Useful accessories for quantitative fluorescence microscopy
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In conventional fluorescence microscopy, there is often a need to perform quantitative measurements on images. Several practical difficulties are inevitable with widefield, i.e. camera-based measurements, particularly if one is interested in performing measurements over an extended period of time, or in comparing measurements from different instruments. In order to ensure that measurements are made in a consistent manner, we need to ensure that:

- The illumination intensity is consistent, i.e. 'flat' over the imaged area and constant over time.
- The sensitivity is constant and repeatable.

We describe here a several aids or procedures that have been found useful towards this.

Flat illumination
In all fluorescence microscopy, it is essential to ensure even or 'flat' illumination of the sample, but this is not easy in practice, whatever the microscope manufacturer's claims. While the modern mercury (Hg) or xenon (Xe) lamps are relatively easy to adjust (centering and focusing), an inevitable consequence of the high operating temperatures of these sources is that the adjustments will drift over time. A greater problem is caused by users – microscope users can be broadly divided into two classes: the group whose motto is:

"it's complicated and I won't fiddle with anything; good grief do you really expect me to read the manual?"

and the group with the mission:

"I am going to fiddle with every control until I get the image I want, it's definitely quicker than reading the manual".

Of course, reality is somewhere in between, but an inevitable consequence in a multi-user installation is that the microscope's light source will not be set up optimally. While observations by eye are rather more tolerant of uneven illumination, when a camera is coupled to the microscope, changes in intensity across the image become noticeable and totally unacceptable for quantitative measurements.

While there is no substitute for careful lamp alignment (particularly with those lamps fitted with a mirror behind the arc), image normalisation in software readily overcomes errors due to uneven illumination. The basic idea is straightforward and well-known:

Acquire an image with the illumination turned off, i.e. with a shutter in the excitation light path: (I_{BLACK}).
Acquire an image representing the illumination intensity profile: (I_{EXC}).
Acquire an image of the sample (I_{SAMPLE}).

The true excitation is thus represented by: $I_{EXC} - I_{BLACK}$
A normalised image can be derived from: $I_{NORM} = (I_{SAMPLE} - I_{BLACK}) / (I_{EXC} - I_{BLACK})$

It is of course necessary to perform this calculation using floating point arithmetic, since the pixels in the image I_{NORM} will assume values ranging from zero to one.

There are a few difficulties, however. The intensity of I_{SAMPLE} is unlikely to be comparable to that of I_{EXC} and how do we get I_{EXC} in the first place? What is needed is a 'perfect', evenly fluorescent reference sample, of comparable thickness to that of the actual sample slide. Any significant change in thickness will result in an incorrect representation of the intensity profile of the illumination source, depending on the depth of focus of the objective. Conventional epi-fluorescence microscopes do not reject out-of-focus light and if a very thick reference sample is used, an illumination profile that is 'flatter' than the actual profile will be generated.

We have found it convenient to use coverslips that have been spin-coated with photoresist (PMMA). This has very broad excitation and emission spectra and is easily excited with visible light. It emits at the red end of the visible spectrum and is thus well-matched to the usual silicon CCD cameras. We commonly use coverslips with a 10 or 30 micron thick coating; these are glued to a standard 25 x 75 glass slide (Figure 1). Such coverslips can be obtained at a modest charge from Agar Scientific Ltd.

It is inevitable that minor imperfections will be present in the PMMA coating, but since the there is a large area to choose from, 'active' areas of sub-mm to mm can be readily found. Irrespective of the quality of the coatings, dust and debris will accumulate and it is thus very useful to 'filter' the I_{EXC} image. Variations in excitation light intensity across the image are highly unlikely to result in the presence of sharp peaks but are likely to be rather smooth. Any sudden variations in intensity are likely to result from dust etc. and applying a median filter across I_{EXC} readily removes these. Examples of 'raw' and 'median-filtered' images and profiles are shown in Figure 2.

