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Nanotechnologies — Performance evaluation requirements for quantifying biomolecules using fluorescent nanoparticles in immunohistochemistry

Nanotechnologies — Exigences d'évaluation des performances pour la quantification de biomolécules en immunohistochimie à l'aide de nanoparticules fluorescentes

PROOF/ÉPREUVE

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

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.

Introduction

Fluorescent nanoparticles are expanding their market into bio-technological and research fields as a labelling material to be used for immunohistochemical staining.

Conventionally, various fluorescent dyes, including FITC (fluorescent isothiocyanate), rhodamine-isothiocyanate and sulforhodamine 101 acid chloride, have been used for immunohistochemical staining. They are still powerful tools for identifying localization of target biomolecules, for example, proteins and sugar chains, mainly for qualitative analyses. They are also applied to quantitative analysis in combination with various algorithms for calculating signal intensity related to the quantity of the target biomolecules. The quantification system generally consists of sample preparation, staining, microscopic observation and photography, and image processing for obtaining quantification results as shown in Figure 1. For reliable measurement results of quantification, fluorescent dyes that are brighter and more photostable by exposure to the excitation light are more appropriate.

Large number of fluorescent nanoparticles are available in the market. Generally, they show higher brightness and are more resistant to photobleaching, compared to the conventional fluorescent dyes. The characteristics of fluorescent nanoparticles can be an advantage for the quantification of target biomolecules by immunohistochemical methods also combining with the same algorithm employed for the quantification with conventional fluorescent dyes.[1][2][3][4]

In this context, various staining kits with fluorescent nanoparticles and various quantification systems have been developed and are available in the market.[5] Thus, the needs to realise the compatibility of various systems are expanding in the research and industrial fields.

In this document the minimum requirements for performance evaluation of products and application using fluorescent nanoparticles is addressed. This document provides information ensuring the comparability of the results of relative quantification by using fluorescent nanoparticles.

This document does not provide industry segment specific performance criteria for the workflow of measuring biomolecules. When applicable, users can also additionally consult existing industry specific standards.

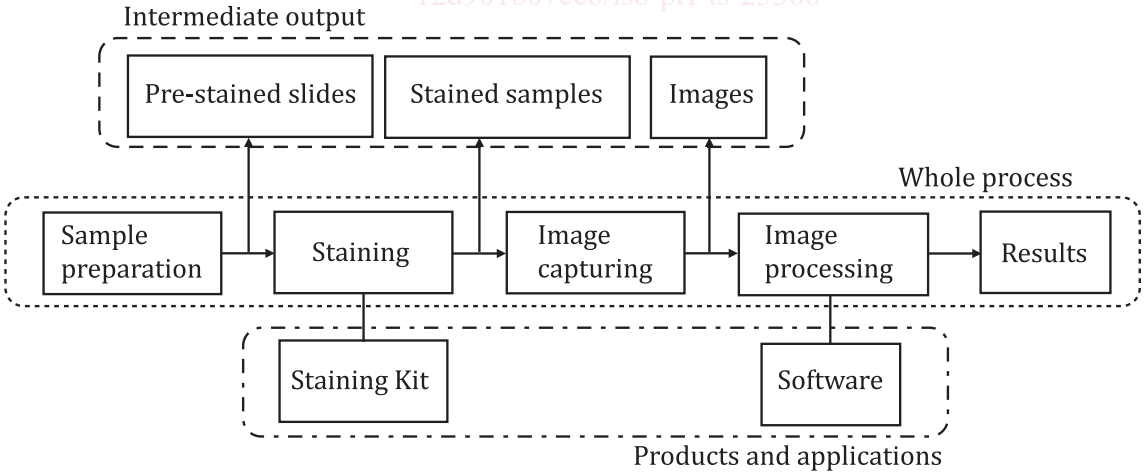

Figure 1 — Target quantification process by using of fluorescent nanoparticles

Nanotechnologies — Performance evaluation requirements for quantifying biomolecules using fluorescent nanoparticles in immunohistochemistry

1 Scope

This document describes minimum requirements for performance evaluation of applying fluorescent nanoparticles in quantitative immuno-histochemistry.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology 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/

3.1
fluorescent nanoparticle
nanoparticle emitting fluorescence excited by light of specific wavelength

3.2
cell block array
paraffin block re-embedded with plural cylinders gouged out from paraffin embedded cell suspension for histopathology

3.3
cell block array section
thin slice of cell block array obtained by cutting cell block array using microtome, and mounted onto a glass slide

3.4
agglomerate
collection of weakly or medium strongly bound particles where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2022, 3.1.2]

3.5
agglomeration
process through which agglomerates form

3.6
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example, covalent or ionic bonds, or those resulting from sintering or complex physical entanglement.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2022, 3.1.3, modified — Note 1 to entry has been adapted.]

