Immunolabelling for detection of endogenous and overexpressed peroxisomal proteins in mammalian cells

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Summary

Peroxisomes are dynamic subcellular organelles in mammals, playing essential roles in cellular lipid metabolism and redox homeostasis. They perform a wide spectrum of functions in human health and disease, with new roles, mechanisms and regulatory pathways still being discovered. Recently elucidated biological roles of peroxisomes include as anti-viral defence hubs, intracellular signalling platforms, immunomodulators, and protective organelles in sensory cells. Furthermore, peroxisomes are part of a complex inter-organelle interaction network, which involves metabolic cooperation and cross-talk via membrane contacts. The detection of endogenous and/or overexpressed proteins within a cell by immunolabelling informs us about the organellar and even sub-organellar localization of both known and putative peroxisomal proteins. In turn, this can be exploited to characterise the effects of experimental manipulations on the morphology, distribution and/or number of peroxisomes in a cell, which are key properties controlling peroxisome function. Here, we present a protocol used successfully in our laboratory for the immunolabelling of peroxisomal proteins in cultured mammalian cells. We present immunofluorescence and transfection techniques as well as reagents to determine the localization of endogenous and overexpressed peroxisomal proteins.

1. Introduction

Peroxisomes are dynamic, multifunctional subcellular organelles that rapidly assemble, multiply and are degraded in response to the metabolic needs of the cell ***(1)***. Peroxisomes play key roles in several anabolic and catabolic cellular pathways including the metabolism of fatty acids and reactive oxygen species, which underlines their importance for human health and development ***(2, 3)***. Additionally, peroxisomes function in a complex network within the cell, cooperating with other membrane-bound organelles via physical membrane contact sites for communicating and performing concerted processes ***(4, 5)***. New peroxisomal functions are constantly being identified, including in viral responses ***(6)***, as intracellular signalling platforms ***(7, 8)*** and as protective organelles in sensory cells, e.g., the hair cells of the inner ear ***(9)***. Recently, the role peroxisomes play in immunity has also begun to be appreciated, via their control of inflammatory responses and immune signalling ***(10)***. Even though peroxisomes are becoming increasingly well characterised, advances in peroxisome purification and (spatial) proteomics analyses ***(11–14)*** are still leading to the identification of novel peroxisomal proteins, or the localization and targeting of previously known proteins to peroxisomes, which were initially detected in the cytoplasm or associated with other organelles ***(15–17)***. Of note, examples of such a dual localization include key components of the organelle division machinery (e.g. DRP1, MFF, FIS1), which are shared by both mitochondria and peroxisomes ***(18)***. Additionally, variability in peroxisomal proteins is detected in individual tissues or organs. The peroxisomal proteome in brain, lung and testis differs significantly from the well-characterized liver and kidney peroxisomes ***(19–22)***, and novel tissue-specific peroxisomal proteins are continuously identified ***(9, 23, 24)***.

A remarkable feature of peroxisomes is their ability to respond to intra- and extracellular changes and stimuli with alterations in their biogenesis, morphology, number, distribution, protein composition and metabolic activity ***(18, 25)***. This dynamism is essential for healthy peroxisomal function, as patients who are deficient in the process of peroxisome growth and

division display developmental and neurological phenotypes, despite the metabolic functions of their peroxisomes being only mildly affected ***(26)***. Understanding the signalling pathways and protein effectors that determine how the form, abundance and distribution of peroxisomes are determined and regulated within the cell is therefore a key question that the field is beginning to answer ***(27–30)***.

The discoveries of the essential cellular roles played by peroxisomes have resulted in a great interest in their physiological functions and dynamics, as well as in the localization of both known and potentially novel candidate proteins at peroxisomes. This chapter is intended to provide detailed information on methods and strategies used successfully in our laboratory for the overexpression and/or immunolabelling of peroxisomal proteins in cultured mammalian cells. We present immunofluorescence and transfection techniques as well as reagents to determine peroxisome-specific targeting and localization of both overexpressed and endogenous proteins. We also discuss how immunofluorescence of endogenous peroxisome marker proteins can report on the cellular complement of peroxisomes in terms of their morphology, number and distribution, in response to stimuli or protein overexpression.

2. Materials

Appropriate aseptic techniques and practices should be applied at all times, and all equipment and reagents used to maintain and process living cells need to be sterile.

2.1 Mammalian Cells and Plasmids

1. Mammalian cell line of interest, here: MFF deficient fibroblasts (human skin fibroblasts; dMFF) (kindly provided by F.S. Alkuraya, King Faisal Specialist Hospital & Research Centre, Kingdom of Saudi Arabia) ***(31)*** (*see* **Note 1**).
2. Plasmid for expression of candidate protein in mammalian cells, here: Myc-PEX11β ***(32)*** (*see* **Note 2**).