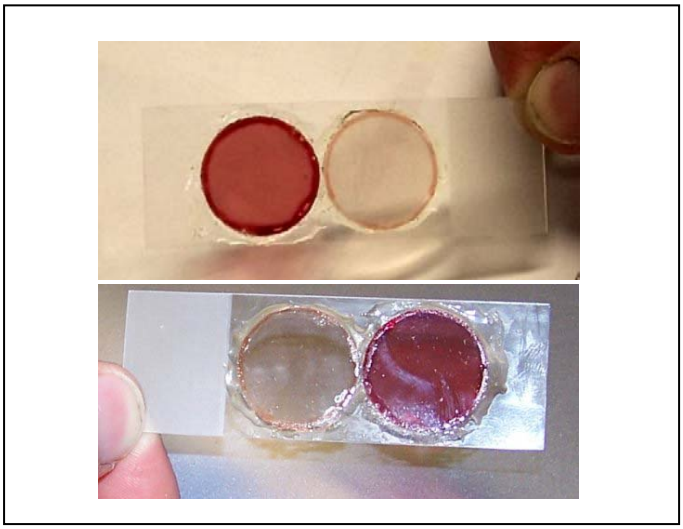


Figure 1: Examples of 10 and 30 micron PMMA-coated reference coverslips, glued to slides.

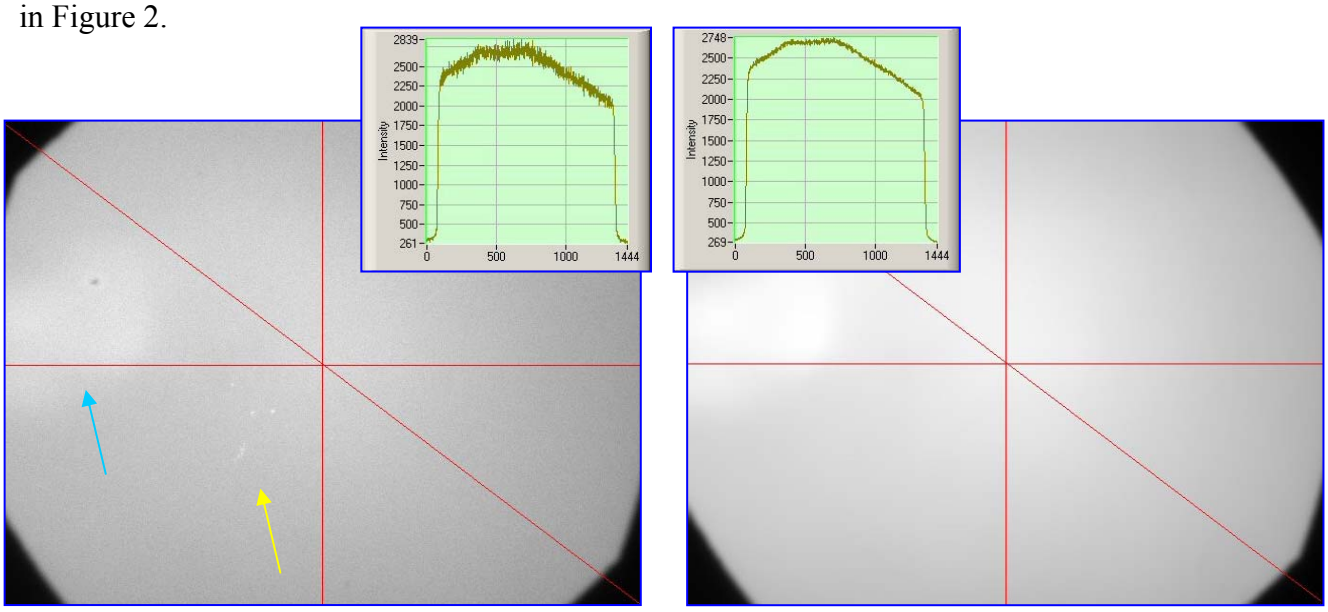


Figure 2: Raw (left panels) and filtered (right panels) images and line profiles, along red diagonal line, from a PMMA reference sample (10 micron thick), taken with a 10x 0.3na objective. Note how specs of dirt, as indicated by yellow arrow have been 'removed', while broad areas of uneven illumination, as indicated by the blue arrow, has been unaffected.