3.7
aggregation
process through which aggregates form

3.8
quantum yield
number of quanta emitted per quantum absorbed

[SOURCE: ISO 22493:2014, 6.6.9]

3.9
molar extinction coefficient
optical density of 1 M fluorescent substance per 1 cm of optical path of absorption cell

3.10
photobleaching
destruction of fluorescing properties of molecules by light, resulting in reduced fluorescence of the sample

[SOURCE: ISO 10934:2020, 3.2.31]

4 Principle

Immunofluorescence methods are based on staining of thin tissue sections with specific antibodies recognizing intended molecules, e.g. proteins and sugars. The specific antibodies are prepared by immunizing animals, e.g. goat, rabbit or mouse, with target molecules. While monoclonal and polyclonal antibodies are used for immunofluorescence methods, antibodies need to be labelled with fluorescent substances, e.g. dyes or nanoparticles. For investigating localization and quantity of the target molecules, tissue sections are stained with fluorescence labelled antibodies. There are two basic methods for staining with labelled antibodies. One is the direct immunofluorescence method, the other is an indirect immunofluorescence method. The direct immunofluorescence method is performed by using a single fluorescence-labelled antibody recognizing the target molecules. The indirect immunofluorescence method is performed with a non-labelled antibody recognizing the target molecules (first antibody) and antibody recognizing the first antibody (second antibody). When the first antibody (e.g. IgG) is produced in mouse, anti-mouse IgG antibody should be selected for the second antibody. In addition, other molecular systems enhancing fluorescence signal are also available in market. For example, the avidin-biotin system is well known for this application. In the avidin-biotin system, the second antibody can be labelled with biotin and reacted with avidin conjugated with fluorescent materials including nanoparticles, e.g. quantum dots (QD), i.e. semiconductor particles with a few nanometres in size. The first antibody-second antibody-avidin-QD complex emits a strong fluorescence signal with less photo bleaching. It is preferred for the quantification of the target molecules by using nanoparticles including QD.

Fluorescence signal from the stained specimen is measured by fluorescence microscopy, which is an optical imaging technique that detects simultaneously the emitted fluorescence from the field of view using a camera. Fluorescence intensity, namely the emitted fluorescence, can be measured as an intensity value in fluorescence microscopy, which is computed by summing together the intensity

values from a group of individual pixels in a digital image acquired using a digital camera. In addition to the measurement of integrated fluorescence intensity, counting bright spots is used to quantify the target biomolecules.

Quantitative comparison of the intensity data or the numbers of the bright spots requires sound experimental design and appropriate operation of the whole quantitative photometric system including a digital camera e.g. a charge coupled device (CCD) or a scientific complementary metal oxide semiconductor (sCMOS). Issues of the quantitative comparison of intensity data involving the digital camera and controller software settings, including collection of dark count images to estimate the offset, flat-field correction, background correction, benchmarking of the excitation lamp and the fluorescent collection optics are described in Reference [15].

For quantitative analysis by immune-fluorescence microscopy, fluorescence intensity or the number of bright spots can be compared to the measured value by enzyme-linked immuno-sorbent assay (ELISA) or fluorescence activated cell sorter (FACS) and a calibration curve drawn (see A.3). Fluorescence microscopy measures the fluorescence intensity or the number of bright spots from immune-stained thin slice of tissues and cells, however, it does not measure the biomolecule number per cell. It measures the fluorescence intensity or the number of bright spots from a slice of a population of the cells although it is corelated to the number of biomolecules in cells when fluorescence intensity or the number of the bright spots is measured from a sufficient number of the cells in a field of view. In this sense, microscopic measurement of fluorescence intensity or the number of bright spots is a relative measurement.

Fluorescence intensity does not in itself have an associated SI unit, because it is a relative measurement. The number of the bright spots has “unit one” but it is also a relative measurement in principle. A relative intensity measurement (RIM) is determined as the ratio of one intensity measurement or bright spot count to another. The fluorescence intensity measurement is an accurate estimate of the ratio of the irradiance from part or all of a specimen, to the irradiance from part or all of the same or another specimen. For the counting of bright spots, the results can be interpreted in a similar way with the integrated fluorescence intensity measurements.

Indices for performance evaluation of quantitative values and application of CBA (cell block array) for realizing comparability among values that form various quantification systems are described in the following clauses.

5 Selection of fluorescent nanoparticles

5.1 General

Nanoparticles shall be selected to fit the purpose of the quantification system. Required performance of nanoparticles varies with respect to quantity of target biomolecules, performance of imaging systems including sensitivity, available excitation wavelengths, colours for staining including multicolour staining. For the selection of nanoparticle-labelled antibodies, including commercially available labelled antibodies, the performance of nanoparticles and the titre of the antibodies should be evaluated (see 5.2).

5.2 Characteristics of nanoparticle

When nanoparticle labelling is performed in the laboratory, the nanoparticle shall be selected based on the characteristics of nanoparticle. The characteristics of nanoparticles to be evaluated shall include but not limited to the following.

a) Brightness.

   Brightness is important for the quantification of biomolecules by immunohistochemistry. Initial brightness can be used as a characteristic for the selection of nanoparticles. It is a relative brightness that is proportional to molar extinction coefficient and quantum yield. For the selection of nanoparticles, these parameters, i.e. molar extinction coefficient and quantum yield, should be evaluated.

