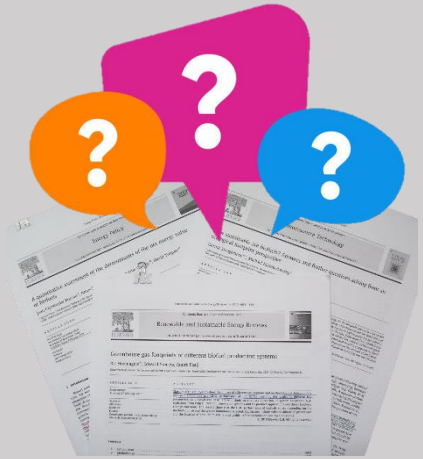
Data reporting in Light Microscopy:
A guide for good practices in Material & Method writing

Have you ever failed to reproduce an experiment that was done by others and was crucial to test your hypothesis?



You come to us with a publication and ask us to check if we can reproduce it. After reading it, it seems we do have the technical capacity to do it but we also realise that **we lack information to do it exactly in the same conditions**.

Science is currently facing a **challenge on reproducibility**.

Both researchers and publishers are demanding a **better description on the reagents used and the techniques performed**.

This is why SMOA publishes this guide, aiming to help you writing the materials and methods (M&M) section and reporting the technical specifications of your **light microscopy experiments**.

1. General information

Some information is **general** to every technique and **it should always** be included

Type of microscope

Manufacturer (Nikon, Zeiss, Leica, Evident), type (confocal, widefield, etc.), specific model and body type (inverted or upright).

Example: Images were acquired on a ZEISS LSM900 confocal vertical system.

Objectives

Manufacturer (Nikon, ZEISS, Leica, Evident), objective corrections (Plan, Apo, etc.), magnification, numerical aperture, working media (air, water, oil, glycerol, silicone).

** State if you use any **extra magnification lens**.

Example: A Nikon Plan Apo 60x 1.4 NA oil immersion objective was used.

Software version and file extension

Brand and version of the software used to acquire the image

Example: ZEN software (3.3 version) was used for the image acquisition.

2. Widefield microscopy

This is the critical information to include when using **widefield microscopy**

Illumination source

Type of source (Mercury lamp, LED), manufacturer, model and used excitation line and filters for each channel.

** You should also add here if your fluorescence has been acquired **simultaneously** OR **sequentially**.

Example: Fluorescence was** excited with a Cool LED pE-300 using green and red lines through 465-495 nm and 550-590 nm bandpass filters for Alexa 488 and Alexa 568 excitation, respectively.

Detection

Camera manufacturer (Hamamatsu, Andor, etc.), type (sCMOS, EMCCD, etc.), model, pixel size, resolution, fluorophores or FP and bandpass of emission filters used for each of them. Always important to include the final pixel size of your image (e.g. 0.15 µm/pixel).

** You should specify if **binning** has been performed (e.g. with no binning, applying a 2x2 binning).

Example: Emission was collected on a Hamamatsu Orca-Fusion sCMOS camera with 16-bit depth, 6.5 μm^2 pixel size and at 1024x1024 pixel resolution**. Alexa 488 and Alexa 568 fluorophore emission signals were collected through a 525-555 nm and a 608-683 nm emission filters, respectively. The final pixel size of the acquired image was 0.15 µm.

Other important data

If any cropping has been performed or any other camera setting has been applied (gains, speed modes, etc.)

If using brightfield, include any phase contrast that has been used (Phase, DIC, etc.)

3. Confocal microscopy

This is the critical information to include when using **confocal microscopy**

Illumination source

Type of laser (diodes, Argon, He-Ne, DPSS, etc.) and used excitation wavelengths.

Example: A 561 nm laser diode was used to excite Alexa 568 fluorophore signal.

Scanning parameters

Scanning mode (sequential or simultaneous), directionality (uni or bidirectional), type of scanner (galvano or resonant), scanning area (1024x1024, 512x512), pixel dwell or speed, averaging and zoom.

Example: Scanning was performed sequentially by unidirectional frame scanning using the galvanometer-based imaging mode with an area size of 1024x1024 and a pixel dwell of 2.4 µs. No averaging or zoom was further used.

Detection

Pinhole size (in A.U.) and for which wavelength was calculated, type of detector (PMT, GaAsP, HyD [S, X R]), fluorophore or FP and bandpass of emission filters used for each of them. Always important to include the final pixel size of your image (e.g. 0.15 µm/pixel).

** You should state if you operate in **photon counting** mode.

Example: For all images the pinhole size was 1.2 A.U. calculated for 647 nm emission. Emission signal of the Alexa 488 and Alexa 568 fluorophores was collected through a 525-555 nm and 608-683 nm emission filters, respectively and detected on GaAsP detectors. The final pixel size of the acquired image was 0.15 µm.