Now that we have obtained a 'perfect' image ($I_{EXC\text{-}MED\text{-}FILT}$ - I_{BLACK}), we have a representation of the true excitation profile, as viewed by the optical system which will acquire the image of the experimental sample, we can scale it to assume values ranging zero to one. The normalisation procedure can then be carried out on the experimental sample image. Results 'before' and 'after' are shown in Figure 3.

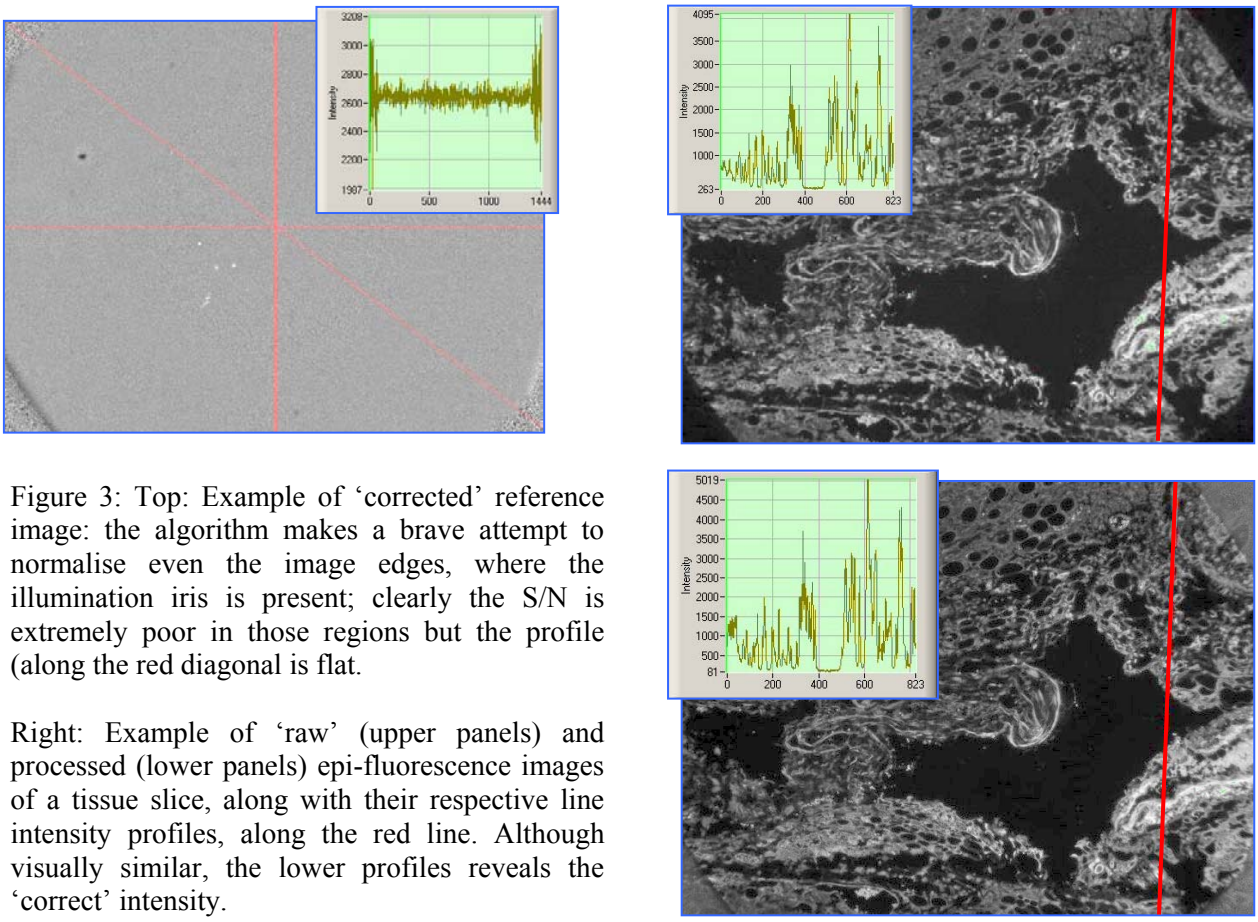


Figure 3: Top: Example of 'corrected' reference image: the algorithm makes a brave attempt to normalise even the image edges, where the illumination iris is present; clearly the S/N is extremely poor in those regions but the profile (along the red diagonal is flat.

