A, Amati-Bonneau P, Bonneau D, **Reynier P**. Perspectives of drug-based neuroprotection targeting mitochondria. ***Rev Neurol (Paris).*** 2014;170:390-400.
- Sarzi E, Angebault C, Seveno M, Gueguen N, Chaix B, Bielicki G, Boddaert N, Mausset-Bonnefont AL, Cazevieille C, Rigau V, Renou JP, Wang J, Delettre C, Brabet P, Puel JL, Hamel CP, **Reynier P**, Lenaers G. The human OPA1delTTAG mutation induces premature age-related systemic neurodegeneration in mouse. ***Brain.*** 2012;135:3599-613.
- Chevrollier A, Cassereau J, Ferré M, Alban J, Desquiret-Dumas V, Gueguen N, Amati-Bonneau P, Procaccio V, Bonneau D, **Reynier P**. Standardized mitochondrial analysis gives new insights into mitochondrial dynamics and OPA1 function. ***Int J Biochem Cell Biol.*** 2012;44:980-8.

David C LOGAN

Professeur des Université, Université d'Angers
Équipe "MitoStress" Institut de Recherche en Horticulture et Semences
www.plantmitochondria.net
Contact: david.logan@univ-angers.fr

My first degree was from the University of Dundee, Scotland, followed by a PhD at University College London. I then had postdoctoral positions in Kent, England and Montpellier, France, in cell physiology and molecular biology before starting research into mitochondrial cell biology as a postdoctoral fellow at Oxford University in 1996. In Oxford I was frustrated with not being able to see mitochondria unambiguously in living tissue so generated stable transgenics of the model plant Arabidopsis expressing mitochondrial targeted GFP. In 1998 I left academic research and worked briefly for the UK government in London before returning to mitochondrial research in 2000 at the University of St Andrews, Scotland. In St Andrews I mutagenized my mito-GFP line and screened, by epifluoresence microscopy, for mutants with altered mitochondrial shape, size, number or cellular distribution. In 2008 I moved to Canada as Associate Professor before coming to Angers in 2012.

Recent publications:

- Candat A, Paszkiewicz G, Neveu M, Gautier R, **Logan DC**, Avelange-Macherel MH, Macherel D (2014) The ubiquitous distribution of LEA proteins across cell compartments offers tailored protection against abiotic stress. ***Plant Cell***, in press.
- Ren M, Venglat P, Qiu S, Feng Li, Cao Y, Wang E, Xiang D, Wang J, Alexander D, Chalivendra S, **Logan DC**, Matoo A, Gopalan S, Datla R (2012) Target of rapamycin signaling regulates metabolism, growth and lifespan in Arabidopsis. ***Plant Cell*** 24: 4850 - 4874.
- Schwarzländer M, **Logan DC**, Johnston IG, Jones NS, Meyer AJ, Fricker MD, Sweetlove LJ (2012) Pulsing of membrane potential in individual mitochondria: A stress-induced mechanism to regulate respiratory bioenergetics in Arabidopsis. ***Plant Cell*** 24: 1188-1201.

Arnaud CHEVROLLIER

Maitre de conférences, Biochimie, Biologie Moléculaire, IUT Génie Biologique, Université d'Angers
arnaud.chevrollier@univ-angers.fr

Equipe mitolab http://mitolab.eu/
Laboratoire de biologie neurovasculaire et mitochondriale intégrée.
UMR CNRS 6214 – INSERM U1083 Bnmi.fr
Adresse : Département de Biochimie, CHU, 4 rue Larrey, 49933 Angers cedex 9.
Contact : + 33 (0) 2 44 68 84 05 arnaud.chevrollier@chu-angers.fr

Mes travaux de recherche portent sur l'étude du métabolisme mitochondrial dans les cellules cancéreuses et dans les cellules fibroblastiques de patients porteurs de mutations de l'ADNmt. La mise en évidence de mutations dans les gènes de la dynamique mitochondriale dans certaines pathologies mitochondriales m'a conduit à visualiser les mitochondries par microscopie. Ces observations m'ont amené à chercher à quantifier l'organisation intracellulaire du réseau mitochondrial et à explorer la relation structure/fonction mitochondriale.

Recent publications:

- Case study: http://www.bitplane.com/learning/
- **Chevrollier A**, Cassereau J, Ferré M, Alban J, Desquiret-Dumas V, Gueguen N, Amati-Bonneau P, Procaccio V, Bonneau D, Reynier P. Standardized mitochondrial analysis gives new insights into mitochondrial dynamics and OPA1 function. (2012) ***International Journal of Biochemistry and Cell Biology.*** 44(6):980-8.
- Guillet V, **Chevrollier A**, Cassereau J, Letournel F, Gueguen N, Richard L, Desquiret V, Verny C, Procaccio V, Amati-Bonneau P, Reynier P, Bonneau D. Ethambutol-induced optic neuropathy linked to OPA1 mutation and mitochondrial toxicity**.** (2010) ***Mitochondrion*****.** 10 (2):115-24.
- **Chevrollier A**, Guillet V, Loiseau D, Gueguen N, de Crescenzo MA, Verny C, Ferre M, Dollfus H, Odent S, Milea D, Goizet C, Amati-Bonneau P, Procaccio V, Bonneau D, Reynier P. Hereditary optic neuropathies share a common mitochondrial coupling defect. (2008) ***Annals of Neurology*****.** 63(6):794-8.
- Lenaers G, Amati-Bonneau P, Delettre C, **Chevrollier A**, Verny C, Miléa D, Procaccio V, Bonneau D, Hamel C, Reynier P. [From yeast to neurodegenerative diseases: ten years of exploration of mitochondrial dynamic disorders]. (2010) ***Médecine/Sciences*** 26(10):836-41. Review. French.

Frédéric JOUBERT

Chargé de recherche CNRS
Laboratoire Jean Perrin, UPMC-CNRS UMR8237
4 place Jussieu, 75005 Paris
Tour 32-33, 4ème étage, pièce 418
tel : (33) (0)1 44 27 90 64
email : frederic.joubert@upmc.fr
web : http://www.labos.upmc.fr/ljp/

Biophysicien de formation, j'ai travaillé dans le champ de la bioénergétique cardiaque pendant presque 15 ans en utilisant la spectroscopie RMN du ^{31}P et les outils de fluorescence. Je me suis plus particulièrement intéressé à la dynamique mitochondriale à partir de 2008, en travaillant notamment sur un modèle de souris hétérozygote pour le gène Opa1. Depuis 2013, je travaille dans un nouveau laboratoire à l'interface de la physique et de la biologie, et j'utilise des systèmes biomimétiques pour rendre compte de certaines propriétés spécifiques des mitochondries.

Recent publications:

- Caffin F, Prola A, Piquereau J, Novotova M, David DJ, Garnier A, Fortin D, Alavi M, Veksler V, Ventura-Clapier R, **Joubert F**. Altered skeletal muscle mitochondrial biogenesis but improved endurance capacity in trained OPA1- deficient mice. (2013) ***J physiol*** 591(Pt 23):6017-37.
- Piquereau J, Caffin F, Novotova M, Prola A, Garnier A, Mateo P, Fortin D, Huynh LH, Nicolas V, Alavi MV, Brenner C, Ventura-Clapier R, Veksler V, **Joubert F**. Down-regulation of OPA1 alters mouse mitochondrial morphology, PTP function, and cardiac adaptation to pressure overload. (2012) ***Cardiovasc Res*** 94(3):408-17.
- Ventura-Clapier R, Garnier A, Veksler V, **Joubert F**. Bioenergetics of the failing heart. (2011) ***BBA - Mol Cell Res*** 1813(7):1360-72.

Manuel ROJO

Directeur de Recherche, DR2 CNRS
Institut de Biochimie et Génétique Cellulaires (IBGC)
UMR 5095 CNRS – Université de Bordeaux
manuel.rojo@ibgc.cnrs.fr

Après des études de Biologie à l'Université de Konstanz, Allemagne, j'ai réalisée une thèse à l'École Polytechnique Fédérale (ETH) de Zürich, Suisse dédiée à l'étude de deux protéines mitochondriales *in vitro.* J'ai ensuite réalisé un stage post-doctoral au Département de Biochimie de l'Université de Genève, Suisse ou j'ai été initié à la biologie cellulaire et moléculaire : j'ai identifié et caractérisé des protéines transmembranaires localisées entre le réticule endoplasmique et l'appareil de Golgi. En 1999, j'ai intégré le CNRS (Équipe A.Lombès, Pitié-Salpêtrière, Paris) et mis en œuvre un projet de recherche dédié à l'étude de la dynamique mitochondriale. En 2008, j'ai fondé l'équipe de recherche « Organisation et Dynamique Mitochondriales » à l'IBGC de Bordeaux.

Publications representatives:

- Sauvanet C., Duvezin-Caubet S., Salin B., David C., Massoni-Laporte A., di Rago JP, **Rojo M.** (2012) Mitochondrial DNA mutations provoke dominant inhibition of mitochondrial inner membrane fusion. **PLoS ONE**, 7(11), e49639.
- Guillery O, Malka F, Frachon P, Milea D, **Rojo M**, Lombès A (2008), Modulation of mitochondrial morphology by bioenergetics defects in primary human fibroblasts, **Neuromuscul. Disord. 18**(4), 319-30.
- Malka F, Guillery O, Cifuentes-Diaz C, Guillou E, Belenguer P, Lombes A, **Rojo M.** (2005) Separate fusion of outer and inner mitochondrial membranes. **EMBO Rep. 6**, 853-859.

Stephan WAGNER

PostDoc in Plant Energy Biology
Chemical Signalling Group, Institute for Crop Sciences and Ressource conservation (INRES), University of Bonn (Germany)

Homepage: http://www.plant-energy.uni-bonn.de/
Contact: stephan.wagner@uni-bonn.de

Using genetically encoded biosensors, we seek to understand how plants as sessile organisms react to a changing environment. Using a combination of *in vitro*, *in vivo* and studies on isolated mitochondria, we use our strong expertise in bioimaging to analyse how specifically redox and calcium signals shape mitochondrial respiration and metabolism. Before joining Markus Schwarzländer's lab in Bonn, I solved three-dimensional structures of proteins that are essential for a plant's resistance against pathogens. I am thus deeply interested in how these signals are mechanistically transduced to and from mitochondria and whether they regulate key mitochondrial proteins through direct modifications.

