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Labelling of peroxisomes for live cell imaging in the filamentous fungus *Ustilago maydis*

Sofia C. Guimarães, Sreedhar Kilaru, Michael Schrader, Martin Schuster*

Biosciences, University of Exeter, Stocker Road, Exeter EX4 4QD, UK

*Address all correspondence to Martin Schuster, College of Life and Environmental Sciences, Biosciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK

E-mail: m.schuster@exeter.ac.uk

Running title: Live cell imaging of peroxisomes in *U. maydis*

Key Words: peroxisomes, motility, *U. maydis*, yeast recombination cloning, *U. maydis* transformation, live cell imaging

Summary

The basidiomycete fungus *Ustilago maydis* has emerged as a powerful model organism to study fundamental biological processes. *U. maydis* shares many important features with human cells but provides the technical advantages of yeast. Recently, *U. maydis* has also been used to investigate fundamental processes in peroxisome biology. Here, we present an efficient yeast recombination-based cloning method to construct and express fluorescent fusion proteins (or conditional mutant protein alleles) which target peroxisomes in the fungus *U. maydis*. *In vivo* analysis is pivotal for understanding the underlying mechanisms of organelle motility. We focus on the *in vivo* labelling of peroxisomes in *U. maydis* and present approaches to analyze peroxisomal motility.

1. Introduction

Filamentous fungi have emerged as powerful model organisms to study cellular mechanisms. The basidiomycete *U. maydis* is a dimorphic fungus which exists in a yeast-like and filamentous form (*1-4*). Over the past decade, *U. maydis* has been introduced as a model system for studying cell biological processes (*5-7*). Remarkably, *U. maydis* shares many important features with human cells (*8*) including i) DNA repair mechanisms (*9*); ii) microtubule organization (*10*); iii) long-distance microtubule-based transport (*11*); iv) polarized growth (*12*); v) open mitosis (*13*) and vi) cooperative peroxisomal and mitochondrial fatty acid beta-oxidation (*14-16*). In addition, *U. maydis* provides the technical advantages of yeast cells (e.g. genetic accessibility, short generation time, simple cultivation methods, and sophisticated molecular tools). The *U. maydis* genome is fully sequenced (*17*) and comprises 20.5 Mb contained in 23 chromosomes. The 6902 genes are listed in the MIPS *U. maydis* database

(http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2GB). Interestingly, *U. maydis* shares many more proteins with humans than with the yeast *Saccharomyces cerevisiae* (*7, 8, 16*).

Over the years, *U. maydis* has become a powerful model system for cell and molecular biology, and suitable molecular tools have been established (*7*). Recently, *U. maydis* has also been used to investigate fundamental processes in peroxisome biology, and exciting new insights in peroxisomal protein composition and metabolism (*16*), the mechanisms of peroxisome motility and organelle distribution (*18, 19*) and peroxisomal targeting via ribosomal stop codon read-through were obtained (*20-22*) with this model fungus.

Peroxisome motility and dynamics are important prerequisites for peroxisome inheritance, proper intracellular distribution, positioning, organelle interactions, and biogenesis (*23-26*). Here, we focus on the *in vivo* labeling of peroxisomes in *U. maydis.* We describe the generation of plasmids encoding fluorescent proteins with a peroxisomal targeting signal through yeast recombination-based cloning (YRBC). We then address laser-based epi-fluorescence microscopy approaches to visualize and analyze peroxisomal motility. A better understanding of peroxisome metabolism, motility and dynamics in *U. maydis* may prove helpful to explain the highly complex phenotypes of peroxisomal disorders in humans.

2. Materials

All solutions are prepared with ultrapure water and kept at room temperature unless stated otherwise.

2.1 For yeast recombination-based cloning (YRBC)

1. *Saccharomyces cerevisiae* strain DS94 (MATα, *ura3-52*, *trp1-1*, *leu2-3*, *his3-111*, and *lys2-801*) (*27*)

2. YPD medium (yeast extract, 10 g/l; peptone, 20 g/l; glucose, 20 g/l; agar, 20 g/l) - Autoclave at 121ºC for 20 min and store at room temperature

3. Yeast synthetic drop-out media (yeast nitrogen base without amino acids and ammonium sulphate, 1.7 g/l; ammonium sulphate, 5 g/l; casein hydrolysate, 5 g/l; tryptophan, 20 mg/l; with 20 g/l agar added for preparing the plates) - Autoclave at 121ºC for 20 min and store at room temperature

4. 6M Sodium iodide - Stored at 4ºC

5. Salmon sperm DNA (Stock solution of 2 µg/µl)

6. 1 M Lithium acetate – Prepare fresh on the day of transformation and filter sterilise

7. 50% PEG 4000 - Prepare fresh on the day of transformation and filter sterilize