2.2 Cell Culture Equipment

1. Class II Biological Safety Cabinet/Tissue Culture Hood (*see* **Note 3**).
2. Humidified CO_2 incubator (95% air, 5% CO_2, 37°C).
3. Inverted light microscope (phase contrast).
4. 37°C water bath.
5. Mammalian cell counter.
6. Vacuum aspiration system.
7. Table top centrifuge equipped with a swing-out rotor for 15 ml conical tubes.
8. Microporator Neon Transfection System, Invitrogen.

2.3 Cell Culture Media, Buffers, and Reagents

1. Complete growth medium: Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (store at 4°C).
2. Phosphate-buffered saline (1×PBS) (without Ca^{2+} and Mg^{2+}): 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl, pH 7.4 (store at 4°C).
3. TrypLE™ Express solution (1×) (Gibco) (store at 4°C) (*see* **Note 4**).
4. 70% (v/v) ethanol.
5. Clean and sterile glass coverslips (19 mm∅, 0.13-0.17 mm thickness) (*see* **Note 5**).
6. Metal tweezers.

2.4 Transfection

1. Neon Transfection Kit, Invitrogen. Store buffers at 4ºC.
2. Plasmid DNA in sterile, deionized water (1 µg/µL) (*see* **Note 6)**.

2.5 Immunofluorescence and Fluorescence Microscopy

1. Fluorescence microscope work station, here: inverted IX-81 fluorescence microscope; 150 Watt Xenon-Arc lamp; 10x ocular lens; PlanApo oil immersion objectives with 60× and 100× magnification; equipped with the appropriate sets of excitation filters, dichromatic mirrors and emission filters; CCD digital black and white camera (CoolSNAP HQ^2) driven by Visitron imaging software.
2. Immersion Oil.
3. 10×PBS (for 1 L): Weigh 80 g of NaCl, 2 g of KCl, 14.4 g of $Na_2HPO_4 \times 2\ H_2O$ and 2 g of KH_2PO_4. Bring volume to 1 L, followed by adjustment of the pH to 7.3. For 1× PBS, dilute 1:10 with dH_2O and adjust pH to 7.4 if necessary. Both solutions are stable at room temperature (RT).
4. PBS-PFA fixative: 4% paraformaldehyde (PFA) in 1×PBS. PFA should be handled under a fume hood and glove protection is required. Weigh 4 g of PFA and add to 90 mL of dH_2O. Add 4 drops of 1 N NaOH, stir and heat solution at 60°C until PFA dissolves. When solution becomes clear, remove flask from the heating plate, cool to RT, add 10mL of 10 x PBS, pH 7.3 and adjust pH to 7.4 (store at 4°C for short term- or at -20°C for long term storage, used at RT).
5. Digitonin stock solution: 1 mg/mL in dH_2O (400×). Store at -20°C.
6. Triton X-100 solution (alternative to digitonin): 0.2% Triton X-100, 1×PBS. Carefully shake until Triton X-100 is completely dissolved. Avoid generating a lot of foam prior to use. This solution is stored at 4°C and used at RT.
7. Blocking solution: 1% Bovine serum albumin (BSA), 1×PBS. Store solution at 4°C or aliquot and freeze. This solution is stable until evidence of bacterial growth.
8. DAPI (diamidino-2-phenylindole, optional): 1 mg/mL in sterile dH_2O (1000×). Store at 4ºC protected from light.

9. Mowiol Mounting Medium (MMM) for immunofluorescence: To prepare MMM stock, dissolve 12 g of Mowiol 4-88 powder in 40 mL of 1×PBS by stirring overnight at RT. The next day add 20 mL of glycerol and stir overnight. Centrifuge the viscous solution for 1 h at 18,500×*g* (Beckman Avanti Centrifuge J-251, 15,000 rpm with a rotor JA 25.50) to pellet any remaining undissolved Mowiol crystals. Dissolve a few crystals of sodium azide in the supernatant to avoid contamination and aliquot stock. Store at 4°C. To prepare the working solution, mix 3 volumes of MMM stock with 1 volume of n-propyl-gallate stock (anti-fading reagent) and store at 4°C. This solution is stable for 4-6 weeks.
10. 2.5% n-propyl-gallate stock (anti-fading reagent): Dissolve 0.625 g of n-propyl-gallate in 12.5 mL 1×PBS (neutralize pH to dissolve n-propyl-gallate). Add 12.5 mL of glycerol and stir at RT until completely dissolved (*see* **Note 7**). Directly add fresh reagent to MMM working solution. Store the solution protected from light at 4°C. This solution is stable for 4-6 weeks and should be replaced when it turns brownish. Prolonged storage can lead to crystal formation within the solution. Do not try to re-suspend, but avoid pipetting crystals.