Recent publications:

- Rietz, S., Stamm, A., Malonek, S., **Wagner, S**., Becker, D., Medina-Escobar, N., Vlot, A. C., Feys, B.J., Niefind, K., and Parker J. E. (2011). Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. ***The NewPhytologist***, 191(1), 107-19.
- **Wagner, S**., Rietz, S., Parker, J. E., and Niefind, K. (2011). Crystallization and preliminary crystallographic analysis of Arabidopsis thaliana EDS1, a key component of plant immunity, in complex with its signalling partner SAG101. ***Acta Crystallographica F***, 67, 245-8.
- **Wagner, S.**, Stuttmann, J., Rietz, S., Guërois, R., Brunstein, E., Bautor, J., Niefind, K., and Parker J. E. (2013). Structural Basis for signaling by exclusive EDS1 Heteromeric Complexes with SAG101 or PAD4 in Arabidopsis Innate Immunity. ***Cell Host & Microbe***, 14(6), 619-30.

Atelier S1 : Réseau mitochondrial

Protocole Imagerie

Marquage structure réseau mitochondrial :

Marquage MitoTracker® Green FM - Special Packaging (Ref: M7514 INVITROGEN: 318 euros)
50µg/100µl DMSO, aliquots -20°C.

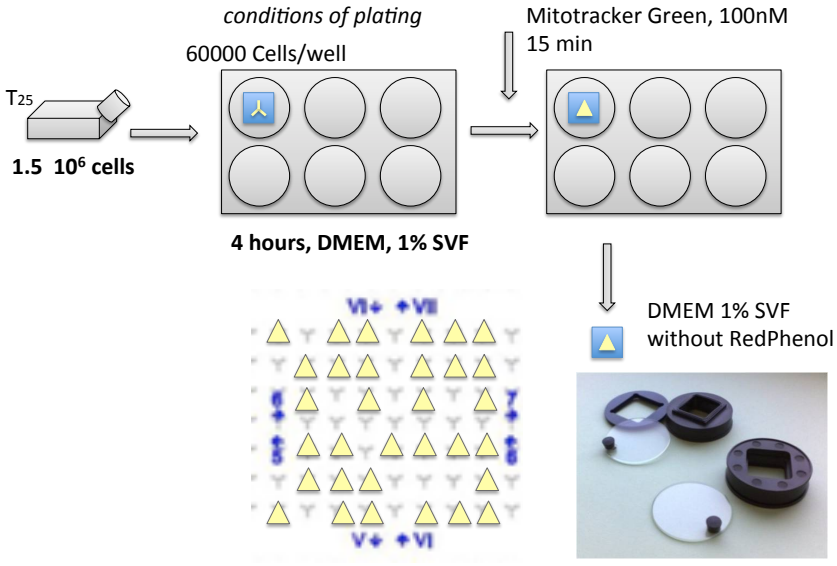
J-1 : les cellules sont ensemencées dans les chambres Labteck coverglass.
Pour les fibroblastes, 35000 cellules/chambre pour les double chambres

J0 : Milieu de marquage : 0.5µl de MitoTracker Green dans 2ml milieu DMEM-F12/5% SVF
J0 : Enlever le milieu de la chambre puis incuber 15mn à 37°C avec le milieu marquage.

Enlèver le milieu, rinçage avec le même milieu sans marquage.

Lecture est faite avec du milieu DMEM sans Red Phenol/1% SVF

protocol



Atelier 2 : Nucléoides, ADNmt

<u>Marquage structure réseau mitochondrial (TMRM) + ADN mito (Picogreen):</u>

Marquage Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) (Ref : T668 INVITROGEN : 219 euros)

25mg/2,5ml DMSO, aliquots 20mM à -20°C.

Marquage Quant-iT™ PicoGreen® dsDNA Assay Kit (Ref : P7589 LIFE TECHNOLOGIES : 536 euros)

Atelier 2 : Nucléoides, ADNmt

J-1 : les cellules sont ensemencées dans les chambres Labteck.
Pour les fibroblastes, 35000 cellules/chambre pour les double chambres

J0 : Milieu de marquage :
TMRM : Première dilution au 1/200ème (1µl dans 199µl) puis reprendre 0.5µl de cette solution dans 2ml de milieu DMEM-F12/5% SVF.

Picogreen : 0.5µl dans les 2ml de milieu contenant le TMRM.

J0 : Enlever le milieu de la chambre puis incuber 20mn à 37°C avec le milieu marquage.

Enlèver le milieu, rinçage avec le même milieu sans marquage.

Lecture est faite avec du milieu DMEM sans Red Phenol/1% SVF.

Atelier 2 : Nucléoides, ADNmt

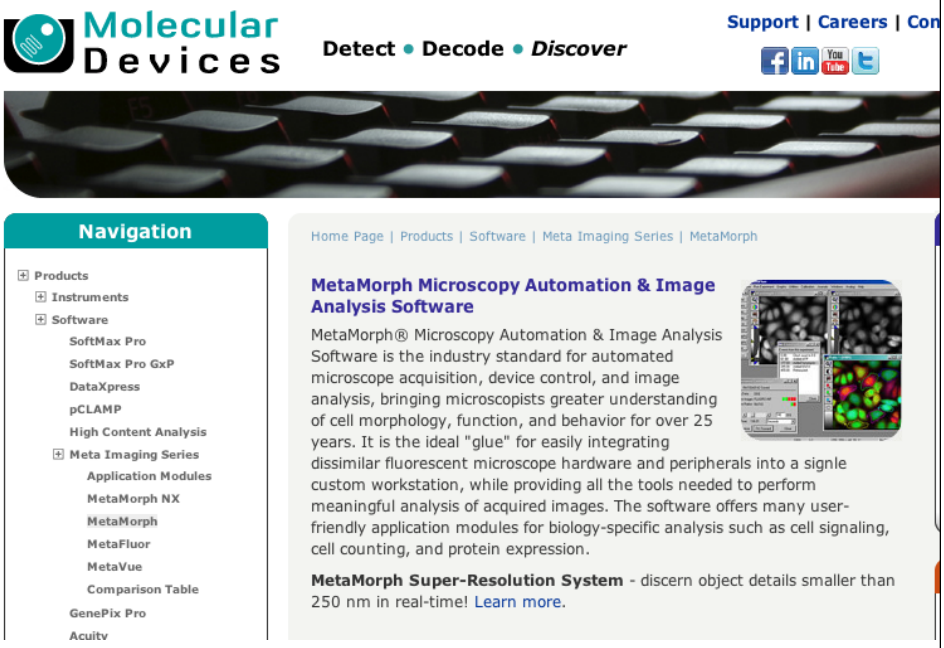
Immunomarquage ADN mito (Anti-DNA):
anti-DNA mouse monoclonal: Ref 61014 PROGEN Biotechnik : 190 euros

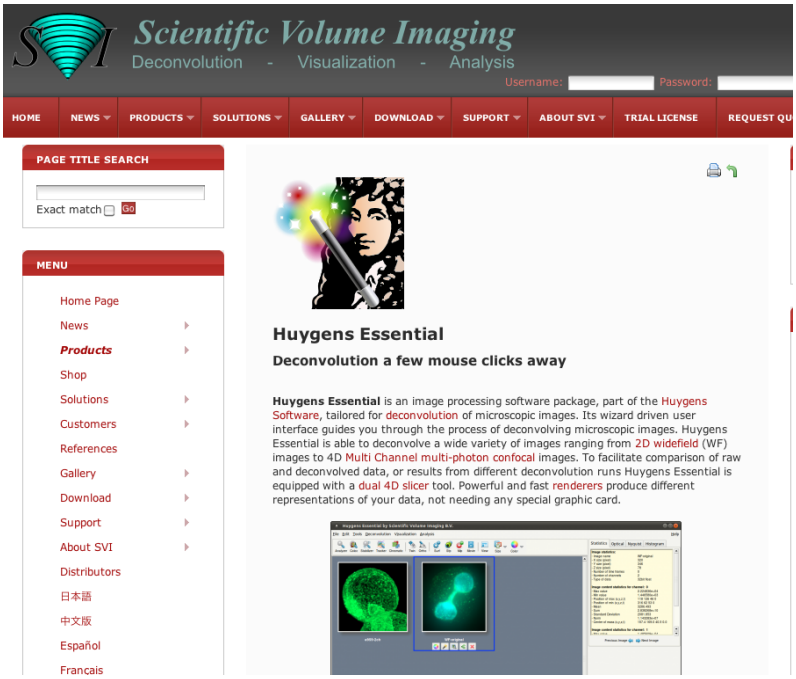
J-1 : les cellules sont ensemencées sur des lamelles préalablement autoclavées en plaque 6 puits. Pour les fibroblastes, 250000 cellules/puit.
Protocole fixation :
J0: enlever le milieu puis faire 1 rinçage PBS 1X, 5mn.
Fixation avec PFA 4% pendant 15mn à RT.
Retirer le PFA puis 3 rinçages PBS 1X.

Mettre Tampon BSA de perméabilisation 20 mn à RT.
Retirer le tampon puis 3 rinçages PBS 1X.
Faire une dilution de l'anti-DNA au 1/200ème dans le tampon BSA overnight à +4°C.

J1 : retirer le tampon puis faire 3 rinçages PBS 1X.
Diluer du DAPI au 1/200ème dans PBS 1X. Mettre le DAPI 5mn à RT.
Retirer et faire 1 rinçage PBS 1X seul.

Montage au *Prolong Antifade kit* **(P7481 chez Molecular Probes)** : Placer une goutte du mélange (tube A + 1ml de tube B) sur une lame en verre préalablement nettoyée à l'alcool, puis mettre à l'aide d'une pince, la lamelle en prenant garde de mettre le coté où il y a les cellules dans le liquide de montage. Stockage dans une boite à 4°C.