4. Spinning disk confocal microscopy

This case will be **a mix** of widefield and confocal reporting. Apart from the general information (type of microscope, objective, software version and file extensions), you will need to state:

-- Illumination (refer to point 3 “Confocal microscopy”)
-- Detection (refer to point 2 “Widefield microscopy”)
-- Other important data (refer to point 2 “Widefield microscopy”)

Besides, **you should also include:**

Spinning disk features

The manufacturer and model of the spinning disk unit used (Yokogawa CSU-X1, Yokogawa CSU-W1), the type of the disk (normal Nipkow or SoRa disk), size (50 um, 25 um), and any magnification lens if used (3.8x magnification lens, normally when SoRa disk is used).

Example: The system was equipped with a Yokogawa CSU-W1 spinning disk unit with a normal Nipkow disk (25 µm pinhole) and no intermediate magnification lens was used.

5. Lightsheet

We are again in a **mixed** case between widefield and confocal reporting. Apart from the general information (type of microscope, detection objectives, software version and file extensions), you will need to state:

-- Illumination (refer to point 3 “Confocal microscopy”)
-- Detection (refer to point 2 “Widefield microscopy”)
-- Other important data (refer to point 2 “Widefield microscopy”)

Besides, **you should also include:**

Illumination objectives

Apart from data on detection objectives, you should also give illumination objectives information including manufacturer (Nikon, ZEISS, Leica, Evident), objective corrections (Plan, Apo, etc.), magnification and numerical aperture.

Example: For the lightsheet illumination we used two ZEISS Plan Apo 10x 0,5 NA objectives

Lightsheet parameters

Lightsheet thickness and side illumination (single or double) should be mentioned.

Example: Lightsheet thickness was adjusted to 3 µm and images were acquired with double side illumination.

6. STED

Here you should report **the same settings as in confocal microscopy** but, apart from that, you should also include:

STED laser features

Depletion laser used (wavelength and intensity) and which fluorophore has been depleted with it.
Number of iterations (accumulations) are also important.

Example: To deplete Alexa 488 fluorophore, a 592 nm depletion laser was used with a 15% of intensity. We performed 10 iterations.

7. Other acquisition parameters

** If a combination of some of these, **state the order** (first tiles, then Z, first Z then tiles, etc.)

When we perform Z stacks, time-lapses, tile-scans, bleaching's or other specific acquisition settings we should also report those special details.

It should not be more difficult than **just explaining what have we have done** when acquiring in such a special scenario:

Tile scan

Motorized stage, number of fields, software used for the stitching, % overlapping and stitching mode.

Example: A fully motorized stage ** allowed XY control to search for an imaging field and collect adjacent frames for tile images. Tile scan was performed acquiring 3x3 fields upon automatic image stitching in nis-elements with a 15% overlap and via blending.

Time lapse

Time interval, duration and usage of any focus keeper (PFS, Definite focus).

Example: A timelapse was performed acquiring one image every 2s during 5 minutes using Zeiss Definite Focus for focus stabilization.

Z-stack

Focusing device, full range acquired and Z-step size

Example: A 5 µm range z stack was performed with a step size of 0.5 µm using a piezo Z-device.

7. Other acquisition parameters

When we perform Z stacks, time-lapses, tile-scans, bleaching's or other specific acquisition settings we should also report those special details.

It should not be more difficult than **just explaining what have we have done** when acquiring in such a special scenario:

Bleaching

Time of pre-bleaching, time or loops and laser used for bleaching and time recorded after bleaching.

Example: Sample was acquired every 10 second for 30 seconds before bleaching, then, bleaching was performed once for 1 second with a 561 diode. Finally, images were recorded during 2 minutes every 10 seconds for after bleaching recovery analysis.

Environmental conditions

Environmental chamber manufacturer and model and environmental conditions (temperature, CO2, humidity, perfusions)

Example: Sample was maintained at 37ºC and 5% CO_2 throughout the imaging using an Oko-Lab chamber and an Oko-lab touch pad.

8. Image analysis parameters

Once obtained, images should be analyced to extract results and conclusions. The processing steps we perform on the images to get them **must also be clearly reported to ensure full reproducibility**.

Pre-processing

Always state any pre-processing to prepare images for analysis (background subtraction, denoising, cropping, deconvolution, etc.) and segmentation parameters (threshold, object filtering, etc.). Also include the brand and version of the software used to analyze the image.

Please report all image **operations in the order** they have been applied, it may be relevant for mathematical reasons!

Example: Image background was first eliminated using subtract background option in Fiji with a rolling ball radius of 50. A Gaussian blur filter was then applied with a sigma of 2. Then, a binary mask was produced applying an Otsu threshold and objects were further filtered by circularity, keeping only those with a value higher than 0,5. Software used was Fiji/ImageJ (2.14.0/1.54f).

Analysis workflows

-- Established workflows: Cite the software, libraries and plugins used along any specific manual intervention (e.g. ROIs) or key specific parameters used (some plugins allow you to choose specific parameters).

-- New workflows: Workflow has to be defined step by step, citing or clearly explaining every individual component used to assemble the novel analysis pipeline. Developed workflow should be published as a code or pipeline. Any macros should be added as suplementary material.