2.6 Controls

Negative controls should be performed to assess the level of general background (i.e., incubation with secondary antibodies only). Negative controls using isotype control antibodies or pre-immune serum from the same species as the primary antibody may also be applied to validate the specificity of staining results.

2.7 Antibody sources

1. Myc: mouse monoclonal Myc-epitope antibody (Santa Cruz, sc-40)
2. PEX14: rabbit polyclonal Peroxin 14 (PEX14) antibody (kindly provided by D. Crane, Brisbane, Australia)
3. Catalase: mouse polyclonal catalase antibody (Abcam, ab88650)

3. Methods

A convenient method to determine the peroxisomal localization of mammalian proteins of interest is by their expression in the form of tagged fusion proteins in mammalian cells and subsequent immunofluorescence microscopy. In the case of previously uncharacterized candidate proteins, this can be used to confirm if the protein is indeed localized to peroxisomes. Colocalization of the tagged fusion protein with a peroxisomal marker is indicative of peroxisomal localization (*see* **Note 8**). As cell-permeable dyes for the *in vivo* staining of mammalian peroxisomes are still not available, the expressed candidate protein and an endogenous peroxisomal marker protein (e.g. PEX14 ***(33)***) are usually detected by antibody staining.

It is recommended, if possible, to confirm the peroxisomal localization of the expressed candidate protein by detection of its endogenous form. When suitable antibodies directed against the candidate protein of interest are available, and the endogenous expression level is high enough, the endogenous protein can be colocalized with a peroxisomal marker protein using immunofluorescence microscopy. It should be considered, however, that the candidate protein might be expressed in a cell type-, tissue- or species-specific manner.

Immunofluorescence of known endogenous peroxisomal proteins can also be informative about peroxisome biology, beyond being simply a marker to determine peroxisomal colocalization. For example, we have used immunofluorescence to show differential localization of endogenous peroxisomal proteins to different regions of highly elongated peroxisomes,

implying different sorting or properties within these domains ***(34)*** (**Figure 1A**). Immunofluorescence of endogenous protein markers can also be used to visualize and characterize parameters of the peroxisomal population within a cell (e.g. morphology, distribution, size, number). Combined with experimental treatments such as cell stimulations or knock-down/overexpression of a protein of interest, this can provide an insight into the process and regulation of peroxisomes dynamics ***(27, 32)*** (**Figure 1B**).