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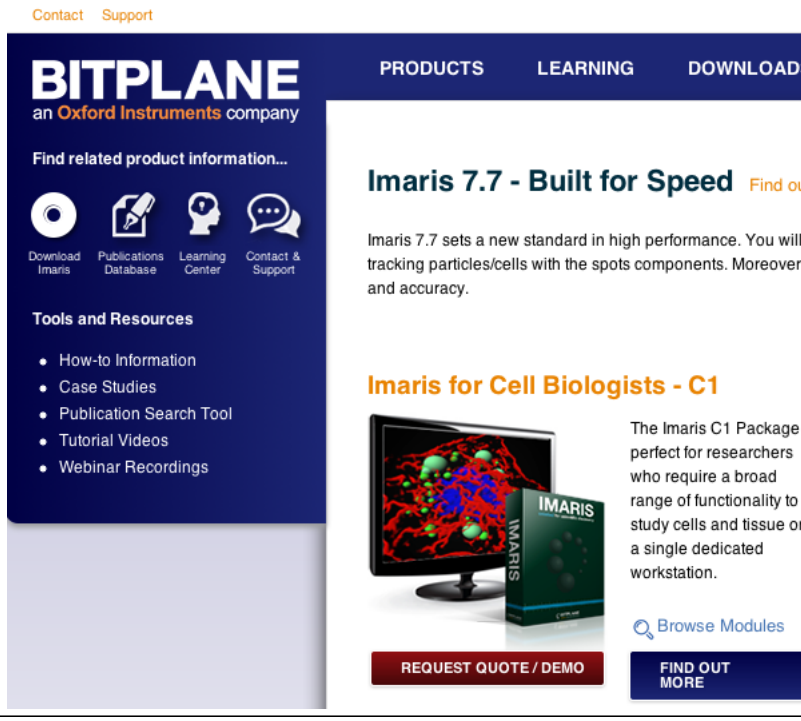
Huygens Essential
Deconvolution a few mouse clicks away
Huygens Essential is an image processing software package, part of the Huygens Software, tailored for deconvolution of microscopic images. Its wizard driven user interface guides you through the process of deconvolving microscopic images. Huygens Essential is able to deconvolve a wide variety of images ranging from 2D widefield (WF) images to 4D Multi Channel multi-photon confocal images. To facilitate comparison of raw and deconvolved data, or results from different deconvolution runs Huygens Essential is equipped with a dual 4D slicer tool. Powerful and fast renderers produce different representations of your data, not needing any special graphic card.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Webinar Recordings
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5e ATELIER DU RESEAU MEETOCHONDRIE

VOIR LA DYNAMIQUE MITOCHONDRIALE

Investigating plant mitochondrial dynamics by wide-field fluorescence microscopy

David C Logan & David Macherel

The purpose of the plant side to the workshop is to compare and contrast confocal and wide-field microscopy of plant mitochondria but using different experimental materials. Whilst the confocal part will focus on the use of mitochondrial targeted photo-convertible mEos (Mathur *et al*, 2010) to investigate inter-mitochondrial interactions, the wide-field component will focus on mitochondria and the actin cytoskeleton, and separately the visualisation of mitochondrial DNA (stained with SYBR Green I) and mitochondria (stable transgenics expressing mitochondrial targeted mCherry fluorescence protein.

Cytoskeleton and mitochondria visualization
To visualize actin filaments and mitochondria, a double transgenic line was generated by crossing mito-GFP (Logan & Leaver, 2000) with a second line (mCherry-mTn) expressing an in-frame fusion of mCherry (Shaner *et al*, 2004) to the C-terminal 197 amino acids of mouse talin which forms the actin binding domain (ABD). The mCherry-mTn line was generated by transformation of Arabidopsis Col-0 with pDCLmcherry-mTn. The backbone of pDCLmcherry-mTn, the pDCLmcherry-X destination vector, was a modified pMDC43 (Curtis & Grossniklaus, 2003) in which mGFP6 was replaced by mCherry via pMDC7 and pMDC24. A cDNA fragment encoding the mTalin ABD was PCR-amplified from the pZP202-GFP-mTn vector (see Kost *et al*, 1998) using attB Gateway primers and recombined into pDONRz. Recombination between pDONRz-mTn and pDCLmCherry-X created pDCLmcherry-mTn. Actin and mitochondria will be imaged using a Zeiss Axioimager Z1.M with Colibri LEDs and a Hamamatsu Orca Flash 4.0 v2 camera. The system is controlled by Zeiss Zen software running on a Dell workstation.

mtDNA and mitochondrial dynamics
A stable transgenic Arabidopsis line expressing mito-mCherry will be used (Candat *et al*. The Plant Cell, in press). Mitochondrial DNA in this line will be stained using SYBR Green I (Invitrogen). Seedlings will be placed in 1 ml of MS culture medium and 0.2 µL of SYBR Green stock will be added (i.e. 1:5000). Incubate for 15 min, wash 2x.

Photoconvertible GFP as tool for mitochondrial dynamics
We will use a stable transgenic line of Arabidopsis constitutively expressing mitochondrial-targeted mEosFP (Mathur *et al*., 2010). EosFP is a coral FP that was genetically engineered to yield a monomeric green-to-red photoconverting FP (Wiendemann *et al*., 2004). Seedlings grown for 7-14 days on 0.5x MS agar medium will be observed using a Nikon confocal A1 system (GFP 488/525, RFP 561/595) and conversion will be induced with a 405 nm laser.

Visualisation of mitochondrial membrane potential with TMRM in Arabidopsis (optional – time permitting)

Transgenic Arabidopsis seedlings constitutively expressing GFP in mitochondria (Logan *et al*. 2000; Candat *et al*., 2014) will be grown in liquid culture for 10 days, and incubated with 20 nM TMRM [Tetramethylrhodamine methyl ester] for 15-30 min. Mitochondria labeled with GFP/TMRM will be observed with the Nikon confocal A1 system (GFP 488/525, RFP 561/595).

Candat A, Paszkiewicz G, Neveu M, Gautier R, Logan DC, Avelange-Macherel MH, Macherel D (2014) The ubiquitous distribution of LEA proteins across cell compartments in Arabidopsis offers tailored protection against abiotic stress. Plant Cell, in press.

Curtis MD & Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol* **133:** 462–469

Kost B, Spielhofer P & Chua NH (1998) A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J* **16:** 393–401

Logan DC & Leaver CJ (2000) Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells. *J Exp Bot* **51:** 865–871

Mathur J, Radhamony R, Sinclair AM, Donoso A, Dunn N, Roach E, Radford D, Mohaghegh PSM, Logan DC, Kokolic K & Mathur N (2010) mEosFP-based green-to-red photoconvertible subcellular probes for plants. *Plant Physiol* **154:** 1573–1587

Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE & Tsien RY (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat Biotechnol* **22:** 1567–1572

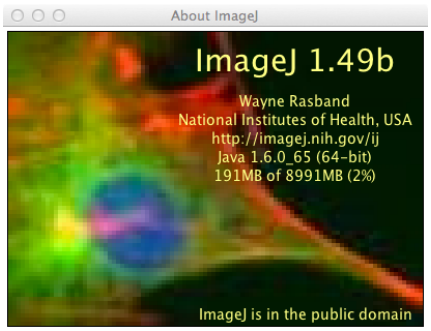
Wiendenmann J, Ivanchenko S, Oswald F, Schmitt F, Rocker C, Salih A, Sîndler KD, Nenhaus GU (2005) EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescnce conversion. Proc Natl Acad Sci USA **101**: 15905-15910

5e ATELIER DU RESEAU MEETOCHONDRIE

VOIR LA DYNAMIQUE MITOCHONDRIALE

Image presentation and analysis with Fiji (ImageJ)

David C Logan



It is recommended to use the latest version of Fiji. The acronym Fiji stands for "Fiji Is Just ImageJ" and is a distribution package of ImageJ complete with many plugins (http://fiji.sc/Fiji).

Contents

1. Opening images.

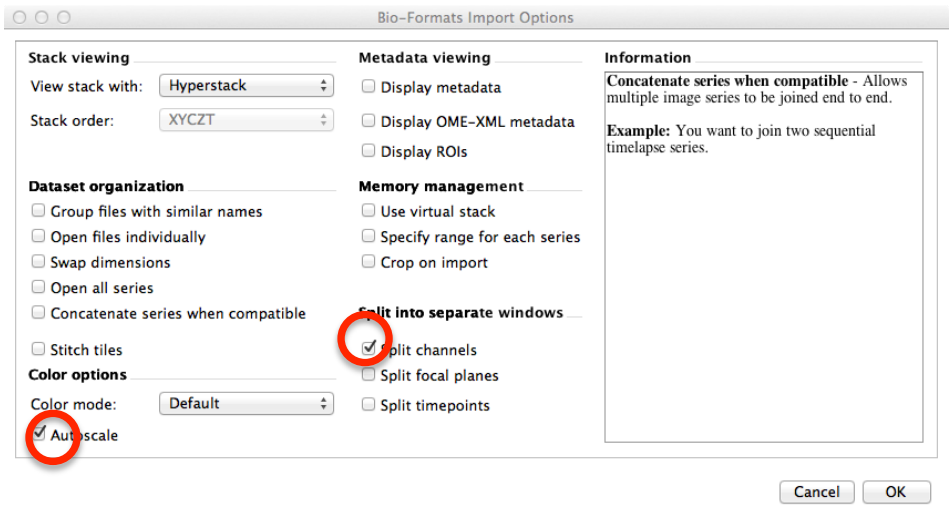
On a Mac, assuming the file suffix has been correctly attributed to Fiji then double clicking on the file icon with open the file.

On a PC, such as in the computer room at the workshop, select “File” then “Open” and then select the correct file. Alternatively, select the file icon, right-click, and choose “Open with” “ImageJ”.

On up to date versions of Fiji different types of file will be opened automatically. However, with older versions of Fiji/ImageJ, Nikon .nd2 files (as saved automatically by the Elements software running the confocal) cannot be opened. Even with the newest version .nd2 files must be viewed as “Standard ImageJ” files (select this option in the “Bio-Formats Import Options” dialogue box, also, make sure to check “Split Channels”.

1.1. Bio-Formats Import Options.

This dialogue box allows you to select various parameters to apply to the image when it is opened in Fiji. The newest version of Fiji (currently 1.49b) can handle most image files as a “Hyperstack” and this is the default setting. Typically, other selections to make are “Autoscale” (bottom left of dialogue) and “Split Channels”, however, the choice of these and other selections will depend on the precise analysis to be performed.



“Autoscale” ensures that the histogram for each channel is stretched to each channels min and max values throughout the stack – adjustments can still be made using “Brightness and Contrast”, but selecting “Autoscale” is a sensible start.
“Split Channels” is useful since often one wants to make a merged image and apply particular LUTs to the separate channels that are to be merged. A LUT (Look Up Table) is simply a colour-map array (palette) used to specify the colour and intensity of the image.

