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Doctoral thesis

DEVELOPMENT OF A REFERENCE METHOD FOR THE ABSOLUTE MEASURING OF FLUORESCENCE IN BIOLOGICAL ANALYSIS

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PhD thesis

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To my family and my nephew.

Abstract

The research presented here was aimed to develop a method traceable to SI to compare the absolute fluorescence intensity measured by different instruments. This is due to the fact that the most common instruments use arbitrary measurement units. Each measure strictly depends on the technical intrinsic properties of the single instrument, hence the use of suitable reference materials is necessary to define its calibration scale.

To overcome this problem, it is necessary to define a reliable and universal measurement unit allowing to measure the absolute fluorescence intensity, whichever system is used, independently from the reference materials which are used.

In the present work, a protocol that permits the comparison of data taken with different fluorescence instruments has been developed

The relationship between the arbitrary unit of these instruments and the molecule numbers of the standard reference material used has been studied.

This allows the transformation from arbitrary unit to MESF, i.e. *MOLECULAR EQUIVALENTS OF SOLUBLE FLUOROCHROME* [1].

The standard reference fluorescence material used was Fluorescein.

Standard reference materials of Fluorescein were used to ensure the traceability of measurements results.

The work goals were to obtain: a method to prepare fluorescein solutions of calibration; characterization of fluorescence instruments ad their calibration; study of a measurement method of the calibration samples and calculation of their uncertainty budget ,expressed in number of molecules of fluorescein, (*NF)*.

The final work goal was to obtain an uncertainty budget for measurements, expressed in equivalent number of molecules of fluorescein (MESF) for each used instrument..

By applying this method It has been possible to compare different measurements, using different fluorescence instruments.

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CHAPTER 1

Introduction

Fluorescence [2] is the emission of light by a substance that has absorbed light or other electromagnetic radiation. It is a form of luminescence. In most cases, the emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation.

However, when the absorbed electromagnetic radiation is intense, it is possible for one electron to absorb two photons; this two-photon absorption can lead to emission of radiation having a shorter wavelength than the absorbed radiation. The emitted radiation may also be of the same wavelength as the absorbed radiation, termed "resonance fluorescence"[3].

The most striking examples of fluorescence occur when the absorbed radiation is in the ultraviolet region of the spectrum, and thus invisible to the human eye, and the emitted light is in the visible region.

Fluorescence has many practical applications, including mineralogy, gemology, chemical sensors (fluorescence spectroscopy), fluorescent labeling, dyes, biological detectors, and, most commonly, fluorescent lamps.

1.1 History of fluorescence

An early observation of fluorescence was described in 1560 by Bernardino de Sahagún and in 1565 by Nicolás Monardes in the infusion known as lignum nephriticum (Latin for "kidney wood"). It was derived from the wood of two tree species, Pterocarpus indicus and Eysenhardtia polystachya [4] [5][6][7]. The chemical compound responsible for this fluorescence is matlaline, which is the oxidation product of one of the flavonoids found in this wood [4]. In 1819 Edward D. Clarke [8] and in 1822 René Just Haüy [9] described fluorescence in fluorites,Sir David Brewster described the phenomenon for chlorophyll in 1833 [10] and Sir John Herschel did the same for quinine in 1845 [11]. In his 1852 paper on the "Refrangibility" (wavelength change) of light, George Gabriel Stokes described the ability of fluorspar and uranium glass to change invisible light beyond the violet end of the visible spectrum into blue light. He named this phenomenon fluorescence : "I am almost inclined to coin a word, and call the appearance fluorescence, from fluor-spar [i.e., fluorite], as the analogous term opalescence is derived from the name of a mineral." [12]

The name was derived from the mineral fluorite (calcium difluoride), some examples of which contain traces of divalent europium, which serves as the fluorescent activator to emit blue light. In a key experiment he used a prism to isolate ultraviolet radiation from sunlight and observed blue light emitted by an ethanol solution of quinine exposed by it [13].

1.2 Physical principles

1.2.1 Photochemistry

Fluorescence occurs when an orbital electron of a molecule, atom or nanostructure relaxes to its ground state by emitting a photon of light after being excited to a higher quantum state by some type of energy [2]:

Excitation: $S_0 + h\nu_{ex} = S_1$ *Eq. 1.1*

Fluorescence (emission): $S_1 = +h\nu_{em} + heat$ *Eq. 1.2*

Here $\boldsymbol{h\nu}$ is a generic term for photon energy with h = Planck's constant and $\boldsymbol{\nu}$ = frequency of light, (The specific frequencies of exciting and emitted light are dependent on the particular system) State S0 is called the ground state of the fluorophore (fluorescent molecule) and S1 is its first (electronically) excited state.

A molecule, S1, can relax by various competing pathways. It can undergo 'non-radiative relaxation' in which the excitation energy is dissipated as heat (vibrations) to the solvent. Excited organic molecules

can also relax via conversion to a triplet state, which may subsequently relax via phosphorescence or by a secondary non-radiative relaxation step.

Relaxation of an S1 state can also occur through interaction with a second molecule through fluorescence quenching. Molecular oxygen(O2) is an extremely efficient quencher of fluorescence just because of its unusual triplet ground state.