Right: Example of 'raw' (upper panels) and processed (lower panels) epi-fluorescence images of a tissue slice, along with their respective line intensity profiles, along the red line. Although visually similar, the lower profiles reveals the 'correct' intensity.

Take care as I_{EXC} is likely to be different for different objectives and the image will be valid only for a particular objective. Although the illumination imperfections due the lamp misalignment or vignetting in the excitation path are nominally independent of objective, the rear apertures of objectives are not identical and it is thus best to repeat these procedures when objectives are changed.

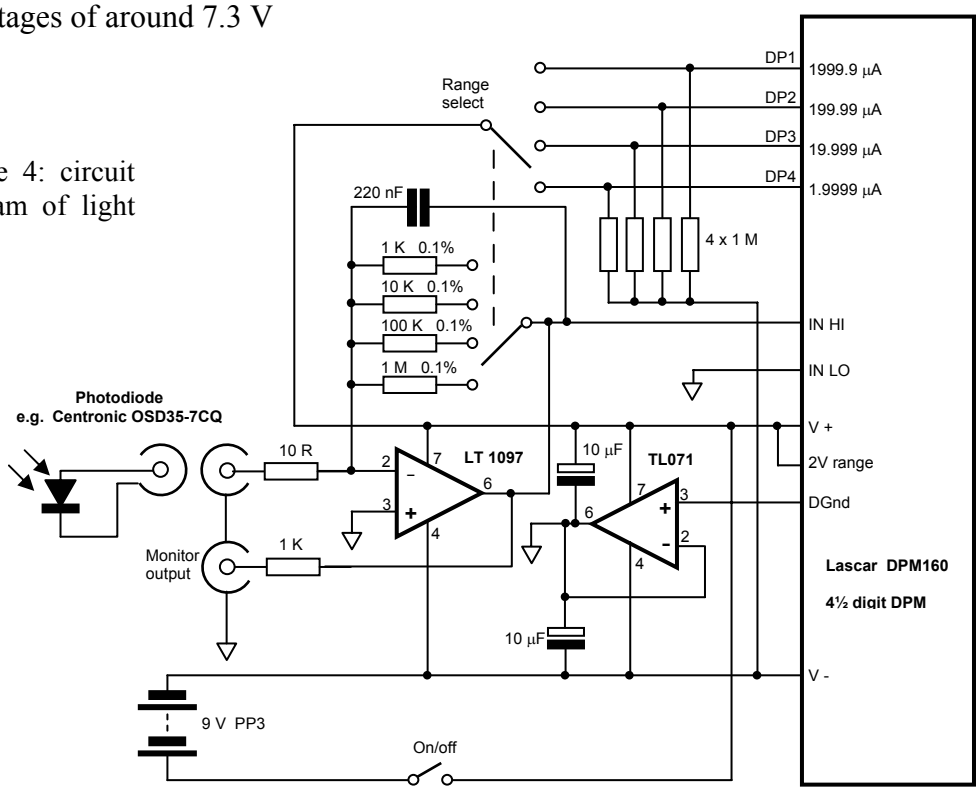
Determining illumination/excitation intensity

It is often necessary to set excitation intensities in a consistent manner, from day-to-day or between different microscopy systems. While absolute measurements (e.g. in terms of luminous flux or flux per unit area) are quite tricky to perform, a simpler approach, using relative measurements with a photometer are often more than adequate. In particular, it is useful to check that appropriate neutral density filters are fitted or that illumination apertures are correctly set.

There are numerous ways of constructing a 'light meter' and here we describe our particular approach, based on a simple generic design for battery-powered multi-range current meter. This was developed to display the output from a silicon photodiode, over f.s.d. ranges of 2 µA to 2 mA, on a 4.5 digit meter. The design (Figure 4) is based around a virtual-earth, current-to-voltage converter, with circuit features to allow a bipolar input capability while still using a single (+9V) supply. The photodiode (Centronic OSD35-7CQ) is presented with a low load by the LT1097 precision transimpedance amplifier, ensuring a linear response to light intensity.