After opening the image one is ready to proceed with preparation of a publication/presentation-ready copy, or some form of image analysis. Each of these will be discussed in turn.

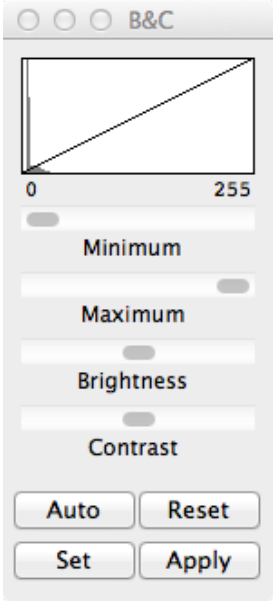
2. Preparation of publication/presentation ready images.

All modifications to an image should be performed on a copy and the original file direct from the image capture software should be saved permanently unaltered. Many changes to images in Fiji cannot be undone and therefore at each stage when a new annotation or adjustment is to be applied it is recommended that a copy of the image or stack is made ("Image", "Duplicate"), make sure the "duplicate stack" click box is selected if duplicating a stack of images. Preparation of publication/presentation ready copies may involve adjustment to the "Brightness and Contrast" of the image, applying colour to a grayscale image, addition of a scale bar, time stamp, or z-position stamp, and annotation of objects within the image.

2.1. Adjustment of "Brightness and Contrast".

The following text is copied from the ImageJ guide:

"Use this tool to interactively alter the brightness and contrast of the active image. With 8-bit images, brightness and contrast are changed by updating the image's lookup table (LUT), so pixel values are unchanged. With 16-bit and 32-bit images, the display is updated by changing the mapping from pixel values to 8-bit display values, so pixel pixel values are also unchanged. Brightness and contrast of RGB images are changed by modifying the pixel values.



Histogram The line graph at the top of the window, which is superimposed on the image's histogram, shows how pixel values are mapped to 8-bit (0--255) display values. The two numbers under the plot are the minimum and maximum displayed pixel values. These two values define the display range, or 'window'. ImageJ displays images by linearly mapping pixel values in the display range to display values in the range 0--255. Pixels with a value less than the minimum are displayed as black and those with a value greater than the maximum are displayed as white.

Minimum and Maximum sliders Control the lower and upper limits of the display range. Holding down Shift will simultaneously adjust all channels of a composite image (e.g., File▷Open Samples▷HeLa Cells (1.3 M, 48-bit RGB)).

Brightness slider Increases or decreases image brightness by moving the display range. Holding down Shift will simultaneously adjust all channels of a composite image.

Contrast slider Increases or decreases contrast by varying the width of the display range.

The narrower the display range, the higher the contrast. Holding down Shift will simultaneously adjust all channels of a composite image.

Auto ImageJ will automatically optimize brightness and contrast based on an analysis of the image's histogram. Create a selection, and the entire image will be optimized based on an analysis of the selection. The optimization is done by allowing a small percentage of pixels in the image to become saturated (displayed as black or white). Each additional click on *Auto* increases the number of saturated pixels and thus the amount of optimization.

Reset Restores the original brightness and contrast settings. The display range is set to the full pixel value range of the image. A resetMinAndMax() macro call is generated if the command recorder is running. Holding down Shift restores original settings in all channels of a composite image.

Set Allows to enter the minimum and maximum display range values in a dialog box. A setMinAndMax() macro call is generated if the command recorder command recorder is running.

> A 16-bit image consists of 65536 possible gray levels. Most of times, however, the relevant image information is contained only within a narrow range of the grayscale. This is the case, e.g., in low light microscopy, in which signal is restricted to the lower end of the grayscale. The *Set Display Range* dialog allows you to choose how to scale the range of gray levels of 16-bit images."

N.B.
(a) A note of caution: be very wary of clicking the "Apply" button. "Apply" will apply the mapping you have chosen in the dialogue box to the pixels (assuming an 8-bit image or stack, or RGB stack).
(b) Sometimes it is useful to alter B&C automatically based on a section (a region of interest – ROI) rather than globally, note that the changes are applied across the whole image but it is the ROI that is used as the basis of this.
(c) For scientific images, changes to B&C must be made equally across the whole image.

2.2. Adding colour to a single grayscale image.

If you wish to add colour to a grayscale image so that you can open it as a colour image in other applications (such as Photoshop, Illustrator, PowerPoint etc) then you need to choose the colour and then save the image in RGB format.

2.2.1. Once the image is open select "Image" then "Lookup Table" then choose your colour. Note that the LUTs "Fire", "Ice" and "Spectrum" are *hot* meaning that colours are assigned based on pixel intensities – this might sometimes be useful. Once you have chosen the LUT the image will change accordingly. However, if saved in this state the colour information will be lost. In order to apply the colour information to the image the image type must be converted to RGB. Select "Image" then "Type" then "RGB Color". Next, save the image in a suitable format

("File", "Save As" and select type).

N.B.
As a general rule images should always be saved as TIFFs. Never save as JPEG format since pixel information will be lost.

2.3. Working with channels – creating a merged image.

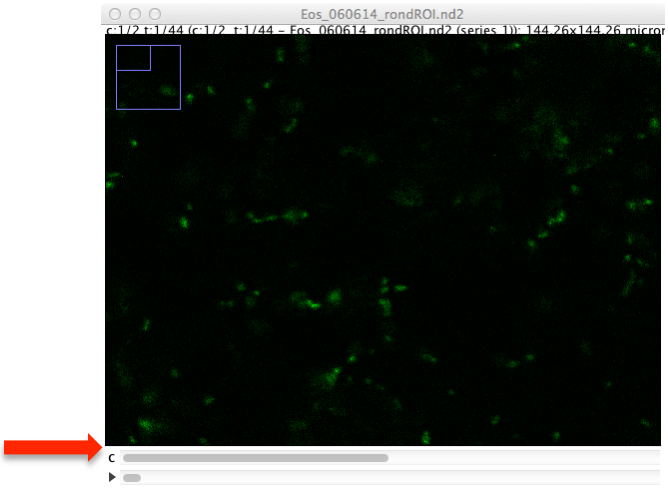
Often the image or stack contains more than one channel (colour) and after optimising B&C (Brightness and Contrast) of each separate channel you will want to create a merged or composite image.

2.3.1. If you opened the file as separate channels then you can create a merged image by choosing "Image", "Color", "Merge Channels".

2.3.2. If you opened the file without splitting the channels then you can first split the channels "Image", "Color" "Split Channels" or create a composite by "Image", "Color", "Make Composite".

2.3.3. Different LUTs can be applied to the channels before creating a composite, while if using "Merge Channels" then a fixed set of LUTs are selectable for each channel to be merged.

2.3.4. "B&C" can be modified for each split channel before merging, or for each channel within a image file than was not split by selecting the channel using the channel slider before altering "B&C".

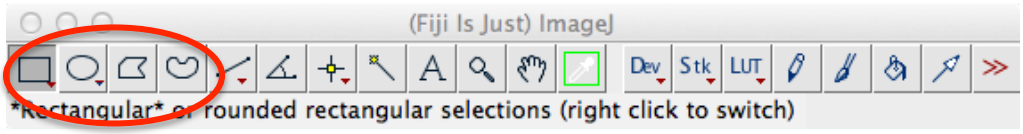


The channel slider is marked with a "C"

2.4. Cropping an image.

Whilst it is desirable to zoom-in on the important information at the time of image capture, it is often necessary to crop images to highlight the important information, remove unimportant background, or to make images have the same proportions for reproduction.

2.4.1. Choose the appropriate crop tool from the menu (one of the first four buttons):



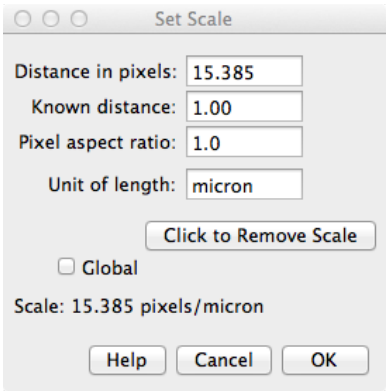
2.4.2. Select the area to be cropped and then select “Image” & “Crop”. Note that once again this cannot be undone so it is best to crop a copy of the image (“Image” & “Duplicate”).

2.4.3. To reuse exactly the same ROI (i.e. selection) on a second open image, click on the second image and then “Edit”, “Selection” and “Restore Selection”. This is very useful when you wish to crop a series of stacks to the exact same size.

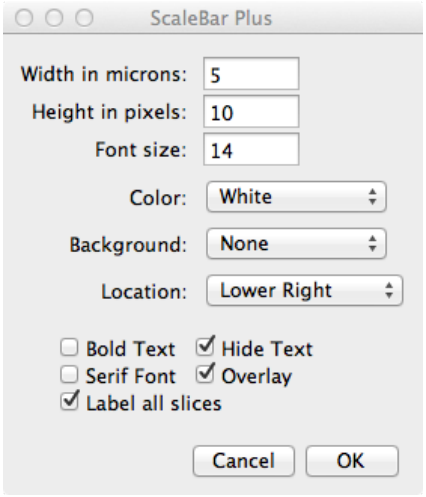
2.5. Addition of a scale bar.

All micrographs should have a scale bar. This is typically very easy to apply in Fiji since the image is loaded with the appropriate metadata (i.e. the size of a pixel in micrometres). In some cases, depending on source of the image and possible bugs in the plugin used to read the file, the metadata may not be read correctly and the scale bar applied may be incorrect. Always look at your images and ask yourself if the scale bar looks correct!

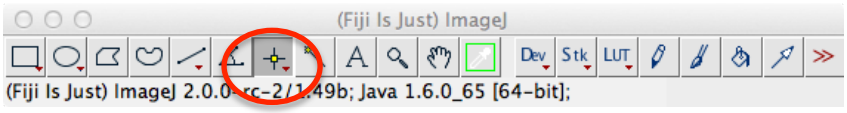
2.5.1. To insert a scale bar first check if a scale has been attributed to the image from the metadata when the image was opened. Select “Analyze” and then “Set Scale”, this dialogue box shows the number of pixels per micron as provided by the metadata. If no metadata is included with the file then the positions within the dialogue box will be empty. Check that the values are as you would expect given the imaging set up. For example, micrograph captured using a camera with pixel size of 6.5 µm x 6.5 µm and a 100X objective each pixel will be 0.065 µm and therefore 1 µm = 15.385 pixels. Once the information in the dialogue box is correct click “OK”. Selecting “Global” will propagate this scale to all open images.



