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**Microscopes — Definition and  
measurement of illumination  
properties —**

Part 3:

**Incident light fluorescence microscopy  
with incoherent light sources**

*Microscopes — Définition et mesurage des propriétés d'éclairage —*

*Partie 3: Microscopie par fluorescence à lumière incidente avec  
sources lumineuses incohérentes*

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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by Technical Committee ISO/TC 172 *Optics and photonics*, Subcommittee SC 5, *Microscopes and endoscopes*.

A list of all parts in the ISO 19056 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

## Introduction

This document defines important quantities and methods to assess the quality of incident light fluorescence illumination for microscopes as well as methods to measure them. Details covered will enable users to compare different microscopes quantitatively with respect to their fluorescence illumination prior to a purchase decision and choose an appropriate instrument according to their application. Data obtained by following these methods also enables users to compare existing instruments with each other or to track performance variations over time, assuring proper performance. Additionally, what information is to be provided to the user is covered.

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# Microscopes — Definition and measurement of illumination properties —

## Part 3: Incident light fluorescence microscopy with incoherent light sources

### 1 Scope

This document specifies procedures for the measurement of illumination brightness, temporal stability and uniformity for incident light fluorescence microscopy. The measurement for uniformity is defined in image planes or intermediate image planes only, when these planes are suitable for detection by electronic imaging devices.

This document defines how illumination brightness, temporal stability and uniformity are measured, and how this information is provided to the user.

**NOTE** The scope is intentionally limited to electronic imaging devices and (intermediate) image planes. The visual observation by means of eyepieces would require a different measurement procedure and hence result in ambiguities in the description of measurement procedures. Nevertheless, this document will give useful estimates for the uniformity with visual observation as in this case an eyepiece is used to observe an intermediate image plane (which is under the scope of this document).

### 2 Normative references

There are no normative references in this document.

### 3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

### 4 Measurands

#### 4.1 General

For obtaining the desired image quality, when using a light microscope, the brightness, temporal stability and uniformity of the image play an important role. This holds true for different applications and various types of instruments. The measurement of illumination brightness and uniformity is mandatory. The temporal stability is an optional measurement because it is only suitable for certain types of light sources (e.g. arc lamps).

#### 4.2 Illumination brightness

The illumination shall be bright enough to allow the detection of the details of the object under investigation.

As this document is based on measurement procedures, the image brightness shall be expressed in the corresponding SI unit. The radiometric unit irradiance shall be used because it is well suited for the essentially monochromatic or narrow-band illumination spectral range used in fluorescence microscopy.

The value is expressed as given by [Formula \(1\)](#):

$$E = \frac{P}{A} \tag{1}$$

where

$E$  is the irradiance averaged over the area of the diaphragm used in W/mm<sup>2</sup>;

$P$  is the radiant flux recorded by the power meter in W;

$A$  is the area of diaphragm used in mm<sup>2</sup>.

### 4.3 Temporal stability

#### 4.3.1 General

The temporal stability of the radiant flux is an important factor when conducting experiments in a time series. If experimental results are to be derived from variations in the image brightness, any variation in illumination brightness due to temporal instability shall not contribute to these results.

The temporal stability shall be expressed for at least one of two different time scales.

#### 4.3.2 Short-term stability of radiant flux

The radiant flux shall be recorded for a time interval of 5 min. During this time interval the detector of the power meter is illuminated continuously and a power reading is recorded every second. The short-term stability of radiant flux,  $S_{\text{short}}$ , is expressed as a percentage by [Formula \(2\)](#):

$$S_{\text{short}} = 100 \times \left( 1 - \frac{P_{\text{max}} - P_{\text{min}}}{P_{\text{max}} + P_{\text{min}}} \right) \tag{2}$$

where

$S_{\text{short}}$  is the short term stability in percent;

$P_{\text{max}}$  is the maximum radiant flux recorded during the time interval of 5 min;

$P_{\text{min}}$  is the minimum radiant flux recorded during the time interval of 5 min.

Care should be taken that the light source is permanently on during the complete time interval of 5 min, because some light sources are only switched on during actual image acquisition.