8. Silica glass suspension (Stock solution of 100 mg/ml) - Store at -20ºC

9. DNA wash buffer (50 mM NaCl, 10 mM Tris HCl pH-7.5, 2.5 mM EDTA and 50% ethanol (v/v)) – Store at 4ºC

10. Yeast-lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH-8.0 and 10 mM EDTA)

11. Phenol:chloroform: isoamylalcohol (25:24:1 v/v)

12. Acid washed glass beads (425–600 µm diameter)

13. TE buffer (10 mM Tris HCl; 1 mM EDTA, pH-8.0)

14. 3 M sodium acetate pH-5.5

15. 4 M ammonium acetate

16. RNaseA (10 mg/ml in water)

17. 100% Ethanol

18. 70% Ethanol

2.2 For *Escherichia coli* transformation

1. *E. coli* strain DH5α

2. DYT medium (tryptone, 16 g/l; yeast extract, 10 g/l; NaCl, 5 g/l; with 20 g/l agar added for preparing the plates) - Autoclave at 121ºC for 20 min

3. 10 mM $MgCl_2$

4. Transformation buffer (250 mM KCl, 15 mM $CaCl_2$, 10 mM PIPES, 55 mM $MnCl_2$)

5. DMSO

6. Liquid nitrogen

7. Ampicillin (Stock concentration 100 mg/ml; filter sterilized)

2.3 For cultivation and genetic transformation of *Ustilago maydis*

1. Vitamin Solution (*28*): 0.1% (w/v) thiamine hydrogen chloride; 0.05% (w/v) riboflavin; 0.05% (w/v) pyridoxine hydrochloride; 0.2% (w/v) D-pantothenic acid hemi calcium salt; 0.2% (w/v) 4-aminobenzoic acid; 0.2% (w/v) nicotinic acid; 0.2% (w/v) choline chloride; 1.0% (w/v) myo-inositol. Use immediately or aliquot and store at -20ºC.

2. Trace Elements (*28*): 0.06% (w/v) boric acid; 0.01% (w/v) ferric acid.6H_2O; 0.4% (w/v) sodium molybdate.2H_2O; 0.04% (w/v) zinc chloride; 0.14% (w/v) manganese (II) chloride.4.H_2O; 0.04% (w/v) copper(II) sulphate.5H_2O. Sterilize by filtration.

3. Salt Solution (*28*): 16% (w/v) monopotassium phosphate; 8.0% (w/v) trace elements (*see* **2.3 point 2**); 1.32% (w/v) calcium chloride.2H_2O; 4.08% (w/v) magnesium

sulphate.7H_2O; 8.0% (w/v) potassium chloride; 4.0% (w/v) sodium sulphate. Sterilize by filtration.

4. CM Medium (*28*): 0.25% (w/v) casamino acids; 0.1% (w/v) yeast extract; 1.0% (w/v) vitamin solution (*see* **2.3 point 1**); 6.25% (w/v) salt solution (*see* **2.3 point 3**); 0.05% (w/v) herring sperm DNA; 0.15% (w/v) ammonium nitrate. Set the pH to 7.0 and autoclave for 20 min at 121°C (*see* **Note 1**). For agar plates, add agar to reach a final concentration of 2%.

5. NM Medium (*28*): 0.3% (w/v) KNO_3, 6.25% (w/v) salt solution (*see* **2.3 point 3)**. Set the pH to 7.0 and autoclave for 20 min at 121°C (*see* **Note 1 and 2**).

6. NSY Glycerol: 0.5% (w/v) sucrose; 0.8% (w/v) bacto nutrient broth; 0.1% (w/v) yeast extract ; 80% (w/v) 87% glycerol (final concentration 69.6%) and autoclave for 20 min at 121°C.

7. SCS buffer: Solution 1: 0.6% (w/v) sodium citrate.2H_2O (final concentration 20 mM); 18.2% (w/v) sorbitol (final concentration 1M). Solution 2: 0.4% (w/v) citric acid.H_2O (final concentration 20 mM); 18.2% (w/v) sorbitol (final concentration 1 M). Add solution 2 to solution 1 until pH 5.8 is reached. Autoclave for 20 min at 121ºC and store at 4 ºC.

8. STC buffer: 50% (w/v) sorbitol; 1% (w/v) 1M Tris-HCl pH 7.5; 10% (w/v) 1M calcium chloride. Autoclave for 20 min at 121ºC and store at 4 ºC.

9. STC/40% PEG: 60% (w/v) of STC buffer; 40% (w/v) PEG 4000. Filter sterilize and store at 4 ºC.

10 $YEPS_{light}$: 1.0 % (w/v) Yeast-Extract; 0.4 % (w/v) Peptone; 0.4 % (w/v) Sucrose. Autoclave for 20 min at 121ºC.