2.5.2. Secondly, choose and stamp the scale bar. Select “Analyze” and then “Tools” followed by “Scale Bar” to show the dialogue box.



Here one chooses the thickness of the bar, its position, colour, presence/absence of text, and font style. Most journals prefer sans-serif fonts so this is default. Specific requirements will determine whether text is required. “Overlay” is recommended otherwise the bar will have the colour of the image. Equally, label all slices is recommended so that the scale is present throughout the image stack if appropriate. The location can be specified automatically in one of four positions, or using the position of the cursor. If you want to set by cursor position then the position should be marked using the point tool before opening the “Scale Bar” dialogue box:



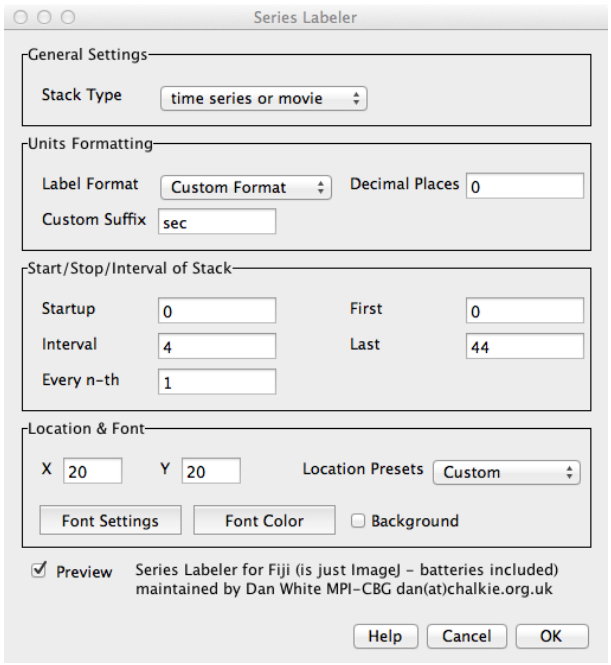
2.6. Addition of a time stamp.

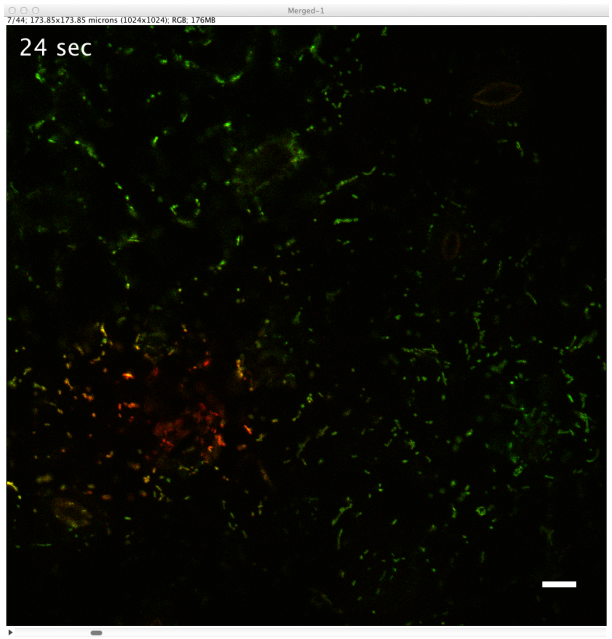
Time-lapse image stacks are best presented with a running clock so that viewers can see the real-time acquisition. There are two options to perform this: “Image”, “Stacks”, “Time Stamper” is one, but is not recommended. There is no preview function so getting the position and size correct is difficult and it is generally less flexible than the second option. The second option is “Image”, “Stacks”, “Series Labeler”. Note that neither of these annotations can be undone (as often the case in Fiji). Therefore, all annotations should be performed on a copy (see paragraph 2 above – “Image” & “Duplicate”). Once the time-lapse stack is labelled it can be exported as a movie or individual images can be saved for presentation as a 2D figure composite.

2.6.1. Open the “Series labeller” dialogue: “Image”, “Stacks”, “Series Labeler”. Choose the appropriate options within the dialogue box and enter the real time values in the text boxes. Select “Preview” at the bottom left of the dialogue box to assist with the size and placement of the time stamp. Generally the start time is “0”, enter the interval between each image and generally choose to label every single image i.e. “Every n-th” is set to “1”. “First” and “Last” are generally set automatically according to the size of

the stack. Remember most journals prefer sans-serif fonts.

NB: If adding both a time stamp and a scale bar, add the time stamp first, otherwise the scale bar will be deleted, or flatten stack after adding scale bar (“Image”, “Overlay”, “Flatten”).





Example of a time series stack (of a photoconversion experiment using mito-mEos) with time stamp in the top left and scale bar in the bottom right.

2.7. Addition of a z-position stamp.

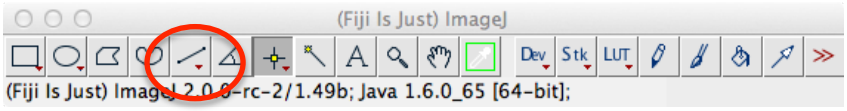
Z-stacks to be presented as a movie are best labelled as described in paragraph 2.4. The only difference is that “z-stack” is chosen in the first drop-down menu

within the “Series Labeler” dialogue box. Again the labelled stack can be presented as a movie or as a series of 2D images in a composite figure.

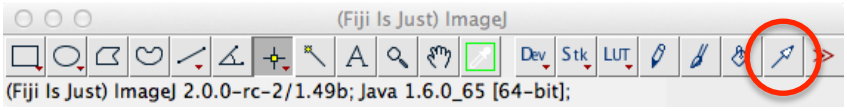
2.8. Adding annotations to the image – using ROI manager.

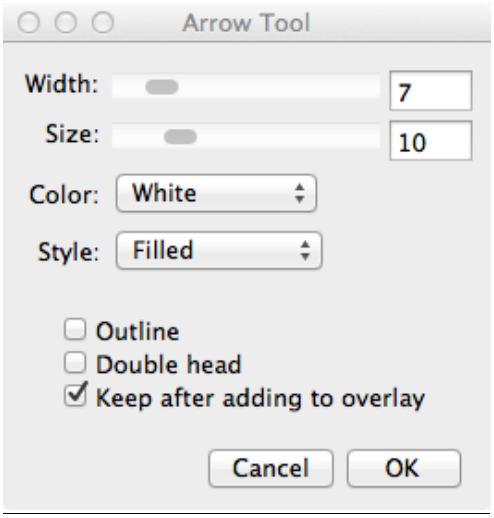
Adding annotations (in this example, arrows) is achieved using the arrow tool and working with the ROI manager. Annotations are added to an overlay and are vector graphics as opposed to raster graphics and therefore are not affected by scaling, meaning they do not become pixelated. Only one ROI can exist at one time without adding them to the ROI manager.

2.8.1. To start it is best to make sure the arrow tool is added to the tool bar by right-clicking on the “line selection tool” which will add the arrow tool to the tool bar if it is not already in place. In the screen capture below, the arrow tool is in place.

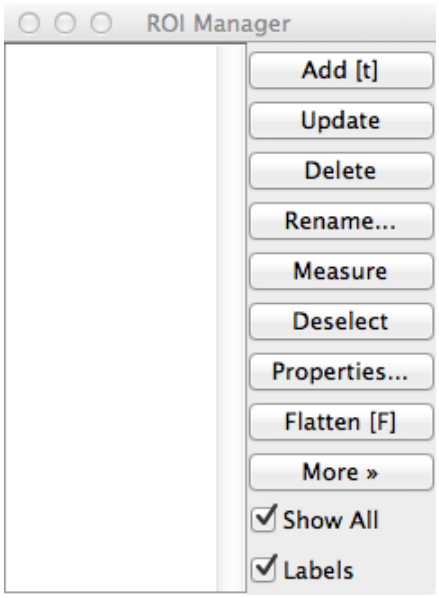


2.8.2. Next, double click on the arrow tool to open the dialogue box:





2.8.3. Now open the ROI manager – “Analyse”, “Tools”, “ROI Manager”. Be sure to select “Show All” in the dialogue box otherwise you will not be able to see each arrow you draw: if “Show All” is not selected you will only see the last arrow added. I also find it useful to select “Labels” to add a number to each arrow while they are being edited.



2.8.4. Draw the arrow you want, and arrange it as required. Now click on the "Add [t]" button, or simply press "t" on the keyboard.

2.8.5. If you want to add more than one arrow to the same slice (image) then you will have to add the ROI to the overlay by pressing "b" on the keyboard.

2.8.6. If you are working with a stack (time series or z) and want to label more slices with the same arrow then you must work with each arrow in turn on the different slices i.e. do not press "b" to add the arrow to the overlay (see bewlow) otherwise it will no longer be an ROI and will not be transferable to another slice. Instead, draw the arrow, position it (it can be positioned as long as it is indicated by little dots: when the arrow is positioned correctly press "t" and then move to the next slice using the ">" key (N.B. This is not an arrow key). The arrow can be re-positioned if necessary and added to the "ROI Manager" by pressing "t". All ROIs are still editable and can be deleted if necessary.

2.8.7. When you are happy with all the annotations then you must add the ROIs as an overlay – this is done by selecting each ROI in "ROI Manager" and typing "b". At this stage, if you save the stack then the overlay will be saved with it. However, the overlay is only seen by ImageJ, not by other programs, i.e. if you open in Photoshop then the arrows will not be present.

2.8.8. To imbed the arrows (overlay) in the image (and here it is final, so work on a copy "Image" & "Duplicate"), then you must click on "Flatten" in the "ROI Manager" or type "f".

2.9. Exporting as a movie file.

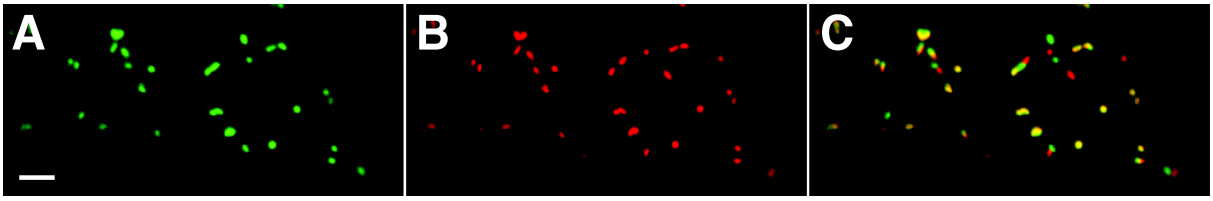
Stacks are easily saved as movies in Fiji: "File", "Save As", "AVI". Uncompressed is recommended but this depends on the downstream use. As a Mac user I always convert my movies to QuickTime movies so any compression is performed at that stage. The only other option is to choose a playback rate (in frames per second). The beauty of having already added the time stamp is that this playback rate is less important (i.e. does not need to be provided to understand the movie) since viewers can see the real-time acquisition rate stamped on the movie. Choosing playback rate may require some trial and error.