#### 4.3.3 Long term stability of radiant flux

The radiant flux shall be recorded for a time interval of 120 min. During this time interval the detector of the power meter is illuminated every 30 s for one second. During the one second illumination of the detector a power reading is recorded. The long term stability of radiant flux,  $S_{\text{long}}$ , is expressed as a percentage by the [Formula \(3\)](#):

$$S_{\text{long}} = 100 \times \left( 1 - \frac{P_{\text{max}} - P_{\text{min}}}{P_{\text{max}} + P_{\text{min}}} \right) \tag{3}$$

where

$S_{\text{long}}$  is the long term stability in percent;

$P_{\text{max}}$  is the maximum radiant flux recorded during the time interval of 120 min;

$P_{\text{min}}$  is the minimum radiant flux recorded during the time interval of 120 min.

#### 4.4 Uniformity

The microscope's optical system shall achieve a certain degree of image uniformity in order to allow the detection of the details of the object under investigation. A severe drop of the image brightness towards the edge of the image field can result in brightness values that are not sufficient in the above mentioned condition.

Furthermore, spatial variations of the image brightness over the image field may not always be distinguished from spatial variations of the properties of the object under investigation.

The uniformity of the brightness,  $U$ , in the image field is expressed as a percentage by [Formula \(4\)](#):

$$U = 100 \times \frac{B_{\text{IF,min}}}{B_{\text{IF,max}}} \quad (4)$$

Where

$U$  is the uniformity of brightness in the image field, in percent;

$B_{\text{IF,min}}$  is the minimum brightness in image field;

$B_{\text{IF,max}}$  is the maximum brightness in image field.

NOTE Depending on the size of the image field, the objective magnification and the size of the sensor pixels, it might be necessary to apply a suitable averaging method in the computation of the uniformity.

## 5 Measurement procedure

### 5.1 General

In addition to defining the measurement geometry and procedure it is necessary to describe essential settings of the microscope in order to eliminate their influence on the measurement of illumination brightness, temporal stability and uniformity.

### 5.2 Microscope settings

The microscope shall be set up in the desired configuration regarding excitation wavelength (band), dichroic filters and/or emission filters.

If the microscope offers an adjustable field stop, it shall be set to the smallest setting for which the field of view of the imaging sensor in [5.5](#) is fully illuminated.

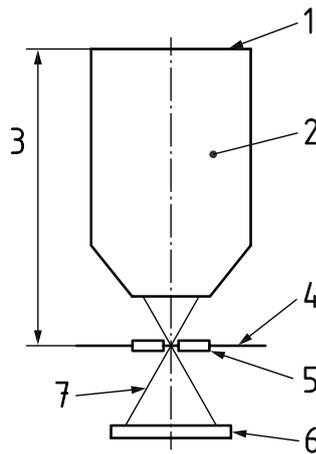
If the microscope offers an adjustable aperture stop, it shall be set to the smallest setting for which the pupil of the objective lens used is overfilled.

For the measurement of illumination brightness and temporal stability the illumination shall be warmed-up according to the manufacturer's specifications. If the manufacturer does not specify a warm-up time, the illumination shall be warmed-up for at least one hour prior to measurement. The light output of the illumination shall be set to its maximum value and, if a filter is used for extinction during measurement, this filter should be specified.

### 5.3 Illumination brightness

The measurement is performed with a 10× objective and a calibrated power meter placed so that the detection area of the power meter is slightly underfilled with light. The detection range of the power meter shall be in the linear range. The detector of the power meter shall not be saturated. The integration time of the power meter shall be between 0,2 s and 1 s.

The focal plane of the objective shall include a circular, centred diaphragm of  $(0,4 \pm 0,1)$  mm in diameter. [Figure 1](#) shows the position of the power meter during the measurement.



#### Key

- 1 objective-locating surface of the nosepiece
- 2 10× objective
- 3 parfocalizing distance of the objective
- 4 focal plane
- 5 circular diaphragm
- 6 detection area of power meter
- 7 marginal ray

**Figure 1 — Position of power meter during measurement**

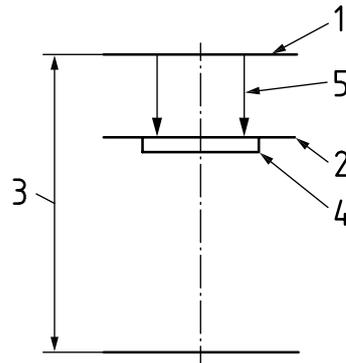
NOTE 1 Because the actual measured value of the area of the diaphragm enters the calculation for illumination brightness in [4.2](#) the diameter need not have a tight tolerance.

NOTE 2 If no diaphragm is used, the measured value of radiant flux can still be used to compare the same instrument at different points in time.