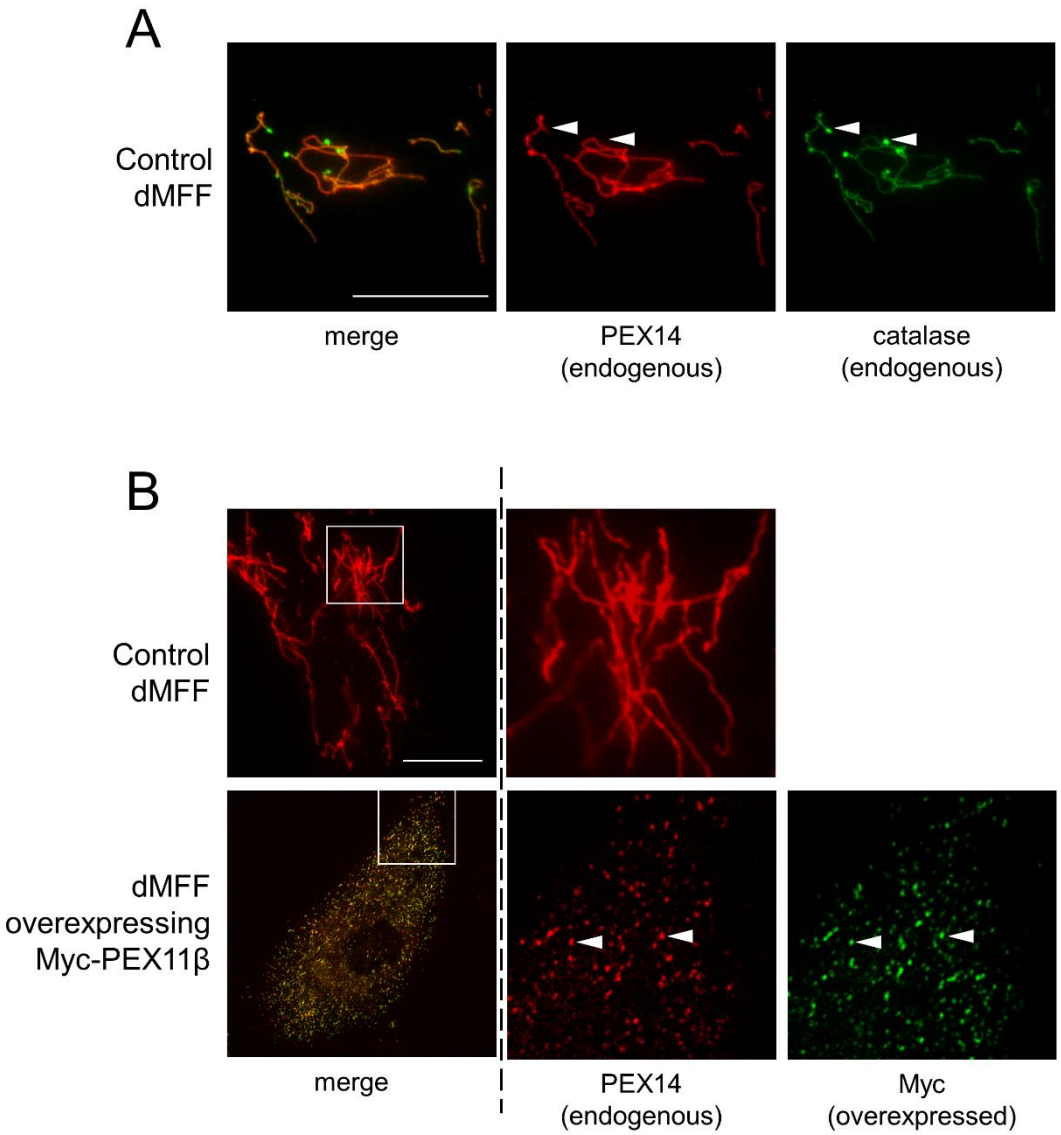

Figure 1.

Figure 1. Localization of endogenous and overexpressed peroxisomal proteins in mammalian cells.

(A) Two endogenous peroxisomal proteins, PEX14 (membrane) and catalase (matrix), are differentially distributed in the highly elongated peroxisomes in dMFF cells. Note that while both proteins are localized to peroxisomes, PEX14 is only present in the tubule, while catalase is enriched in the globular peroxisome body (indicated by arrowheads). dMFF cells were processed for immunofluorescence and stained with antibodies to PEX14 (red) and catalase (green). Overlays (merge) shown on the left. Bars = 20 μm. Adapted from ***(34)***.

(B) Overexpression of Myc-PEX11β causes division of highly elongated peroxisomes in dMFF cells. As expected, Myc-PEX11β is localized to peroxisomes, as shown by colocalization with the peroxisomal membrane marker PEX14 (indicated by arrowheads). Note that the endogenous PEX14 signal shows a dramatic change in peroxisomal morphology between control dMFF cells (top row, highly elongated) and dMFF cells overexpressing Myc-PEX11β (bottom row, small spherical structures), indicating PEX11β-dependent peroxisome division. dMFF cells were transfected with Myc-PEX11β by microporation, processed for immunofluorescence and stained with antibodies to PEX14 (red) and the Myc-epitope (green). Overlays (merge) shown on the left; boxed areas in the left panel are expanded in the right panels. Bars = 20 μm. Adapted from ***(27)***.

3.1 Cell Culture

1. Perform all cell culture related work in a Class II Biological Safety Cabinet/Tissue Culture Hood and disinfect the work surface and materials (e.g., pipettes) with 70% (v/v) ethanol.
2. Grow dMFF cells in complete growth medium (10 cm ∅ cell culture dishes) (*see* **Note 9**) in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C).
3. Refresh the cell culture medium every 2-3 days.
4. Split the cells before they reach 100% confluency (*see* **steps 5** to **14**).
5. Pre-warm 1×PBS, TrypLE™ Express solution, and complete growth medium to 37°C.
6. Remove all medium from the cell culture dish with a Pasteur pipette by vacuum aspiration and wash the cells once with 3 mL of 1×PBS.
7. Add TrypLE™ Express solution to the cells and gently tilt to cover the surface (1 mL/10 cm dish).
8. Incubate the cells for 2-5 min at 37°C (*see* **Note 10**).
9. Upon detachment, harvest the cells in complete growth medium (10 mL/10 cm dish) (*see* **Note 11**).
10. Carefully resuspend the cells by pipetting the cell suspension 2-3 times up and down and further detach remaining cells from the surface of the dish. Repeat in case the cells still form aggregates or clumps.
11. Transfer the cells to a 15 mL conical tube and take an aliquot of the suspension for cell counting.
12. Pellet the cells by centrifugation (500×*g*, 3 min at RT).
13. Resuspend the cell pellet in 10 mL of complete medium.
14. For maintenance, transfer the required amount of cells (approx. 5×10^5 cells) to a new 10 cm cell culture dish containing 10 mL of complete medium (*see* **step 4** and **Note 12**). For immunofluorescence of endogenous proteins, seed 2.5 x 10^5 cells into a 6 cm Ø dish