3. Analysis of mitochondrial dynamics – qualitative and quantitative.

The capture of good quality images to show mitochondrial morphology and dynamics is itself a challenge but beyond this the community is becoming more demanding in requesting quantification of morphology and dynamics. Some quantification can be relatively simple to achieve i.e. numbers of mitochondria, numbers of long versus round mitochondria, number of mitochondrial clusters versus number of single mitochondria. Some of these can be obtained by simple counting, but it is best if reproducible non-biased criteria can be applied. ImageJ can be used for these measurements but there is no time to go into all possibilities. Here two quantitative assays are presented – co-localisation to quantify the extent of mitochondrial movement; and particle counting to measure the numbers, sizes and morphology indicators of mitochondria.

3.1. Co-localisation for qualitative and quantitative representation of dynamics.

3.1.1. Qualitative representation: in it's simplest qualitative form, one can capture two images a precise time frame apart, apply different LUTs to each image and then merge them. This method gives a visually very striking representation of mitochondrial motility.



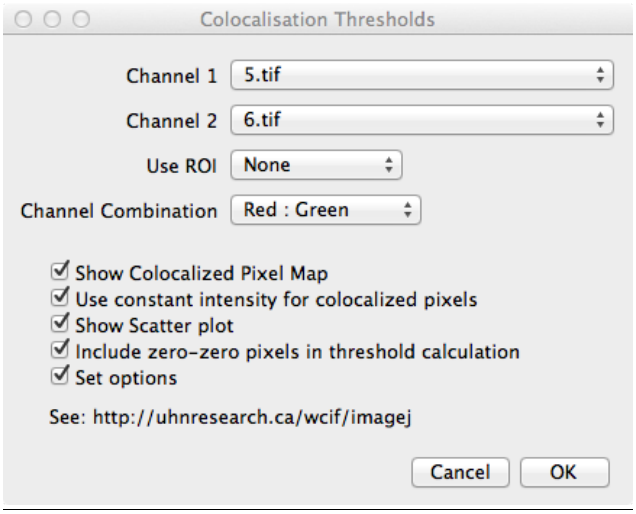
In the figure above two images were captured, 1 second apart, of mitochondria moving in an Arabidopsis leaf cell. The first image (A) was false-coloured green and the second (B) false-coloured red, using LUTs (see section 2.2). The resulting merged image is shown in (C) where mitochondria that moved within the 1 s period are coloured green or red while those in the same position are yellow.

3.1.2. In order to compare treatments or conditions that may affect motility it is beneficial to be able to generate quantitative data. One relatively simple method to do this is to use co-localisation algorithms within ImageJ, for example "Analyze", "Colocalization", "Colocalization Threshold". This method provides colocalisation statistics and an image similar to that above but with colocalised pixels in white. Although one of the biggest problems with co-localisation is thresholding of the images this is not relevant here since we are using two images captured with exactly the same parameters and not the more usual colocalisation of one fluorophore with another that would typically be captured with different settings (illumination and detector).

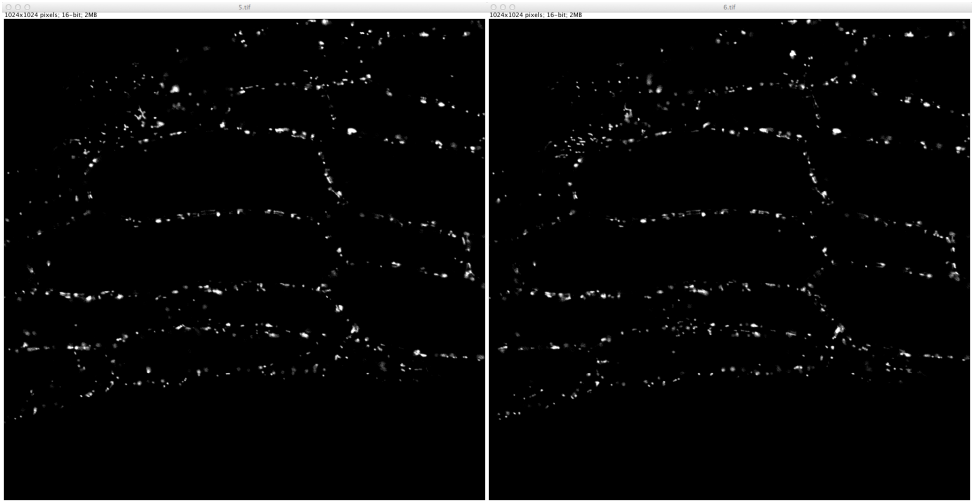
3.1.3. Open the two images to be compared, i.e. images captured at two defined time points, or extract these single time points from a stack (this can be achieved easily using "Image", "Stacks", "Tools", "Slice Keeper"

and defining the slice to be copied from the stack as a single image – do this for each of the two images to be used for colocalisation analysis).

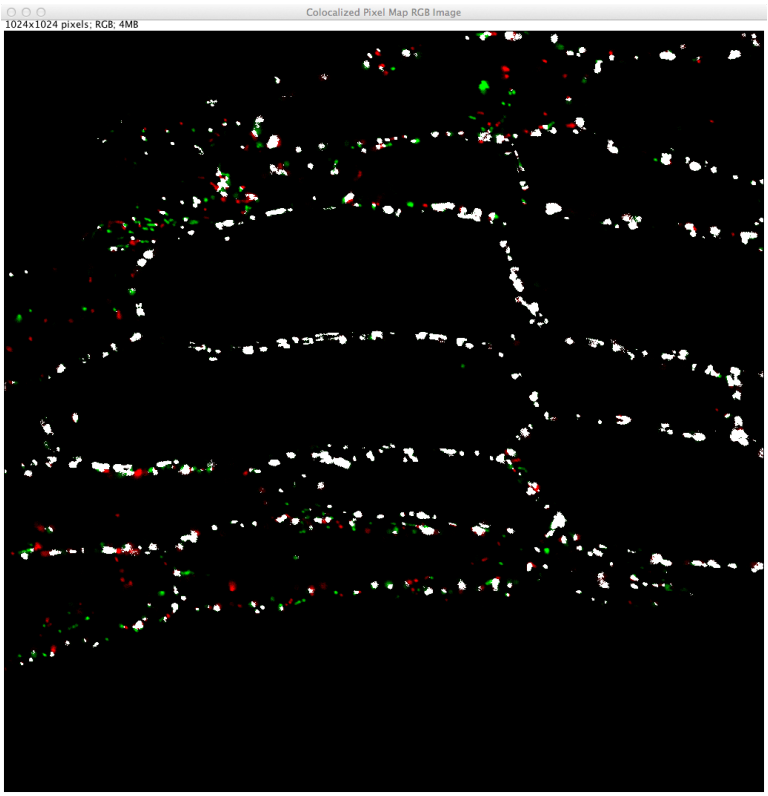
3.1.4. Perform colocalisation “Analyze”, “Colocalization”, “Colocalization Threshold” - the following dialogue box will appear:



3.1.5. Select the two images as “Channel 1” and “Channel 2” and select all other options, for example, “Set options” must be enabled to view the statistics. Selecting “Use constant intensity for colocalised pixels” means that they will appear white in the “Colocalised Pixel Map”.



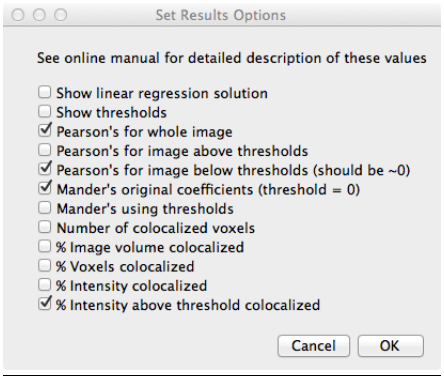
Above, the two images to be subject to colocalisation analysis, note that the images do not need to be coloured, grayscale works fine. Below, the “Colocalised Pixel Map”:



Results

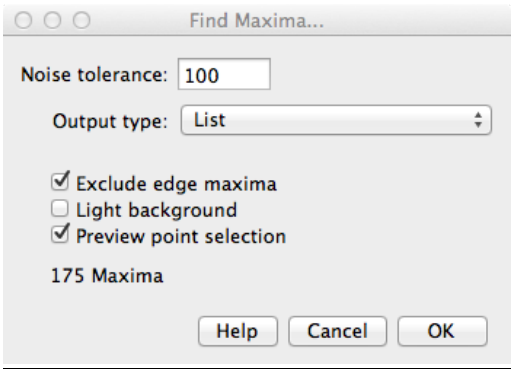
Images	Mask	ZeroZero	Rtotal	R<threshold	M1	M2	%Ch1 Int > thresh	%Ch2 Int >thresh
5.tif & 6.tif	ROI0	incl.	0.519	0.006	0.7674	0.7742	72.67%	73.39%