Switched feedback resistors around the operational amplifier provide an output voltage of 2 V full-scale which is presented to a commonly available 4½ digit panel meter (Lascar DPM160). The voltage follower (TL071) is used to derive a 'split' supply from the floating PP3 battery. In our application, four input sensitivities cover the range of typical photodiode output currents, but feedback resistors can be varied to suit, bearing in mind that larger area diodes have leakage currents of the order of a small fractions of a nanoamp. For increased sensitivity, an FET amplifier is preferable in place of the LT1097 but input offset voltage trimming will then be required. We have found it convenient to use a single integrating capacitor (220 nF), in effect increasing the time constant up to 220 ms in the most sensitive range, which is most prone to interference from room lights. Current drain is around 5 mA and the circuit operates down to battery voltages of around 7.3 V

Figure 4: circuit diagram of light meter



The whole circuit is constructed in a small plastic box with the photodiode mounted within a brass plate (75 x 25 mm), backed with a thin fibreglass printed circuit board. This system is shown in Figure 5. With the particular photodiode used, approximate responsivities at different wavelengths are presented below:

Figure 5: The completed current detector

Typical detector sensitivities

0.12 A/W @190 nm
0.14 A/W @ 254 nm
0.15 A/W @ 340 nm
0.20 A/W @ 400 nm
0.27 A/W @ 500 nm
0.32 A/W @ 600 nm
0.37 A/W @ 700 nm
0.42 A/W @ 800 nm
0.46 A/W @ 900 nm

Determining detection sensitivity

A related problem in fluorescence microscopy is the standardisation of the sensitivity of image acquisition devices. To characterise this fully, over the complete spectral range is of course time-consuming and difficult if absolute measures are required; however once again, relative measurements are readily performed using a surface-mount light emitting diode (LED) behind a pinhole. All we then need to do is to pass a stable current through the LED, the emission wavelength of which is chosen to suit the particular experiment. A generic design for a wide-range, calibrated current source is presented here to power light-emitting diodes. The design (Figure 6) is based around a voltage-to-current converter, where the high collector impedance of a transistor is used to provide the 'current' output. The operational amplifier is used to regulate the set current, which can be varied over a 20 μA to 20 mA f.s.d. range and monitored on a 3.5 digit DVM. A simple 'gating' circuit is also incorporated and is useful for checking camera synchronisation timings in triggered acquisitions, in conjunction with an external pulse generator.