containing four sterile, clean, round glass coverslips and 4 mL complete growth medium. Allow 24 h for the cells to attach to the coverslips.

15. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C).

3.2 Transfection of human skin fibroblasts (dMFF) using microporation (optional)

1. Perform all transfection related work in a Class II Biological Safety Cabinet/Tissue Culture Hood (*see* **protocol 3.1, step 1** and **Note 3**).
2. Split an appropriate amount of cultured human fibroblasts 24h before transfection and seed 4 x 10^5 cells per Ø 10cm culture dish. This amount of cells is required for one transfection using the 100 µL Neon tip (*see* **Note 13**). Additional 10 cm dishes are prepared as required. It is highly recommended to prepare one or two additional dishes as a backup (4x10^5 cells / 10cm dish).
3. On the day of transfection, add 4 mL complete growth medium (without antibiotics!) into a 6 cm Ø dish and place in the incubator (*see* **Note 14**). For each microporation, one 6 cm Ø dish is required. For immunofluorescence analysis, add four sterile, clean, round glass coverslips (19mm Ø, 0.13-0.17 mm thickness) to the dish prior adding the complete medium using sterile tweezers (*see* **Notes 15-17**). Coverslips are not required for immunoblotting analysis.
4. Harvest the cells by trypsinization, pool them and centrifuge at 500 x *g* for 3 min.
5. Aspirate the supernatant, resuspend the cell pellet in 10 mL 1x PBS, and centrifuge as above.
6. During the centrifugation, place the Neon Microporation device in the biological safety cabinet. Fill a Neon tube with 3 mL of electrolyte buffer E2 and insert the tube into the Pipette station. Set the pulse conditions (here: 1400 V, 20 ms pulse width, 1 pulse) on the device (*see* **Note 18**).

7. Aspirate the supernatant, and resuspend the pellet in R-buffer (add 110 µL R-buffer per $4x10^5$ cells) (*see* **Note 19**). Transfer the cells gently into a sterile 1.5 mL microcentrifuge tube.
8. Add 10 µg of plasmid DNA into another sterile 1.5 mL microcentrifuge tube (*see* **Note 20**).
9. Gently add the 110 µL of cell suspension (in R-buffer) and pipette one or two times up and down.
10. Fit a 100 µL Neon tip onto the Neon pipette.
11. Immerse the tip into the cell-plasmid DNA mixture and slowly take up 100 µL of the sample. Avoid generating air bubbles in the tip.
12. Insert the pipette into the E2 buffer-containing tube in the Pipette station, and press start on the touch screen.
13. After the electric pulse was delivered, remove the pipette from the Pipette station and transfer the cells from the tip to the 6cm Ø dish containing the pre-warmed growth medium (without antibiotics) (*see* **Note 21**)
14. Move the dish horizontally and vertically to evenly distribute the cells (*see* **Note 22**).
15. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37 ^oC) for 24h or 48h to allow cell attachment and efficient protein expression (*see* **Note 23)**.
16. Discard the Neon tip in an appropriate biological hazardous waste container and repeat **steps 8 – 16** for the remaining samples (*see* **Note 24**).

3.3 Detection of expressed (or endogenous) peroxisomal proteins

1. Prepare dMFF cells as described (*see* **protocol 3.1**). For overexpression of a protein of interest (optional), after 24 h microporate the dMFF cells with a mammalian expression vector encoding a tagged version of your peroxisomal protein of interest (here, Myc-PEX11β) (*see* **protocol 3.2** and **Note 25)**. When designing an expression construct for a

peroxisomal membrane protein, such as PEX11β in this case, ensure that the tag does not interfere with membrane insertion of the fusion protein. Expression of both N- and C-terminally tagged fusion proteins may be required. When designing an expression construct for a peroxisomal matrix protein, ensure the tag is on the N-terminus of the fusion protein. As the vast majority of the mammalian peroxisomal matrix proteins contain a peroxisomal targeting signal (PTS1) at the very C-terminus, tagging at the C-terminal domain (for example with GFP) interferes with peroxisomal targeting via the peroxisomal import receptor PEX5. However, C-terminally tagged versions can be used as negative controls ***(35)*** (*see* **Note 26**).