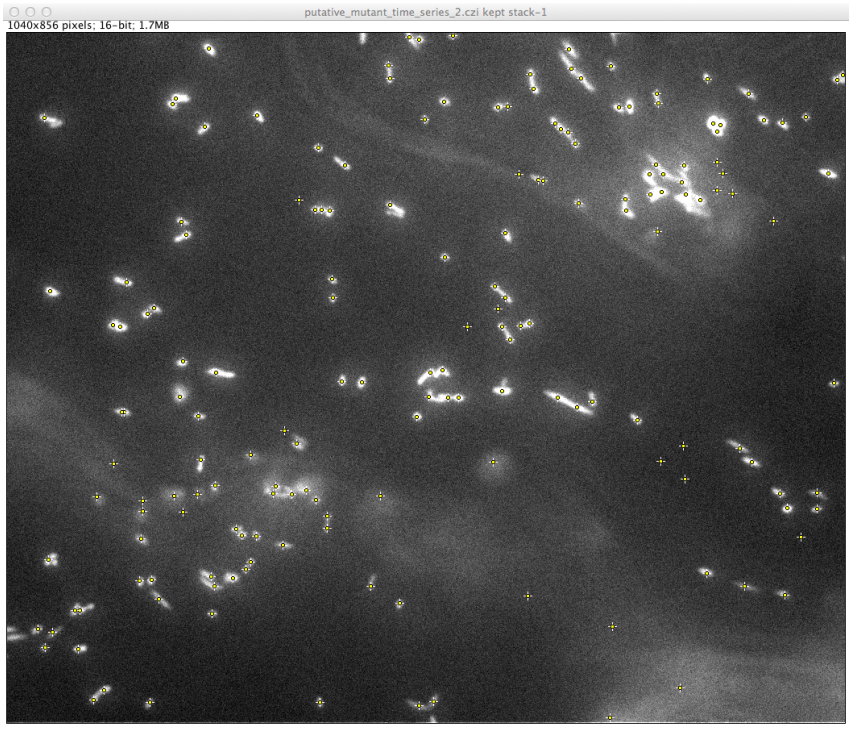
3.1.6. A variety of statistics can be generated and output to a results sheet. See http://www.uhnresearch.ca/facilities/wcif/imagej/colour_analysis.htm for a description of these. For the purposes of quantifying mitochondrial motility we can quote Pearson's or Mander's coefficient and the "% Intensity above threshold colocalized". This later value, like the Mander's coefficient, is presented for each channel and is likely to be different in the two cases since the two images (channels = time points in this case) are of course different. To provide a value for colocalisation of mitochondria as an indicator of lack of movement an average of the two channel values can be used. For the example image, showing little movement, this value is 73.03%.



3.2. Counting and measuring particles (mitochondria).

3.2.1. To simply count particles in an image one can use the “Process” “Find Maxima” command. The settings require optimisation of the “Noise Tolerance” value for each image – simply vary this value while previewing point selection (i.e. have “Preview point selection” checked).





3.2.2. Selecting “Count” from the “Output type” dropdown menu returns the number of maxima found. Selecting “List” returns the x and y coordinates of each maximum. “Find Maxima” does not work on stacks but the “Find Stack Maxima” macro can be used.

3.2.3. Select “Plugins”, “Macros”, “Record” and paste the macro below (also available here: http://imagej.nih.gov/ij/macros/FindStackMaxima.txt into the window then click on “Create” to open the editor. Then “File” “Save As” to save the macro. Then select “Plugins” “Macros” “Install” to find and install the macro file you created. This macro should then appear when you select “Plugins”, “Macros”.

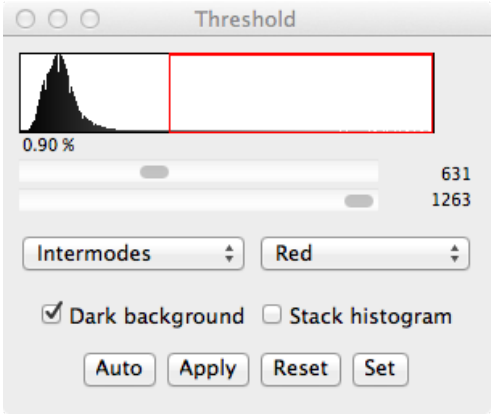
```
// Find Stack Maxima
//
// This macro runs the Process>Binary>Find Maxima
// command on all the images in a stack.

  Dialog.create("Find Maxima");
  Dialog.addNumber("Noise Tolerance:", 5);
  Dialog.addChoice("Output Type:", newArray("Single
Points", "Maxima Within Tolerance", "Segmented Particles",
"Count"));
  Dialog.addCheckbox("Exclude Edge Maxima", false);
  Dialog.addCheckbox("Light Background", false);
  Dialog.show();
  tolerance = Dialog.getNumber();
  type = Dialog.getChoice();
  exclude = Dialog.getCheckbox();
  light = Dialog.getCheckbox();
  options = "";
  if (exclude) options = options + " exclude";
  if (light) options = options + " light";
  setBatchMode(true);
  input = getImageID();
  n = nSlices();
  for (i=1; i<=n; i++) {
     showProgress(i, n);
     selectImage(input);
     setSlice(i);
     run("Find Maxima...", "noise="+ tolerance +"
output=["+type+"]"+options);
     if (i==1)
        output = getImageID();
    else if (type!="Count") {
       run("Select All");
       run("Copy");
       close();
       selectImage(output);
       run("Add Slice");
       run("Paste");
    }
  }
  run("Select None");
  setBatchMode(false);
```

3.2.4. To count and measure particles one can use “Analyze”, “Analyze Particles”.

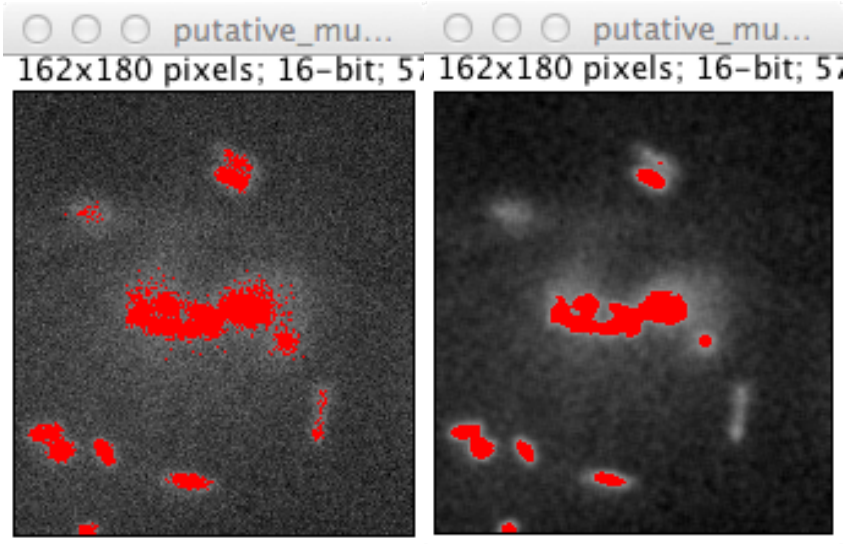
3.2.5. First, the image must be subjected to threshold using “Image” “Adjust” “Threshold”. Thresholding is not easy to perform and almost every image

is different, at least when imaging plant tissue. I imagine thinner, flatter animal cells in culture are much easier to threshold. As ever, the ease of thresholding and success of the method depends on the cleanliness and contrast of the starting image. Images with uniformly bright objects against a uniformly dark background are the easiest to threshold. Such images are not easy to capture! It is unlikely all objects (mitochondria) will be included after thresholding but the analysis of multiple images and averaging of the results should overcome this problem. Equally, thresholding smaller ROIs within the image may work better if trying to threshold large bright objects and small dim objects in the same image.

3.2.6. Threshold image: “Image”, “Adjust”, “Threshold”. In this example red is chosen to highlight the particles to be retained. Once the appropriate threshold is set, click “Apply”. It is rare that “Auto” results in an acceptable threshold. Clicking “Set” allows you to input values for the upper and lower thresholds rather than using the sliders.

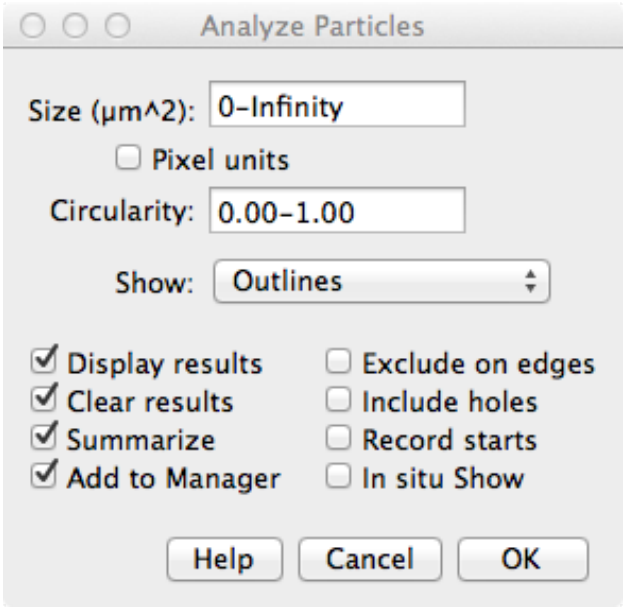


It is often helpful to “Process”, “Smooth” the image before thresholding to reduce the chances that small speckled areas will be identified:



Original Smoothed

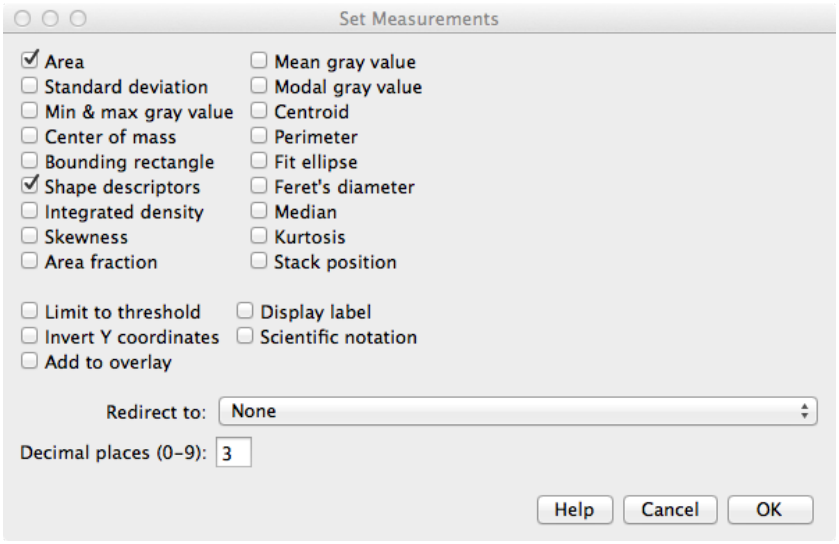
3.2.7. Now you can “Analyze”, “Analyze Particles”:



If the image has been calibrated for size then the size of the particles to be included in the analysis can be defined in the first text box. This is useful to exclude wrongly identified due to thresholding or to exclude clumps of particles that cannot be resolved as individual particles.