Molar extinction coefficient is defined as the optical density of 1 M fluorescent substance per 1 cm of optical path of absorption cell, measured by absorption photometry. Quantum yield is defined as the number of quanta emitted per quantum absorbed and can be measured with a fluorospectrophotometer.

b) Photobleaching time.

When fluorescent dyes or nanoparticles are continuously irradiated by excitation light, their emission output decreases and is eventually bleached. It can be characterized by the half-time of bleaching when the number of emitted photons per hour is halved.

c) Particle size and size distribution.

Average particle size should be analysed with coefficient of variation (CV). Dynamic light scattering (DLS) and scanning electron microscopy (SEM) can be used for this analysis. For SEM analysis some standards[16] are helpful.

d) Aggregation and agglomeration.

Agglomeration is the process or degree to form agglomerates that are a collection of weakly or medium strongly bound particles. In agglomeration, the resulting external surface area is similar to the sum of the surface areas of the individual particles. Aggregation is the process or degree to form aggregates that are particles comprising strongly bonded or fused particles. In aggregation, the resulting external surface area is significantly smaller than the sum of the surface areas of the individual particles (see Clause 3).

An example of an evaluation method of aggregation is shown in Annex B.

NOTE That includes QD aggregate and agglomerate in biological media[6] as well as the other types of nanoparticles.[7][8][9][10] The excess aggregated QDs can be separated and removed.[11]

e) Non-specific absorption of nanoparticles.

Nonspecific absorption of fluorescent nanoparticles can be a part of the background noise, diminish the quality of fluoromicroscopic images, and hinder the relative-quantification analysis. Nonspecific absorption of nanoparticles should be evaluated (see 7.1).

f) Uniformity of nanoparticle(s).

When nanoparticles are used as fluorescent substances, variation in particle size can reduce the level of correlation between quantity of biomolecules and fluorescence intensity. Particle size shall be evaluated along with CV and reported.

6 Quantification system

6.1 Overall design

When designing a quantification system for selected fluorescent nanoparticles, the intended use shall be defined and documented. The quantification system described in this document uses technology of immunohistochemistry that is an application staining thin sections of tissues with an antibody specifically recognizing a target biomolecule. The thin section can be prepared from formalin fixed paraffin embedded and frozen tissues. They are subsequently stained with antibodies linking to nanoparticles. In some cases, another molecular system including biotin-avidin conjugation can be used to detect the antibody binding to the target molecules (see 6.2.1). For the quantification, stained slides are observed with fluorescence microscope and images of tissues are captured with a digital camera, followed by quantification of the fluorescence signals from the tissue image with the image analysis software. The design of the quantification system should cover all the steps of the process, from the prepared tissue sections and the experimentally measured values, to the image processing and final quantification of the data with the processing software.

The design description shall contain but not be limited to the following specifications: the fluorescent nanoparticle to be used and its performance, antibodies including nanoparticle labelled and non-labelled antibodies when used, staining conditions, microscope system with imaging equipment, spectral characteristics of the light source used, and image analysis software. The design shall be reviewed to ensure conformity to the requirements of the system.

For selecting image processing software, requirements of the software shall be described. Minimum exposure time for imaging is critical for the quality of image processing by the software as well as other instrument configurations stated in 6.4. Staining procedure, imaging and image processing software are not independent of each other. When quantification analysis method is newly developed or changed, the design description shall be reviewed.

6.2 Antibody

6.2.1 General

Various antibodies are used in the immunohistochemistry. For example, a single labelled antibody for direct immunofluorescence methods and a set of two antibodies comprising primary antibody recognizing a target biomolecule and labelled second antibody recognizing the first antibody molecule. The labelled antibody is conjugated with a nanoparticle directly or molecules attached to the other molecules, for example biotin attached to avidin. By any method, the antibody recognizing the target biomolecule and nanoparticle are linked after staining to mark the target biomolecules.

NOTE Various labelling designs can be employed for immunohistochemistry. Typical labelling schemes are as follows.

a) Primary antibody - Biotinylated secondary antibody -avidinated nanoparticles.

b) Biotinylated antibody -avidinated nanoparticles.

c) Nanoparticles with primary antibody bound to the surface.

Antibodies shall be selected based on various characteristics including antibody type, target antigen, applicability to method, animal species, immunoglobulin type, titre and affinity. The specific issues described below should be considered.

6.2.2 Antibody type

Antibody type, i.e. monoclonal vs polyclonal should be considered for the choice of the quantification method.

Monoclonal antibodies generally bind to a single recognition site (single epitope), leading to 1:1 ratio with respect to biomarker molecules, and allowing direct quantification (e.g. by fluorescence intensity).

On the contrary, polyclonal antibodies can bind to multiple epitopes within the same biomarker, providing some advantages in terms of tagging efficiency but precluding any intensity-based quantification. Depending on spatial resolution, polyclonal antibodies can still be used for quantification of biomolecules by using the method of counting the number of fluorescent points in the image (see 6.4.2).

6.2.3 Target antigen

The target antigen is selected according to the role of the antibody used in the staining process.

For antibody to be used for recognizing biomolecules, e.g. antibodies for the direct immunofluorescence method and primary antibodies for the indirect immunofluorescence method, the antigen shall be confirmed as the target biomolecule.