2. All following steps can be performed under non-sterile conditions. Aspirate the culture medium from the 6 cm dish and briefly wash the cells (on glass coverslips) with 1×PBS to remove serum proteins. Take care that the cells never get dry during the experimental procedure.
3. Fix the cells immediately with fresh 4% paraformaldehyde (PFA) for 20 min at RT (*see* **Note 27**).
4. Wash samples 3 times in 1×PBS for 2-5 min.
5. Permeabilize cells with 2.5 µg/mL digitonin in 1×PBS for 5 min at RT (*see* **Note 28**).
6. Wash samples 3 times in 1×PBS for 2-5 min.
7. Block in 1% BSA in 1×PBS for 10 min at RT.
8. Wash samples 3 times in 1×PBS for 2-5 min.
9. Using tweezers, place the coverslips in a humid chamber, cell-side up (*see* **Notes 29** and **30**) Apply minimal forces, as the cover slip can break easily.
10. Apply antibodies diluted in 1% BSA/1×PBS (here, mouse monoclonal anti-Myc, 1:200; rabbit polyclonal anti PEX14, 1:1400) and incubate for 1 h at RT (*see* **Notes 31** and **32**).
11. Wash samples 3 times in 1×PBS for 2-5 min.

12. Incubate cells with fluorophore-conjugated secondary antibodies diluted in 1×PBS for 1 h at RT (here, donkey anti-rabbit-Alexa594, 1:1000; donkey anti-mouse-Alexa488, 1:400). During the incubation, the humid chamber should be covered (e.g. with aluminium foil or a box) to avoid exposure to room light and potential photobleaching of the fluorophores. Keep the antibody dilutions on ice and in the dark prior to use.
13. Wash samples 3 times in 1×PBS for 2-5 min (*see* **Note 33**).
14. Apply a drop of Mowiol 4-88 on a glass microscope slide. Glass slides should be clean and dust-free. Label the slides with a waterproof marker or pencil (*see* **Note 34**).
15. Using tweezers dip the cover slip briefly into dH_2O and remove excess water by holding a piece of filter paper close to the rim of the cover slip.
16. Quickly place the cover slip (cell-side down!) onto the drop of Mowiol Mounting Medium (MMM) on the glass slide. Avoid enclosing air bubbles underneath. Using the tips of the tweezers, minimal pressure can be applied to the top of the cover slip to remove air bubbles. Remove excess MMM (*see* **Note 35**).
17. Put slides in a lightproof slide box and let MMM dry for 2-3 h. Store slides at 4°C in the dark until analysis (*see* **Note 36**).
18. Microscopic analysis: choose the appropriate microscope settings to image both the peroxisomal marker protein and the exogenously expressed (or endogenous) candidate protein of interest.
19. Adjust the exposure times according to the brightness of the fluorescence signals and collect multicolor images (**Figure 1**).
20. In case of protein overexpression, the transfection efficiency can be determined by calculating the fraction of double-labelled cells (count at least 50 randomly selected cells) (*see* **Note 37**).

4. Notes

1. Primary cells such as the patient skin fibroblasts used here are difficult to transfect, but high transfection rates have been achieved by the microporation protocol outlined in this Chapter (*see* **protocol 3.2**). If primary cells are not required (e.g. for determining localization of a candidate peroxisomal protein), a permanent, adherent cell line can be selected which is easy to transfect with plasmid DNA, provided it contains an elaborate peroxisomal compartment. As peroxisomes are very prominent in liver and kidney, permanent hepatocyte- or kidney-derived cell lines are suitable cell models. We have successfully used HepG2 cells (human hepatoblastoma cells, ATCC HB-8065) and COS-7 cells (African green monkey kidney fibroblast-like cells, ATCC CCL-70) for our studies ***(36, 37)***. They can be transfected by DEAE-Dextran ***(38)***, PEI ***(16)***, lipofection or electroporation ***(39, 40)***.
2. Plasmids designed for the generation of tagged fluorescent or non-fluorescent fusion proteins, or for mammalian expression of fluorescent peroxisomal marker proteins which target the peroxisomal matrix, are commercially available.
3. Follow the biosafety and GMO guidelines of your institution.
4. TrypLE™ Express (12604013, Gibco) is an animal origin-free, RT-stable, recombinant enzyme suitable for the dissociation of a wide range of adherent mammalian cells. It cleaves peptide bonds on the C-terminal sides of lysine and arginine, and is a direct replacement for trypsin. Its high purity increases specificity and reduces damage to cells that can be caused by other enzymes present in some trypsin extracts. Alternatively, Trypsin/EDTA solution (1x) can be used: 0.05% (w/v) trypsin, 0.68 mM EDTA, 5.5 mM glucose, 137.93 mM NaCl, 5.36 mM KCl, 6.9 mM $NaHCO_3$ (store at -20°C).
5. For sterilization, glass coverslips are put in a glass petri dish, wrapped in tin foil and dry-sterilized for 6 h at 180°C.