11. Regeneration (REG) agar: 1.0% (w/v) yeast extract; 2.0% (w/v) peptone; 2.0% (w/v) sucrose; 18.22% (w/v) sorbitol, 1.5% (w/v) agar. Autoclave for 20 min at 121ºC.

2.4 Equipment

2.4.1 For molecular biological work and growth of cells

- Autoclave
- Small table top centrifuge (for microcentrifuge tubes)
- Large table top centrifuge (for 15 and 50 ml conical tubes); we use a Heraeus Biofuge Stratos Benchtop centrifuge (Kendro Laboratory Products, Osterode, Germany)
- PCR machine
- Water bath
- Vibrax, (we use IKA Vibrax VXR, IKA-Werke, Staufen, Germany)
- Clean bench
- 50 ml flasks
- 28 and 37°C incubators (standing and shaking)

2.4.2 For live cell imaging

- Standard wide field microscope (upright or inverted; *see* **3.3.1** for our setup) with fluorescent light source and appropriate filter sets to image GFP and RFP
- Microscope slides
- Cover slips
- Microwave
- Stirring hot plate

3. Methods

We have developed vectors to label peroxisomes in *U. maydis* using yeast recombination-based cloning (YRBC), which enables assembly of multiple overlapping

DNA fragments in a single cloning step. The YRBC method avoids the dependency on the availability of restriction enzyme sites in the DNA sequence as required for conventional restriction/ligase based cloning approaches. Instead, multiple DNA fragments, with 30 bp overlap sequences, are transformed into *S. cerevisiae*, where upon homologous recombination the vector is generated in a single step. As an example, we illustrate the generation of paGFP-SKL, a vector encoding a fusion of the photo-activatable green fluorescent protein paGFP (or monomeric green or red fluorescent protein eGFP/mCherry) to the C-terminal peroxisome targeting signal peptide SKL (**Fig. 1**). The vector is integrated into the succinate dehydrogenase (*sdi1*) locus of the *U. maydis* AB33 strain (*U. maydis* strains reviewed in (*7*)), avoiding untargeted ectopic integration and the risk of unwanted side effects such as gene disruptions or even alteration of gene expression levels. The resulting strain enables the visualization of peroxisomes in both yeast-like and hyphal cells of *U. maydis*.

3.1 Generation of plasmid through yeast recombination-based cloning (YRBC)

The plasmid pCpaGFP-SKL (*18*) (**Fig 1**) was generated through yeast recombination-based cloning (YRBC) in *S. cerevisiae*, following published procedures (*28*). YRBC involves ten major steps (**Fig. 2**). In brief, the cloning vector needs to be linearized with a suitable restriction enzyme which can be chosen freely and independently of the DNA fragment to be cloned. Primer design is the key step in generating the vectors using YRBC. The 30 bp overlapping sequences to the next DNA fragment need to be incorporated in the 5′ end of the 20–25 bp primer sequence, which results in a total primer length of about 50–55 bp. Likewise, primers GD110, GD111, GD112 and GD113 (**Table 1**) were synthesized and used to amplify the desired DNA fragments using Phusion high-fidelity DNA polymerase (Thermo Scientific, Leicestershire, UK).

The PCR reagents and cycling parameters are described in **Table 2** and **Table 3**, respectively. The DNA bands of interest are excised and purified from the gel as described below.

Place Figs. 1 and 2 here

Place Tables 1 and 2 and 3 here

3.1.1 Purification of DNA fragments from agarose gels

DNA fragments of interest are purified using silica glass suspension as described previously (*30*).

1. Run the PCR products on an agarose gel and cut the corresponding fragments (*see* **Note 3**).

2. Melt the gel slice at 55 °C for 5 min with 3 volumes of 6 M sodium iodide (*see* **Note 4**), followed by further incubation for 5 min at 55 °C with 20 µl silica glass suspension (100 mg/ml stock solution).

3. Centrifuge the reaction mixture at 13,000 rpm for 30 s and discard the supernatant.

4. Wash the pellet with DNA wash buffer for 3 times (*see* **Note 5**).

5. Finally, elute the DNA from the glass beads by adding 10 µl water and incubation at 55 °C for 10 min.

3.1.2 Preparation of yeast competent cells and transformation

Transformation of DNA fragments into *S. cerevisiae DS94* is performed as described previously (*29, 31*).

1. Grow *S. cerevisiae* DS94 cells overnight in 3 ml YPD medium at 28°C in a shaking incubator (200 rpm).

2. Transfer the overnight culture to 50 ml YPD and grown for 5 h at 28 °C in a shaking incubator (200 rpm).

3. Harvest the cells by centrifugation at 2200 rpm for 5 min and wash the cells with 5 ml sterile water.

4. Suspend the cells in 300 µl water and keep at room temperature for further use.

5. Add 4 µl each of purified DNA fragments (linearized vector, and PCR products obtained with primers GD110, GD111, GD112 and GD113 (**Table 1**) to a sterile microcentrifuge tube and add 50 µl salmon sperm DNA (2 µg/µl stock), 50 µl *S. cerevisiae* cells, 32 µl 1 M lithium acetate and 240 µl 50% PEG 4000.