-- AI workflows: If a pre-trained model has been used, it must be cited and clearly identified. If trained from scratch, you should describe the method and its creation steps and, at least, give access to it. Ideally, you would facilitate access to the training data.

9. Sample preparation parameters

It is as well crucial to correctly report **sample preparation parameters**. For that, sample collection, fixation, permeabilization or labelling parameters (concentrations, times, temperatures or other specificities) must be reported. This is slightly beyond our technical realm, but we would like to remind you about four elements that are essential for **optical microscopy samples**.

Sample preparation

- Fluorescent Proteins (FP): It is very important to state which FP are you using, small modifications or FP variants may impact a lot their signal, maturation, location etc. (eGFP, sfGFP, mGFPmut3 are all GFP-based proteins, but definitely not the same one!)

- Coverslips: We normally use 0.17 mm coverslips (we call them #1.5), but for superresolution we recommend #1.5H instead. These diferencies should be reported, so coverslip thikness or number should be included.

- Mounting media: It can affect your fluorophore signal, cause aberrations and alter your final resolution. Thus, please state which one have you used to mount your sample (Fluoromount-G, Vectashield, Mowiol, Prolong Glass etc.)

- Imaging media (for live cells): Please also report the medium in which you perform the imaging of your cells. It may well be that you dont need CO_2 as you are including HEPES, or you are correctly using phenol-red free DMEM or some antioxidants. Their names, working concentratons and any specificity should be reported.

10. Core facility acknowledgement

It is also important to **acknowledge your core facility**, not only because they have helped you and because this will help them to **maintain and expand funding and motivation**, but also for **simple transparency** and information (anyone can then ask your core facility to report on calibration, quality controls etc.).

Core facility acknowledgement

A simple sentence stating the name of the facility

Example: This work has been performed in the Advanced Light Microscopy Facility at the Centro de Biología Molecular Severo Ochoa (Madrid, Spain).

Where to find the information

Of course, the SMOA team can **advise you on or directly give you** the required information.

We are aware this is **a lot of information** but you have several ways to find it:

Image Metadata

Common commercial software (Nis-elements [Nikon], ZEN [ZEISS], LasX [Leica], Cell Sense [Olympus-Evident]) give the possibility to interrogate about your images metadata. Once acquired and opened in the correspondent software, you will probably have the option to get “image properties” (right click). In Fiji you can use the “show info” option as well.

SMOA technical information

Each of the SMOA systems has a “technical sheet” which includes the most important features of the system (name, filters, objectives etc.). If you know your main experimental conditions, this sheet will allow you to obtain some more specific technical data.

Other resources

Given the importance of correct microscopy data reporting, you also have useful tools that may help:

- MethodsJ helps you to write the material and methods section of a scientific article, by automatically inspecting the metadata of an image acquired on a scientific microscope and generating some boilerplate text you can use in the paper.

- MicCheck is an easily usable tool that allows you to introduce your microscopy experimental parameters and tells you what should be included in your material and methods section.

Want to read more about?

If you are becoming concerned about this issue and want to **read more about it**, here you have some of the most interesting papers in microscopy data reporting, where you can also **learn more tools** and **get deeper** into its important implications.

-- Marqués G, Pengo T, Sanders MA. **Imaging methods are vastly underreported in biomedical research**. Elife. 2020 Aug.

-- Foucs Editorial. **Minding microscopy metadata**. Nat Methods. 2021 Dec.

-- Montero Llopis P, Senft RA, Ross-Elliott TJ, Stephansky R, Keeley DP, Koshar P, Marqués G, Gao YS, Carlson BR, Pengo T, Sanders MA, Cameron LA, Itano MS. **Best practices and tools for reporting reproducible fluorescence microscopy methods**. Nat Methods. 2021 Dec.

-- Hammer M, Huisman M, Rigano A, Boehm U, Chambers JJ, Gaudreault N, North AJ, Pimentel JA, Sudar D, Bajcsy P, Brown CM, Corbett AD, Faklaris O, Lacoste J, Laude A, Nelson G, Nitschke R, Farzam F, Smith CS, Grunwald D, Strambio-De-Castillia C. **Towards community-driven metadata standards for light microscopy: tiered specifications extending the OME model**. Nat Methods. 2021 Dec.

-- Aaron J, Chew TL. **A guide to accurate reporting in digital image processing - can anyone reproduce your quantitative analysis?** J Cell Sci. 2021 Mar.

-- Heddleston JM, Aaron JS, Khuon S, Chew TL. **A guide to accurate reporting in digital image acquisition - can anyone replicate your microscopy data?** J Cell Sci. 2021 Mar.

We wish you the best of luck with your imaging and we hope
to see you around the SMOA!

Disclosure

We acknowledge that M&M sections are **often limited in many of the journals**, but we also realise that many of those journals offer (and sometimes even demand) a **supplementary M&M section** which is **way less limited**.

Is in this specific section where you should include all the relevant information we have just herein discussed.