6. Prepare transfection-quality plasmid DNA with your method of choice. Plasmid DNA is diluted in sterile, deionized water (highly recommended for transfection by microporation).
7. We usually stir for 2-3 h at RT, but stirring overnight is sometimes recommended.
8. Alternatively, a fluorescent candidate protein can be co-expressed with a fluorescent peroxisomal marker protein and examined for colocalization by fixed or live cell imaging, omitting the immunofluorescence. The latter requires specific equipment (inverted microscope, heated microscope stage, temperature and CO_2 control, live cell imaging chamber to insert either cover slips or glass bottom dishes). A multitude of constructs encoding for peroxisome-targeted fluorescent fusion proteins are now commercially available (e.g. EGFP-, DsRed- or mCherry-tagged peroxisomal fusion proteins). EGFP (and its derivatives) bearing a peroxisomal targeting signal 1 (PTS1) composed of the amino acids SKL at the very C-terminus (EGFP-PTS1) is commonly used to label the peroxisomal matrix. Fluorescent proteins (either candidate fusions or marker proteins) can also be used in conjunction with immunofluorescence depending on the experimental set-up.
9. Alternatively, 5 mL/T-25 or 15 mL/T-75 cell culture flasks can be used.
10. Carefully check on an inverted microscope when the cells start to detach. You can gently tap against the side of the dish to improve detachment; however, tapping may stress sensitive cells. It is recommended to further detach cells by gently pipetting complete medium against the surface of the dish while resuspending the cells. Note that extended trypsinization decreases cell viability; the cells should only be exposed to the trypsinization solution until they start to detach.
11. FBS contains trypsin inhibitors, which inhibit trypsin activity to prevent further cell damage.
12. Regularly check that the cells are not contaminated with bacteria (culture medium quickly turns yellow (acidic) and cloudy!), yeast, fungi (microscopic evaluation) or mycoplasma. It is recommended to perform a mycoplasma test on a regular basis. Tests based on DNA-

staining (e.g., with DAPI or Hoechst H33258) can be inconclusive; PCR-based tests are more reliable. Always ensure that the cultured cells are healthy and actively growing (recommended confluency: 70-80%), as all the subsequent steps strongly depend on healthy cells and good cell culture practice.

13. Splitting the cells 24 h prior to transfection is important to avoid stress due to high cell density. Furthermore, cell counting on the day of transfection is not required anymore and will reduce the handling time of the cells during the transfection procedure.
14. The addition of antibiotics to the growth medium can drastically reduce the viability of freshly transfected cells.
15. Some cell lines do not attach or spread well on glass coverslips. To improve attachment and cell growth, coverslips can be coated with poly-L-lysine, collagen or an extract of extracellular matrix proteins (e.g., matrigel) ***(41)***.
16. Depending on the application, glass coverslips of different sizes (e.g., smaller to fit into multi-well plates) and forms (e.g. square ones) can be used. Smaller coverslips require less antibody solution. We prefer slightly bigger coverslips, which harbour more cells, and are thus well suited for morphological quantification of cellular (organelle) parameters.
17. Ensure that the glass coverslips do not float or overlap. The latter will result in areas without attached cells. Adding the coverslips before the medium helps to keep them at the bottom of the dish. Use sterile tweezers to adjust floating or overlapping coverslips.

18. A detailed instruction manual of how to use the Neon device can be downloaded from the supplier's website. (http://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFSAssets%2FLSG%2Fmanuals%2Fneon_device_man.pdf). Pulse conditions for other cell types need to be optimized.