“Circularity” refers to how round the particles are – this value will be reported in the “Results” window (“Window”, “Results”) and is useful for assigning a quantitative value to the mitochondrial morphology. Particles are identified with an ellipse; a value of 1 is an ellipse that is a perfect circle, while a value of 0 is a straight line. If you were dealing with uniform particles this could be a means to filter, for mitochondria it is instead a useful measure of morphology.

The results to be reported are selected using the “Analyze”, “Set Measurements” dialogue box:



The following is taken from the ImageJ user guide

http://rsbweb.nih.gov/ij/docs/guide/146.html

« ***Area*** Area of selection in square pixels or in calibrated square units (e.g., mm2, μm2, etc.) if Analyze▷ Set Scale…↓ was used to spatially calibrate the image.

Mean gray value Average gray value within the selection. This is the sum of the gray values of all the pixels in the selection divided by the number of pixels. Reported in calibrated units (e.g., optical density) if Analyze▷ Calibrate…↓ was used to calibrate the image. For RGB images, the mean is calculated by converting each pixel to grayscale using the formula $\text{gray} = (\text{red} + \text{green} + \text{blue})/3$ or $\text{gray} = 0.299 \times \text{red} + 0.587 \times \text{green} + 0.114 \times \text{blue}$ if *Weighted RGB Conversions* is checked in Edit▷ Options▷ Conversions…↑

Standard deviation Standard deviation of the gray values used to generate the mean gray value. Uses the Results Table↑ heading **StdDev**.

Modal gray value Most frequently occurring gray value within the selection. Corresponds to the highest peak in the histogram. Uses the heading **Mode**.

Min & max gray level Minimum and maximum gray values within the selection.

Centroid The center point of the selection. This is the average of the x and y coordinates of all of the pixels in the image or selection. Uses the **X** and **Y** headings.

Center of mass This is the brightness-weighted average of the x and y coordinates all pixels in the image or selection. Uses the **XM** and **YM** headings. These coordinates are the first order spatial moments.

Perimeter The length of the outside boundary of the selection. Uses the heading **Perim.**. With IJ 1.44f and later, the perimeter of a composite selection is calculated by decomposing it into individual selections. Note that the composite perimeter and the sum of the individual perimeters may be different due to use of different calculation methods.

Bounding rectangle The smallest rectangle enclosing the selection. Uses the headings **BX**, **BY**, **Width** and **Height**, where **BX** and **BY** are the coordinates of the upper left corner of the rectangle.
Fit ellipse Fits an ellipse to the selection. Uses the headings **Major**, **Minor** and **Angle**. **Major** and **Minor** are the primary and secondary axis of the best fitting ellipse. **Angle** is the angle between the primary axis and a line parallel to the X-axis of the image. The coordinates of the center of the ellipse are displayed as **X** and **Y** if Centroid is checked. Note that ImageJ cannot calculate the major and minor axis lengths if *Pixel Aspect Ratio* in the Analyze▷ Set Scale…↓ dialog is not 1.0. There are several ways to view the fitted ellipse:

1. The Edit▷Selection▷Fit Ellipse↑ command replaces an area selection with the best fit ellipse.

2. The DrawEllipse macro draws (destructively) the best fit ellipse and the major and minor axis.

3. Select *Ellipses* from the *Show:* drop-down menu in the particle analyzer (Analyze▷Analyze Particles…↑) and it will draw the ellipse for each particle in a separate window.

Shape descriptors Calculates and displays the following shape descriptors:

- ***Circularity*** $4\pi \times [Area][Perimeter]2$with a value of 1.0 indicating a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape. Values may not be valid for very small particles. Uses the heading **Circ**. ***Aspect ratio*** The aspect ratio of the particle's fitted ellipse, i.e., $[Major\ Axis][Minor\ Axis]$. If ***Fit Ellipse*** is selected the **Major** and **Minor** axis are displayed. Uses the heading **AR**. ***Roundness*** $4 \times [Area]\pi \times [Major\ axis]2$or the inverse of *Aspect Ratio*. Uses the heading **Round**. ***Solidity*** $[Area][Convex\ area]$; Note that the Edit▷Selection▷Convex Hull↑ command makes an area selection convex.

Feret's diameter The longest distance between any two points along the selection boundary, also known as maximum caliper. Uses the heading **Feret**. The angle (0--180 degrees) of the Feret's diameter is displayed as **FeretAngle**, as well as the minimum caliper diameter (**MinFeret**). The starting coordinates of the Feret diameter (**FeretX** and **FeretY**) are also displayed (*see also* Feret's Diameter macro and Chamfer distances and Geodesic diameters plugin).
Integrated density The sum of the values of the pixels in the image or selection. This is equivalent to the product of **Area** and **Mean Gray Value**. With IJ 1.44c and later, **Raw integrated density** (sum of pixel values) is displayed under the heading **RawIntDen** when **Integrated density** is enabled. The Dot Blot Analysis tutorial demonstrates how to use this option to analyze a dot blot assay.

Median The median value of the pixels in the image or selection.

Skewness The third order moment about the mean. The documentation for the Moment Calculator plugin explains how to interpret spatial moments. Uses the heading **Skew**.

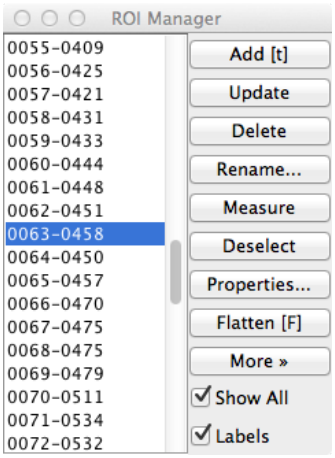
Kurtosis The fourth order moment about the mean. Uses the heading **Kurt**.

Area fraction For thresholded images is the percentage of pixels in the image or selection that have been highlighted in red using Image▷Adjust▷Threshold… [T]↑. For non-thresholded images is the percentage of non-zero pixels. Uses the heading **%Area**.

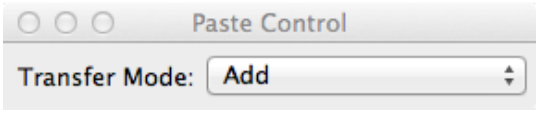
Stack position The position (slice, channel and frame) in the stack or hyperstack of the selection. Uses the headings **Slice**, **Ch** and **Frame**.

N.B.: For line selections the heading **Length** is created. For straight line selections, **Angle** is recorded even if ***Fit Ellipse*** is unchecked. Also, note that measurements that do not apply to certain selection types may be listed as *NaN*, *Infinity* or *– Infinity*. »

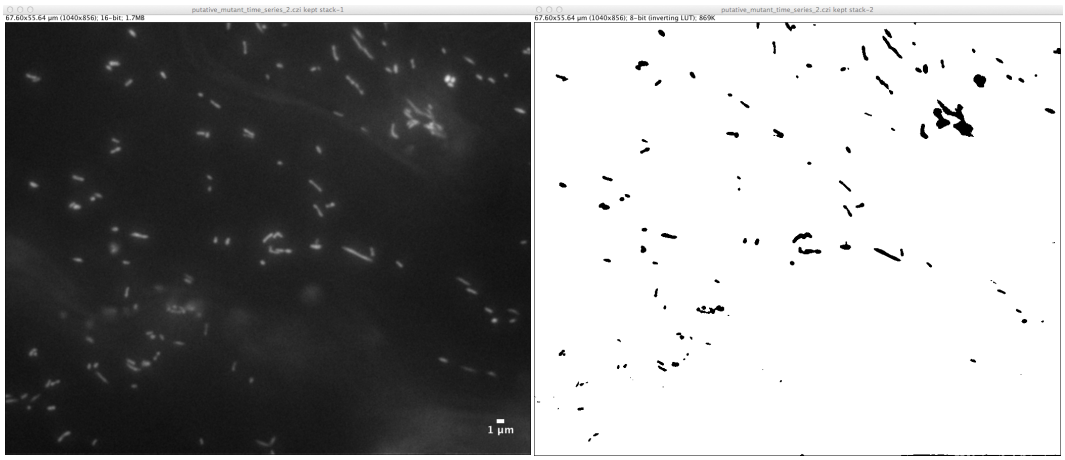
3.2.8. By selecting “Add to Manager” the identified particles are added to “Analyze”, “Tools”, “ROI Manager”. Selecting one of the labelled particles in “ROI Manager” will highlight the corresponding particle in the overlay image. Mis-identified particles can be deleted using this dialogue box.



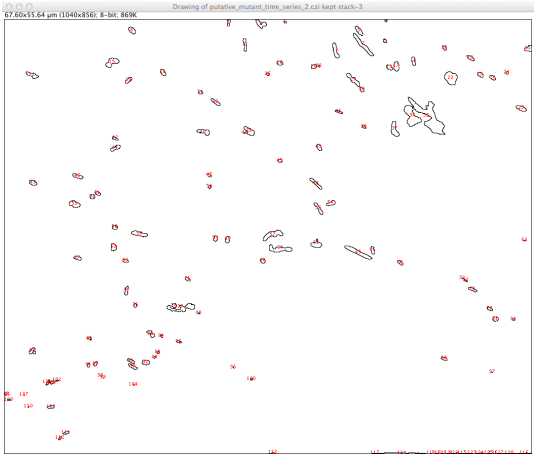
3.2.9. To make a composite image of the original and the identified particles outline image, copy the outline, “Edit”, “Copy”, then “Edit”, “Paste Control” and select “Add” from the pulldown menu:



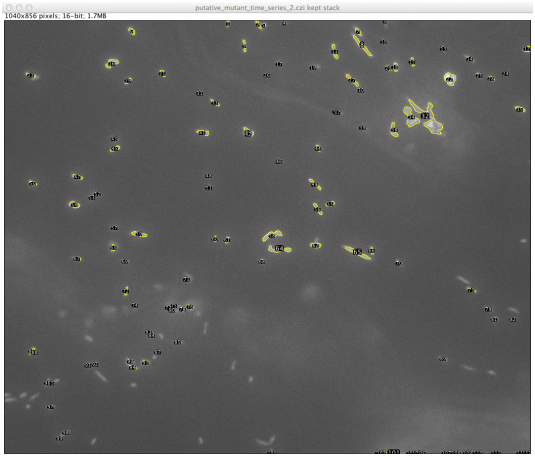
Then click on the original image and “Edit”, “Paste”. Finally, select “Image”, “Overlay”, “Show Overlay”.



Original Threshold



Outline



Outline + original combined