6. Mix the components gently by inverting the tubes for a few times and incubate at 28 °C for 30 min.

7. Perform a heat shock at 45°C for 15 min and centrifuge tubes at 2000 rpm for 2 min.

8. Gently remove the supernatant and suspended the pellet in 150 µl water.

9. Plate the cell suspension, in two dilutions (20% and 80% of the cells) onto two yeast synthetic drop-out medium plates which lack uracil and incubate at 28°C for 2 days.

3.1.3 Colony PCR on yeast colonies and plasmid DNA isolation from yeast cells

Colony PCR is performed on yeast cells by using DreamTaq DNA polymerase (Thermo Scientific, Leicestershire, UK) in 20 µl total volume. PCR reagents and cycling parameters are described in **Table 4** and **Table 5,** respectively. One primer (SK41) which binds to the vector and another primer (GD113) which binds to the insert

(**Table 1**) are used to identify the positive clones; the expected band sizes are of 1800 bp. Plasmid DNA is isolated from the positive yeast colonies as described previously with slight modification (*32*).

1. Grow the recombinant *S. cerevisiae* cells overnight in 15 ml yeast synthetic drop-out medium at 28 °C and harvest the cells by centrifugation at 3000 rpm for 5 min.

2. Add 200 µl yeast-lysis buffer, 200 µl phenol:chloroform: isoamylalcohol (25:24:1 v/v) and 0.3 g acid washed glass beads to the tubes.

3. Vortexed for 5 min using IKA Vibrax shaker.

4. Add 200 µl TE buffer (pH-8.0) and centrifuge for 5 min at 13,000 rpm.

5. Carefully transfer the upper aqueous layer to a sterile microcentrifuge tube and add 50 µl 3 M sodium acetate pH-5.5 and 1 ml ethanol.

6. Keep the tubes at −20°C for 15 min and centrifuge at 13,000 rpm for 20 min.

7. Suspend the cell pellet in 400 µl TE buffer containing RNaseA (100 µg/ml final concentration) and incubate at 37°C for 15 min.

8. Precipitate the DNA by addition of 10 µl 4 M ammonium acetate and 1 ml 100% ethanol.

9. Centrifuge the tubes for 5 min at 13,000 rpm and wash DNA with 70% ethanol.

10. Remove the residual ethanol by incubating the tubes at 37°C for 10 min and suspend the DNA in 20 µl water.

Place Tables 4 and 5 here

3.1.3 *E. coli* transformation and isolation of plasmid DNA

Transform 10 µl of DNA isolated from *S. cerevisiae* into competent *E. coli* DH5α cells. Isolate the plasmid DNA from the transformed *E. coli* colonies and further confirm by

restriction analysis and sequencing. For transformation and isolation of plasmid DNA, use established standard protocols for molecular biology.

1. For preparation of competent cells, grow a single *E. coli* colony overnight in 20 ml DYT medium at 37 °C in a shaking incubator (200 rpm).

2. Add 100 µl overnight culture to 100 ml fresh DYT medium containing 10 mM $MgCl_2$ and incubate at 18 °C with 100 rpm until the optical density reaches 0.25 (approx. 48 h).

3. Chill the cells in ice water for 10 min and centrifuge at 4 °C for 10 min at 5000 rpm.

4. Discard the supernatant and suspend the pellet in 60 ml ice cold transformation buffer.

5. Centrifuge the cell suspension at 4 °C for 10 min at 5000 rpm and suspend the cell pellet in 16 ml transformation buffer.

6. Add 1.2 ml DMSO and prepare 50 µl aliquots.

7. Freeze aliquots in liquid nitrogen and store the competent cells at −80 °C.

8. Use 10 µl of isolated yeast DNA and add 50 µl of *E. coli* competent cells. Keep on ice for 10 min. Heat shock for 45-60 s at 42ºC. Leave on ice for 2-3 min, add 900 µl of DYT and incubate for 1 h at 37ºC, 200 rpm.

9. Spread the cells on DYT plates containing 100 µg/ml ampicillin, and incubate overnight at 37ºC.

10. For the plasmid isolation pick a single colony and inoculate in 5ml DYT and incubate overnight at 37ºC.

11. To isolate the plasmid from *E. coli* use a commercial plasmid extraction kit (*see* **Note 6**) or other method available in the laboratory.