19. We use 10% more R-buffer (110 µL instead 100 µL) to compensate for pipetting errors and to avoid aspiration of air.
20. Use conical 1.5 mL microcentrifuge tubes instead of round bottom 2.0 mL microcentrifuge tubes to avoid generation of air bubbles. The amount of plasmid DNA added is decreased to 5 µg when a fluorescently tagged protein is used.
21. In case of an electrical spark caused by air bubbles during the electrical pulse, discard the cell-containing tip and repeat the microporation using the back-up cells.
22. Do not swirl, as this will lead to accumulation of cells in the centre of the dish.
23. Based on our experience, a change of growth medium during incubation time is not required. Incubation times depend on the expression level and efficiency of peroxisomal targeting of the expressed protein of interest.
24. The Neon pipette tips and tubes can be regenerated and reused as described ***(42)***.
25. It is recommended to use small tags such as Myc or FLAG. However, larger fluorescent tags such as GFP can be detected by anti-GFP-specific antibodies to increase the fluorescent signal if required. Besides wild type proteins, mutated versions can also be used to gather information on specific protein functions as well as on targeting signals.
26. It is also possible that candidate proteins are dually targeted to peroxisomes (via a C-terminal PTS1) and mitochondria (via an N-terminal mitochondrial targeting signal). In cases where both peroxisomal and mitochondrial targeting information is predicted (using PTS1 predictor algorithms and Mitoprot2 or Predotar1.03), N- and C-terminally tagged versions can be expressed: N-terminal tagging should permit peroxisomal targeting (but inhibit mitochondrial localization), whereas C-terminal tagging should permit mitochondrial targeting (but inhibit peroxisomal localization).
27. Fixation with alcoholic fixatives only (e.g., ethanol or methanol) is not recommended, as it interferes with peroxisome morphology ***(43)***. Some primary antibodies, however, do not work after PFA fixation. In those cases, a combined PFA-methanol fixation can be

performed. Cells are first fixed with 4% PFA as indicated, washed 3 times with 1×PBS, and are afterwards post-fixed (and permeabilized) with absolute methanol (-20°C) for 5 minutes. A further permeabilization step can be omitted. In addition, denaturing methods to expose hidden antigens in PFA-fixed cultured cells can be applied ***(44)***.

28. Instead of 2.5 µg/mL digitonin, cells can be permeabilized with 0.2% Triton X-100 in 1×PBS for 10 min at RT. This, unlike digitonin, will permeabilize the peroxisomal membrane so is recommended for the localization of endogenous/overexpressed matrix proteins, or membrane proteins where the antibody epitope will be in the peroxisomal matrix. Expressed mammalian PEX11β is extracted from peroxisomal membranes of fixed cells during Triton X-100 permeabilization ***(45)***. When permeabilizing with digitonin, ensure that the peroxisomal marker protein is a membrane protein (here, anti-PEX14), and that the antibody for detection is directed against an epitope which is accessible on the cytosolic surface of the peroxisomal membrane ***(46)***.
29. A humid environment is necessary to prevent evaporation of reagents during incubations. Inexpensive humidity chambers can be generated by putting a moistened filter paper into the lid of a cell culture dish or multi-well plate.
30. Cells grown on coverslips can be incubated with antibodies cell-side up in uni- or multi-well plastic dishes. Alternatively, coverslips can be placed cell-side down on a drop of antibody solution applied on parafilm.
31. Antibody dilutions should be optimized using recommended concentrations as a guideline. A dilution series of all primary and secondary antibodies can be performed.
32. Primary antibody incubations can also be performed at 4°C overnight in a refrigerator or a cold room. In urgent cases, antibody incubations can be performed at 37°C for 30 min in an incubator. In both cases, evaporation of the antibody solution should be minimized (e.g. by covering with parafilm or by increasing the volume of the antibody solution applied).

33. At this step, cellular nuclei can be stained by incubating the cells with the cell-permeable blue fluorescent nucleic acid stains DAPI or Hoechst H33258. After washing the cells following the secondary antibody, incubate the sample for 1-3 min in DAPI working solution (dilute DAPI stock solution 1:1000 in 1×PBS). Aspirate the DAPI solution from the dish and wash the cells briefly 2-3 times with 1×PBS before proceeding. The excitation/emission wavelengths for DAPI are 360/460 nm.
34. If necessary, glass microscope slides can be cleaned with 70% ethanol, dried and cleaned with (lens) paper.
35. Excess MMM can be removed either by aspiration with a vacuum pump using a pasteur pipette or plastic pipetting tip or, less elegantly, by using a piece of filter paper. Do not leave MMM on top of the coverslip as it gets dry and interferes with microscopic observation.
36. Ensure that the MMM has dried and is no longer fluid before you add immersion oil and start microscopic analysis. Proper mounting in MMM (containing fresh n-propyl-gallate as antifading reagent) and proper storage at 4°C in the dark can protect the samples for several months or even years.
37. In general, transfection efficiencies between 70-90% are obtained.

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