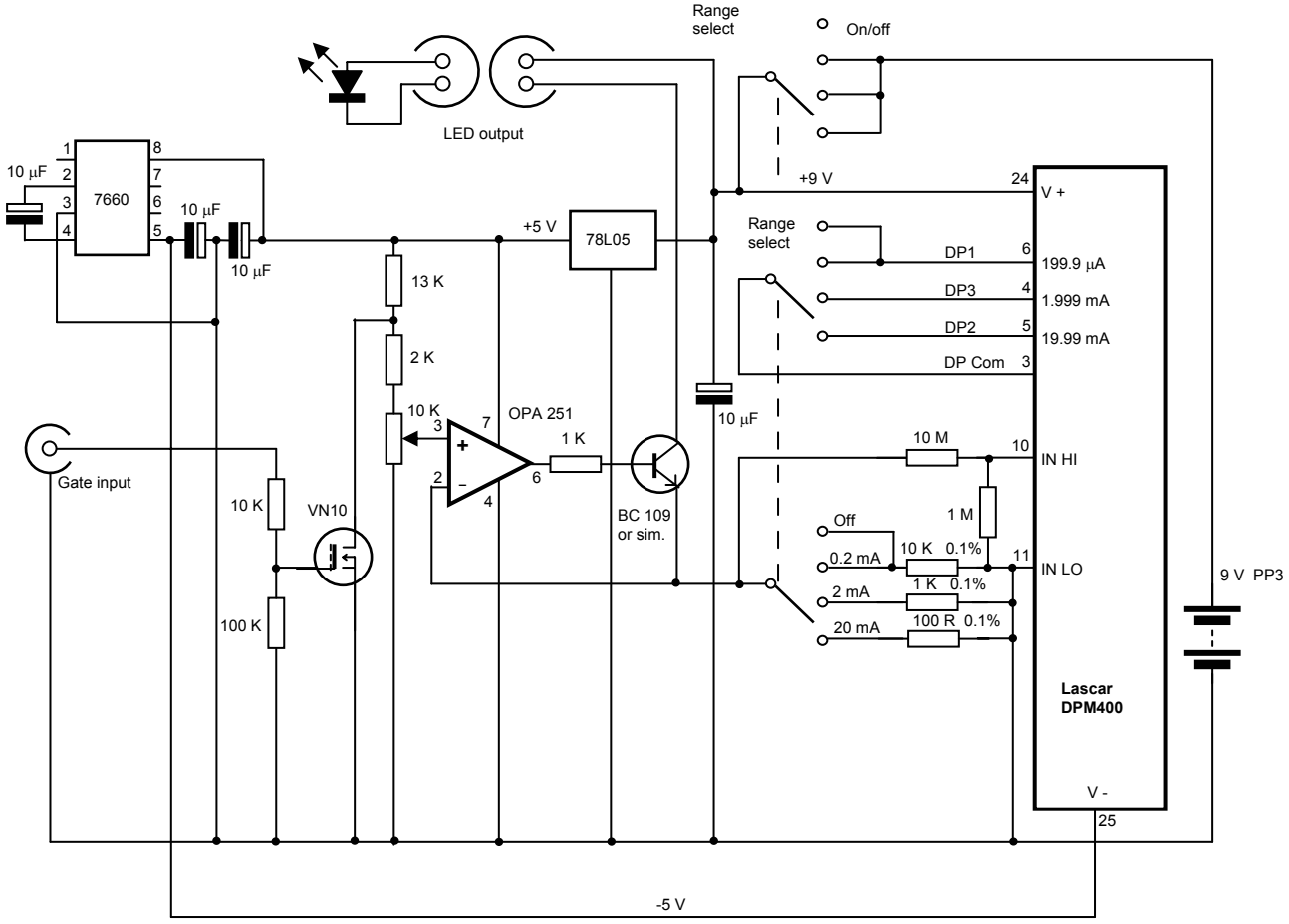


Figure 6: A simple variable current source is used to pass a known current through an LED; the current magnitude, derived from the voltage drop across the source emitter resistor, is read on a 3.5 digit DVM.

A range of 16 mm mounted pinholes is used, in turn mounted in brass plates 75 x 25 mm. Circularly-mounted 2-10 μm diameter pinholes are readily available from a number of optical suppliers; we use devices from Comar Instruments, as listed in the parts list.

The particular DPM used does require a negative supply, provided by the 7660 switched capacitor inverter, but other DPMs are available which do not, thereby simplifying the design; we just happened to have the particular units to hand. A 3 pole - 4 way switch switches emitter

resistors for the different ranges and provides the on-off function. Battery current drain clearly depends on LED current, and a battery charge state is indicated on the meter.

Figure 7: The completed current source.

This light source should not be considered as being equivalent to a true 'point' source and used for point-spread-function measurements for example. Nevertheless, it is useful to determine the imaging performance of the microscope (Figure 8) and definitely very useful as an aid to align cameras to the microscope's output port: by stage-moving the source left-right within the 'scope, i.e. camera field of view, the camera can be quickly rotated to ensure orthogonality with the stage.

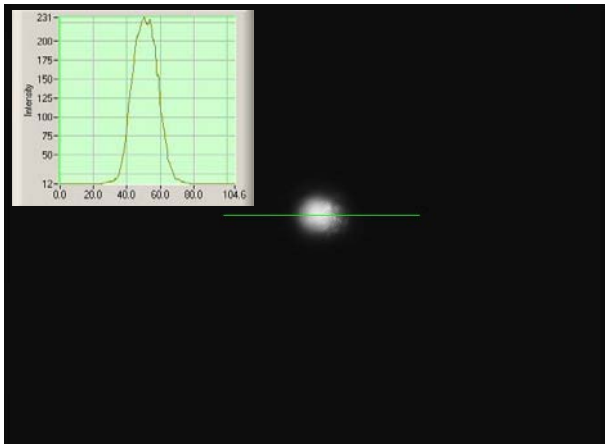


Image of 20 micron pinhole source and intensity line profile.
Image acquired with 40x, 0.7 na objective.