3.2 Transformation of *U. maydis*

3.2.1 Generation of Protoplasts (adapted from *(33)*)

1. Grow the *U. maydis* strain of interest (here, AB33) overnight in 50 ml YEPS $_{light}$ medium at 28ºC, 200 rpm. The OD_{600nm} should be in the 0.6-0.8 range (*see* **Note 7**).

2. Spin down the culture for 10 min at 3,000 rpm. Remove the supernatant and resuspend the pellet in 25 ml SCS buffer. Centrifuge for 10 min at 3,000 rpm and remove the supernatant.

3. Resuspend the pellet in 2 ml SCS buffer containing 7 mg/ml of lysing enzymes from *Trichoderma harzianum* (containing β-glucanase, cellulase, protease and chitinase activities) (Sigma–Aldrich, Gillingham, UK) and leave at room temperature for 10-15 min until 30-40% of the cells become rounded (protoplast formation is monitored under the microscope). From this step forward, perform everything on ICE (!).

4. Add 10 ml of cold SCS buffer and centrifuge at 2,200 rpm for 7 min at 4ºC. Remove the supernatant. Repeat the washing step twice. Centrifuge the final wash at 2,100 rpm for 12 min. Resuspend the pellet in 500 µl of ice cold STC buffer. Prepare aliquots of 50 µl and either store immediately at -80ºC or use directly for transformation.

3.2.2 Linearization of plasmid poGSKL for *U. maydis* transformation

1. Linearize the plasmid of interest (here, poGSKL) (*34*) by restriction endonuclease digestion (here, *Ssp*I).

2. Run a test gel with 1 µl of the digestion mixture. To the reaction tube add $1/10^{th}$ of the volume of 3 M sodium acetate and 2.5 volumes of 96% ethanol. Vortex and leave for at least 30 min at -20ºC.

3. Spin down the mixture at 13,300 rpm for 20 min, 4ºC. Discard the supernatant and add 1 ml of 70% ethanol. Spin down at 13,300 for 7 min. Remove the supernatant and let the DNA pellet air dry. Resuspend in 15-50 µl double distilled water (depending on the amount of DNA in the gel - *see* **step 2**).

3.2.3 *U. maydis* transformation

1. Thaw a 50 µl *U. maydis* aliquot on ice (*see* **3.2.1, step 4**) and add 1-5 µg of linearized plasmid DNA and 1 µl of heparin (1 mg/ml). Incubate on ice for 10 min.

2. Prepare the Reg-agar plates which are composed of two layers. In a 100 mm petri dish, add a bottom layer with selectable antibiotic (15-20 ml) and leave it to solidify. Then, add the top layer without antibiotic (!!!) (15-20 ml) (*see* **Note 8**).

2. Add 500 µl PEG (40% (w/v) in STC buffer (filter sterilized) on top of the protoplasts and carefully mix by pipetting up and down. Leave on ice for 15 min.

3. Carefully mix and streak the mixture onto 2 plates of Reg-agar (80% and 20% of the mixture). Incubate the plates for 4-7 days at 28ºC.

4. Singularize the transformants on CM-agar plates with the appropriate antibiotic. Confirm peroxisomal targeting by microscopy. For this, prepare a glass slide with a drop of water (around 1µl) and streak a singularized colony from the plate. Add a coverslip and examine for fluorescence. After confirmation, strains should be grown in YEPS $_{light}$ medium. Mix 1ml of the cell culture with 1ml NSY-glycerol and store at -80ºC (*see* **Note 9**).

3.3 Live cell imaging of *U. maydis*

3.3.1 Our microscope setup

We use an inverted microscope (IX81; Olympus, Hamburg, Germany) with Plan-Apochromat 100×/1.45 NA oil total internal reflection fluorescence microscopy or UPlan-SApochromat 60×/1.35 NA oil objective lenses (Olympus, Hamburg, Germany). The fluorescent tags are excited using a VS-LMS4 Laser Merge System (Visitron) with 70-mW solid-state lasers at 488 and 561 nm (*see* **Note 10**). Images are captured using a fast, high-resolution camera - CoolSNAP HQ2 (Photometrics/Roper Scientific, Ottobrunn, Germany). For GFP detection the microscope is equipped with an eGFP ET filter-set (470/40 Et Bandpass filter, Beamsplitter T495 LPXR and 525/50 ET Bandpass filter (Chroma Technology GmbH, Olching, Germany)). For co-visualization studies of two different proteins labeled with GFP and mCherry, for example mCherry-SKL and Kinesin3-GFP, we use Dual-View imager (Dual-View Micro; Photometrics) equipped with a dual-line beam splitter (z491/561; Chroma Technology Corp, Olching, Germany) with an emission beam splitter (565 DCXR; Chroma Technology Corp, Olching, Germany), an ET-Band pass 525/50 (Chroma Technology Corp.), and a single band pass filter (BrightLine HC 617/73; Semrock, Rochester, USA). Photo-bleaching experiments are preformed using a 405-nm/60-mW diode laser, which is decreased by a neutral density 0.6 filter, resulting in 15-mW output power, coupled into the light path by an adaptor (OSI-IX 71; Visitron Systems, Munich, Germany). The 405-nm laser is controlled by UGA-40 controller (Rapp OptoElectronic, Hamburg, Germany) and VisiFRAP 2D FRAP control software from MetaMorph Series 7.5. (Visitron Systems, Munich, Germany). For microscope acquisition, device control and image analysis is assured by the MetaMorph® Software

(Molecular Devices, Downingtown, PA). This software allows a complete control of the system plus diverse applications for image analysis.