### 5.4 Temporal stability

The measurement is performed with a 10× objective and a power meter placed so that the detection area of the power meter is underfilled with light. The detection range of the power meter shall be in the linear range. If a 10× objective is not available, the measurement shall be performed without an objective and the power meter shall be placed at the position of the pupil of the illumination, thus the objective needs to be removed. The power meter shall be mounted in a fixed position with regard to the objective or the objective nosepiece respectively. External influences on the measurement, e.g. changes in room temperature or changes in the ambient lighting conditions, have to be eliminated. [Figure 2](#) shows an alternative position of the power meter when used without objective.

The detector of the power meter shall not be saturated. The integration time of the power meter shall be between 0,2 s and 1 s.

**Key**

- 1 objective-locating surface of the nosepiece
- 2 pupil position of illumination
- 3 parfocalizing distance
- 4 detection area of power meter
- 5 marginal ray of illumination (arrow indicates propagation direction)

**Figure 2 — Alternative position of power meter without objective**

## 5.5 Uniformity

The measurement for uniformity shall be performed by using an electronic image sensor that can capture the specified image field. This electronic image sensor shall consist of at least 50 rows and columns of individual elements (pixels). Furthermore, the image sensor shall be designed for an entrance pupil at infinity.

If the mechanical design of the microscope does not allow the image sensor to be placed in the plane of the image field, no auxiliary optics that would be part of the measurement setup shall be used. In this case an uniformity value cannot be measured according to this document.

This image sensor does not need to be calibrated in radiometric units as its output can be related to the brightness measurement described in 4.2. However, gamma correction shall not be applied to the signal from the image sensor in order to retain a linear relationship between irradiance and sensor signal (hence  $\gamma = 1$ ).

The specimen shall be a homogeneous fluorescent specimen, e.g. a solution of a fluorescent marker or a broadband fluorescent polymer slide. Solutions of markers are generally preferable over solid samples, since bleached fluorophores in the object field are replaced by unbleached fluorophores due to diffusion.

While performing the measurement, it shall be ensured that the complete data recorded originates from the homogeneous fluorescent region of the specimen to avoid misjudgement, which might be caused, e.g. by field of curvature of the optics or a tilted specimen. The signal-to-noise ratio shall be sufficient to clearly determine the uniformity. The signal-to-noise ratio is considered sufficient if the uniformity varies less than 5 percentage points when the illumination brightness is increased by a factor of at least 1,5.

[Table 1](#) shows examples for measurements, where signal-to-noise ratio is sufficient for determination of uniformity.

**Table 1 — Example of uniformity measurements**

<b>Illumination brightness</b> arbitrary units, e.g. "%" or "W/mm <sup>2</sup> "	<b>Uniformity</b> <i>U</i> %	<b>signal-to-noise ratio sufficient for determination of uniformity</b>
10	65	—
15	72	no
22,5	75	yes
33,75	76	yes

**5.6 Spectral information**

The illumination brightness and uniformity shall be referred to a specific spectral range. The spectral range shall be given as the wavelength of maximum brightness together with the two wavelengths at which the brightness is 50 % of the maximum value. If the brightness value of 50 % is achieved for more than two wavelengths, all spectral ranges shall be indicated. This spectral information can be measured in the focus plane of the objective or it can be calculated from theoretical values of the individual components (light source, filters, transmission of optical components). Examples are given in [Annex A](#).

**6 Information provided to the user**

If information on illumination properties is provided to the user, information on illumination brightness and uniformity are mandatory; information on temporal stability is optional. Information shall be given according to the definitions given in [4.2](#), [4.3](#) and [4.4](#) and shall contain the following additional data:

- a) information on the dimensions and shape of the image field;
- b) information on the microscope configuration, especially additional optical elements (like filters, objective lens, etc.) that can be exchanged by the user but were part of the light train when measuring the illumination brightness;
- c) information on the illuminating light source type used and its relevant parameters (spectral properties, voltage or current, etc.);
- d) microscope setting of the field and aperture diaphragm;
- e) information on the spectral range that the measured illumination brightness and uniformity are reported in;

The spectral range should include at least one of the listed wavelength ranges:

- (385 ± 25) nm;
- (475 ± 25) nm;
- (550 ± 30) nm;

NOTE The listed wavelength ranges may include more or other wavelength ranges than indicated here.

Examples of information provided to the user are given in [Annex A](#).