Figure 8: Typical image and profile from the source.

Specialist suppliers

Agar Scientific Ltd
66A, Cambridge Road
Stanstead, Essex, CM24 8DA
Tel: 01279-813-519, 01279-8134-919
Fax: 01279-815-106
http://www.agarscientific.com/

Comar Instruments
70, Hartington Grove.
Cambridge, CB1 4UH,
Tel: 01223 245470

Lascar Electronics Ltd.
Module House, Whiteparish, Wiltshire, SP5 2SJ
Tel: + 44 1794 884 567
Fax: + 44 1794 884 616
http://www.lascarelectronics.com/

For those interested in duplicating the systems, here are lists of parts used:

Components used in photodiode current meter

Amplifier / display unit

DPM160 / Lascar		Farnell	178-651	1 off
Case black		RS	262-6424	1 off
3P 4W switch	T&B MRJE3-4FN	Farnell	958-761	1 off
Control knob:	Nut cover	Farnell	320-766	1 off
	Knob	Farnell	320-626	1 off
	Cap	Farnell	320-808	1 off
On-off switch		Farnell	986-124	1 off
LTC 1097CN8		Farnell	295-966	1 off
TL071		Farnell	400-646	1 off
SMB pcb r/a socket		Rapid	16-1508	1 off
Battery clip		Rapid	18-0092	1 off
1 K 0.1%		Rapid	63-1236	1 off
10 K 0.1 %		Rapid	63-1448	1 off
100 K 0.1%		Rapid	63-1658	1 off
1 M 0.1 %		Rapid	63-1756	1 off
1 M 1%		Rapid	62-2742	3 off
10 R 1%		Rapid	62-2502	1 off
1 K 1%		Rapid	62-2598	1 off
220 nF ceramic		Rapid	08-0280	1 off
10 µF tantalum		Rapid	11-1022	2 off
Printed circuit board	in house n/a		n/a	

Detector 'slide'

Brass 'slide' – 75 x 25 mm	in house	n/a	1 off
Slide cover	1.6 mm PCB		1 off
Input cable RG178 / 1.5 m	Farnell	149-071	1 off per detector
Cable connector RG178 BNC	RS	304-5242	1 off
Photodiode Centronic OSD35-7CQ	RS	564-037	1 off
33·6mm^2 active area			

Total cost < £150

Components used in LED current source unit

Case grey		RS	262-6430	1 off
3P 4W switch	T&B MRJE3-4FN	Farnell	958-761	1 off
Control knob:	Nut cover	Farnell	320-766	2 off
	Knob	Farnell	320-626	2 off
	Cap	Farnell	320-808	2 off
10 K pot. Spectrol 192JB-103		Farnell	328-2624	1 off
OPA 251 op-amp		Farnell	102-260	1 off
ICL7660SCPA switched cap. dc-dc		Farnell	408-566	1 off
DPM400/ Lascar		Farnell	175-944	1 off
3 pin output socket		Farnell	150-605	1 off
SMB panel socket		Rapid	16-1506	1 off
10 µF/16V electrolytic		Rapid	11-0825	4 off
100 R 1%		Rapid	64-0440	1 off
1K 1%		Rapid	64-0500	2 off
10 K 1%		Rapid	64-0560	2 off
100 K 1%		Rapid	64-0620	1 off
10M 1%		Rapid	62-7638	1 off
1 M 1%		Rapid	64-0680	1 off
Battery clip		Rapid	18-0092	1 off
Spacers	in house 5 mm long			4 off
Printed circuit board	in house n/a	n/a		1 off

LED/pinhole assembly

3 pin output plug		Farnell	150-496	1 off per LED
Output cable RG178 / 1.5 m		Farnell	149-071	1 off per LED
LED red/surface mount		RS	307-6533	1 off
Brass 'slide' – 75 x 25 mm		in house	n/a	1 off
Slide cover		1.6 mm PCB		1 off
10 micron pinhole		Comar	10 HL 16	1 off OR
5 micron pinhole		Comar	05 HL 16	1 off OR
2 micron pinhole		Comar	02 HL 16	1 off OR

Total cost < £150