3.3.2 Culture preparation for microscopy

1. From the -80ºC glycerol stock streak the strain of interest (here, AB33_eGFP-SKL) on a CM-plate supplemented with glucose. The strain should be incubated at 28ºC and should be properly grown after 1-2 days.
2. Prepare a 100 ml flask with 20 ml of complete medium (CM) supplemented with 1% glucose. From the plate remove a small amount of cells with a 100 µl tip to add into the flask (the tip can go into the flask) (*see* **Note 11**). Incubate overnight (or more than 8 hours) at 28ºC, 200 rpm.

   For hyphal formation (AB33_GFP-SKL), cells are grown in a 100 ml flask with 20 ml of complete medium (CM) supplemented with 1% glucose over the day or overnight. Spin down 15ml of the cell culture at 3,000 rpm for 10 min in a 15 ml conical tube. Discard the supernatant and rinse the tube with nitrate minimal medium (NM). Re-suspend the cell pellet in NM containing 1% glucose and incubate for 6-8 h or overnight at 28ºC, 200 rpm (**Fig. 3**) (*see* **Note 12**).

   Place Fig. 3 here

3.3.3 Live cell imaging of peroxisomes in *U. maydis*

1. First prepare an agarose cushion. To do so, prepare a 2% solution of agarose in water and melt it in a microwave. Next, prepare 2 microscope slides with masking tape on one side. Place those slides on a flat table and put a third slide between them. Put a drop (50-100 µl) of the agarose solution on the middle slide

and cover it with a fourth slide (*see* **Fig. 4**). Keep the agarose close to the microscope facility/setup on a stirring hot plate at approx. 60-70ºC. This way, new slides can be constantly generated. Preparing 5-10 slides in advance will allow you to perform your experiments in an appropriate timeframe under similar conditions (*see* **Notes 13 and 14**).

Place Fig. 4 here

2. For live cell imaging add 1 µl of your cell culture to an agarose cushion and cover with a coverslip. Peroxisome fluorescence signals (GFP-SKL) are very intense (*see* **Note 15**). To capture single images of fluorescent peroxisomes, we routinely use an exposure time of 100 to150 ms with binning 1 and digital gain 3. To capture all peroxisomes within the cell, we generate Z-stacks with the above settings and a Z step size of 200 nm. We usually use a total number of 31 steps covering the whole cell.

   To visualize and quantify motility behavior of peroxisomes (**Fig. 5**), we acquire continuous streams using 150 ms of exposure time with binning 1 and digital gain 3. The total number of plains acquired range from 75 to 200.

   Place Fig. 5 here

3. For co-localization or co-motility analysis (for example, mCherry-SKL and Kinesin3-GFP) we perform synchronized observations of red (mCherry or RFP) and green (GFP) fluorescence signals (*see* **Note 16**) using a Dual-View imager.

We capture single images or continuous streams using 150 ms of exposure time with binning 1 and digital gain 3. The total number of plains acquired range from 75 to 150 (*see* **Note 17**). The amount of signals in both channels makes it difficult to get clear information from those streams. In this case, parts of the cell can be photo-bleached using the 405 nm laser before stream acquisition (*see* **Note 18**) (*see* **Fig. 6** for an example and compare to kymograph in **Fig. 5**).

Place Fig. 6 here

3.3.4 Visualization and analysis

1. We use MetaMorph software for analysis and image processing. This can also be done with ImageJ or other image processing software.

Motility events of peroxisomes are visualized in kymographs which represent time over distance. To generate a kymograph in MetaMorph, draw a line (single or multipoint) over the total length of the cell or region of interest. Then, in the main menu go to Stack → Kymograph. In the newly opened window, the width of the line can be chosen to cover the whole cell or only the path of a single motility event. By pressing “Create” a kymograph over the chosen area is generated. In the kymograph, each pixel line represents one frame of the original movement so that the velocity, flux and run length can be analyzed (*see* **Note 19**). As shown in **Fig. 5**, kymographs generated over the desired length of the hyphal cell can give you an overview of the type and characteristics of the motile events (run length, velocities, direction, pausing time, and frequency).

2. To analyze the distribution of peroxisomes, acquire z-stacks of fungal cells (*see* **3.3.3, step 2**). From these stacks, generate maximum projections using MetaMorph. Draw a line over the length of individual hyphal cells and use the command linescan (Measure → Linescan) to analyze a fluorescent intensity profile. The profile can be transferred into the software Excel. An individual intensity profile can be used or the mean intensity of multiple intensity profiles can be calculated (**Fig. 7**).

Place Fig. 7 here

3. To visualize co-motility, generate a kymograph of each channel as described above, and align (Process → Color Align) the two kymographs (**Fig. 6**).

4. Notes

1. Before use, the medium needs to be supplemented with 1% glucose (50% (w/v) stock). As the CM-Glucose medium gets easily contaminated, it is recommended to work under sterile conditions and to use a burner or clean bench. The NM-Glucose medium degrades over time and loses its ability to induce hyphal growth. NM medium without Glucose is stable!

2. NM medium becomes cloudy in the autoclave and needs to be mixed until it has been cooled down and turns clear again.

3. To facilitate detection of the PCR fragments, run a side lane with 0.5-1 µl of the PCR product. If required, the PCR fragments can be stored at -20ºC for 2-3 days.

4. It is important to completely dissolve the agarose. Increase incubation time (up to 10 min) if the agarose has not dissolved properly.

5. When pipetting the wash buffer, cover the beads and do not vortex.

6. We use GeneJET Plasmid Miniprep Kit from Thermo Sientific, Paisley, UK.

7. If overgrown in the morning, you can dilute the culture to an OD_{600nm} 0.2 and incubate until the desired OD_{600nm} is reached. Do not use cultures with $OD_{600nm} > 0.8$.

8. For each transformation, two Reg-Agar plates are required.

9. For cell stocks we use 2 ml Micro tubes with screw tops (e.g. Sarstedt, Nümbrecht, Germany).

10. The advantage of using lasers as an excitation light source is that the output power can be regulated. As the peroxisome signals are very strong, it is important to reduce the power to avoid saturation of the camera. This can also be achieved by putting neutral density lens filters into the light path.

11. It requires experience to select/pick the right amount of cells. Initially, select different amounts of cells and monitor for optimal growth on the following day. Preferably initiate the cultures in the late afternoon/evening to avoid overgrowth. On the next day, you should have an optimal OD_{600nm} of around 0.8. When the culture is very transparent, it is too diluted and it will be difficult to find cells for imaging. However, if it is very dense and turbid, the cells may be stressed and should not be used. If required, you can dilute the culture by adding fresh medium (i.e., remove some of the culture and add the same amount of medium to refresh nutrients). If you do this, the culture needs at least 1 hour incubation in a shaking incubator before starting microscopy.

12. In case of an "over day" culture, you do not have to rinse the conical tube. Try to shift the cells as late as possible into nitrate minimal medium (NM). The cells should not be kept longer than 18 h in nitrate minimal medium (NM) as they get stressed.

13. The 2% agarose solution can be reused over several days. If it becomes too solid, prepare a fresh solution. The agarose cushions prevent the cells from moving around and supply them with water and oxygen at the same time.

14. Keep in mind that observation under the microscope should not exceed 15 min (!) due to oxygen depletion processes.

15. We reduce the laser power to 2-6% due to intense fluorescence. If you cannot reduce the intensity of your light source, try to dim it using a neutral-density filter to avoid over-exposure.

16. For this kind of experiment it is important to image both channels simultaneously to capture transient interactions. Normally, the red label is weaker than the green one; thus the peroxisomes should be labeled with mCherry and the second protein of interest (here, Kinesin 3) should be labeled with GFP. It is also helpful if you can regulate the excitation power separately for each channel.

17. The length of the stream depends on the signal stability in both channels. Normally, the red fluorescent tags are less photo-stable and bleach faster than the green fluorescent tags (*35, 36*).

18. Photo-bleaching can be performed over different lengths of the cell, ranging from 1 μm up to 40 μm. However, keep in mind that using the 405 nm laser will not only bleach the fluorescent tags but can also damage other molecules in the cell.

19. Analysis of the velocity works only in newly generated kymographs. Use the “single line” tool and trace one motility event. The velocity is shown in the kymograph window and can be transferred into Excel via the “open Log” button in the kymograph window.

Acknowledgments

We would like to thank G. Steinberg for his support and the opportunity to publish this method chapter. This work was supported by the Portuguese Foundation for Science and Technology and FEDER/COMPETE (SFRH/BD/73532/2010 to S.C. Guimarães) and CRUP/Treaty of Windsor (ACÇÕES INTEGRADAS 2009, B-33/09 to G. Steinberg and M. Schrader). M. Schrader acknowledges support from the Marie Curie Initial Training Network (ITN) action (FP7-2012-PERFUME-316723).

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Figure Legends

Figure 1. Vector for integration of peroxisome marker protein into the genome of *U. maydis*. The vector pCpaGFP-SKL confers carboxin resistance and is designed for integration of a paGFP-SKL fusion protein into the genome of *U. maydis*. The vector contains a H253L point mutation in the succinate dehydrogenase (*sdi1*) gene sequence, which confers carboxin resistance and allows targeted integration into the *sdi1* locus of *U. maydis.* Note that fragments are not drawn to scale.

Figure 2. Flow chart illustrating the different steps involved in yeast recombination based cloning (YRBC). See main text for details (adapted from (*37)*).

Figure 3. The dimorphic fungus *U. maydis*. The yeast-like form (upper panel; Bar, 5 µm) and the filamentous hyphal cell (lower panel; Bar, 10 µm) are depicted.

Figure 4. Preparation of the agarose cushion. a) Place a drop of liquid 2% agarose (60ºC) on a glass slide. b) Gently, but immediately, cover with a glass slide as indicated in the Figure. Let it solidify and when dry (15-20 min), carefully remove the upper slide and (c) add 1 µl of *U. maydis* culture. d) Cover the agarose cushion with a coverslip and observe under the microscope.

Figure 5. Morphology and dynamics of peroxisomes in *U. maydis*. Upper panel: Peroxisome morphology in the *U. maydis* hyphal cell labelled with eGFP-SKL. The cell edge is indicated in blue. Bar, 15 µm. Lower panel: Different types of peroxisome motility visible in the kymograph: directed transport (green arrow); diffusional (purple

arrow head) and stationary (dashed grey rectangle). The stream was acquired over 300 frames with 150 ms exposure. Bars, 5 µm, 6 s.

Figure 6. Co-mobility of Kinesin3 and peroxisomes after photobleaching. Bleach the hyphal cell in a specific area(s) of interest; afterwards acquire time series to observe co-motility events within the bleached region. By overlapping the two channels, the motility of the organelles/protein of interest can be visualized. For a more graphical and quantitative observation, generate a kymograph that allows the measurement of velocity, distance and duration of the events. This was used in (*18*) to identify the molecular motor involved in peroxisome motility in *U. maydis* and to analyze the relative localization of the motor protein to the peroxisome.

Figure 7. Peroxisome distribution in *U. maydis*. Upper panel: 2D-deconvolved maximum projection of a Z-axis stack, adjusted in brightness, contrast, and gamma settings. Bar, 5 µm. Lower panel: Fluorescent intensity profiles of eGFP-SKL in AB33 hyphal cells. Position of cell tips is indicated (Tip). Each data point represents the mean ± SEM (60 cells, two experiments). This kind of experiment was used in (*18*) to show even distribution of PO throughout the wild type hyphal cell and the mis-localisation of peroxisomes in the absence of early endosome motility.

Table 1. Primers used in this study

Primer	Sequence (5' to 3')
GD110	TAAGCTGTCAAACATGAGAATTCATCGATGGCGGCCGCACGGGGATCTTC
GD111	CTTAATTAAGGATCCGGCGCGCCGCGGCCGCACGCTAAGTGGAGTTGTCC
GD112	TATTTGAGAAGATGCGGCCAGCAAAACTAACTGAAGCTTGCATGCCTGCA
GD113	TGCAGCCGGGCGGCCGCTTTAAAGCTTCGACTTGTACAGCTCGTCCATGC
SK41	GTGGATGATGTGGTCTCTACAGG

Table 2. PCR mix for fragment amplification

	Volume
Template DNA	1 µl
10 µM fw primer	2 µl
10 µM rv primer	2µl
5x Phusion HF buffer	10µl
10 mM dNTPs	1 µl
dH_2O	33.5 µl
Phusion polymerase	0.5 µl
Total	50 µl

Table 3. PCR cycling parameters for fragment amplification

Step	Temperature	Volume
1	98 ºC	30 s
2	98 ºC	10 s
3	60 ºC	20 s
4	72 ºC	30 s/ 1kb
5	10 ºC	10 min
6	10 ºC	∞
Go to step 2 and repeat for 34 cycles		

Table 4. PCR mix for Colony PCR

	Volume
Yeast Colony	--
10 μM fw primer	1 μl
10 μM rv primer	1μl
dH_2O	8 μl
2xDream Taq Green PCR Master Mix	10 μl
V_T	20 μl

Table 5. PCR cycling parameters for Colony PCR

Step	**Temperature**	**Time**
1	95 ºC	5 min
2	95 ºC	30 s
3	60 ºC	20 s
4	72 ºC	60 s/ 1kb
5	10 ºC	10 min
6	10 ºC	∞
Go to step 2 and repeat for 34 cycles		