Molecules that are excited through light absorption or via a different process (e.g. as the product of a reaction) can transfer energy to a second 'sensitized' molecule, which is converted to its excited state and can then fluoresce. This process is used in lightsticks to produce different colors.

1.2.2 Quantum yield

The fluorescence quantum yield gives the efficiency of the fluorescence process. It is defined as the ratio of the number of photons emitted to the number of photons absorbed [14] [15].

$$\Phi = \frac{Number\ of\ photons\ emitted}{Number\ of\ photons\ absorbed} \qquad Eq.\ 1.3$$

The maximum fluorescence quantum yield is 1.0 (100%); every photon absorbed results in a photon emitted. Compounds with quantum yields of 0.10 are still considered quite fluorescent. Another way to define the quantum yield of fluorescence, is by the rate of excited state decay:

$$\Phi = \frac{k_f}{\sum k_i} \qquad Eq.\ 1.4$$

where k_f is the rate of spontaneous emission of radiation and $\sum k_i$ is the sum of all rates of excited state decay. Other rates of excited state decay are caused by mechanisms other than photon emission and are, therefore, often called "non-radiative rates", which can include: dynamic collisional quenching, near-field dipole-dipole interaction (or resonance energy transfer), internal conversion, and intersystem crossing. Thus, if the rate of any pathway changes, both the excited state lifetime and the fluorescence quantum yield will be affected.

Fluorescence quantum yields are measured by comparison to a standard. The quinine salt quinine sulfate in a sulfuric acid solution is a common fluorescence standard.

1.2.3 Lifetime

The fluorescence lifetime refers to the average time the molecule stays in its excited state before emitting a photon. Fluorescence typically follows first-order kinetics:

$$[S1] = [S1]_0 e^{-\Gamma t} \qquad \textit{Eq. 1.5}$$

where $[\boldsymbol{S1}]$ is the concentration of excited state molecules at time $\boldsymbol{t}$, $[\boldsymbol{S1}]_{\boldsymbol{0}}$ is the initial concentration and Γ is the decay rate or the inverse of the fluorescence lifetime. This is an instance of exponential decay. Various radiative and non-radiative processes can de-populate the excited state. In such case the total decay rate is the sum over all rates:

$$\Gamma_{tot} = \Gamma_{rad} + \Gamma_{nrad} \qquad \textit{Eq. 1.6}$$

where $\boldsymbol{\Gamma_{tot}}$ is the total decay rate, $\boldsymbol{\Gamma_{rad}}$ the radiative decay rate and $\boldsymbol{\Gamma_{nrad}}$ the non-radiative decay rate. It is similar to a first-order chemical reaction in which the first-order rate constant is the sum of all of the rates (a parallel kinetic model). If the rate of spontaneous emission, or any of the other rates are fast, the lifetime is short. For commonly used fluorescent compounds, typical excited state decay times for photon emissions with energies from the UV to near infrared are within the range of 0.5 to 20 nanoseconds. The fluorescence lifetime is an important parameter for practical applications of fluorescence such as fluorescence resonance energy transfer and Fluorescence-lifetime imaging microscopy.

1.3 Jablonski diagram

The Jablonski [2] diagram describes most of the relaxation mechanisms for excited state molecules. After an electron absorbs a high energy photon the system is excited electronically and vibrationally. The system relaxes vibrationally, and eventually fluoresces at a longer wavelength.

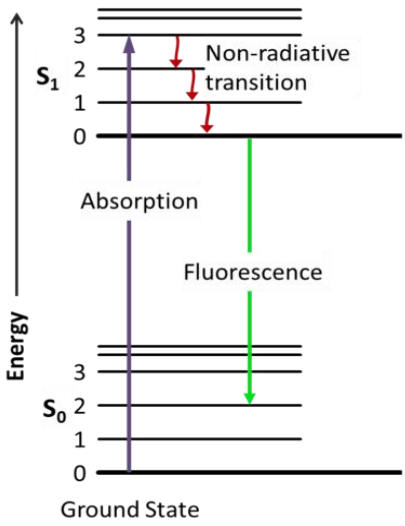

Fig. 1.1 Jablonski diagram [2].

1.4 Fluorescence anisotropy

Fluorophores are more likely to be excited by photons if the transition moment of the fluorophore is parallel to the electric vector of the photon [16]. The polarization of the emitted light will also depend on the transition moment. The transition moment is dependent on the physical orientation of the fluorophore molecule. For fluorophores in solution this means that the intensity and polarization of the emitted light is dependent on rotational diffusion. Therefore, anisotropy measurements can be used to investigate how freely a fluorescent molecule moves in a particular environment.

Fluorescence anisotropy can be defined quantitatively as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad \textit{Eq. 1.7}$$

where $I_{\parallel}$ is the emitted intensity parallel to polarization of the excitation light and $I_{\perp}$ is the emitted intensity perpendicular to the polarization of the excitation light [17].

1.5 Stokes shift

In general, emitted fluorescent light has a longer wavelength and lower energy than the absorbed light [18]. This phenomenon, known as Stokes shift, is due to the energy loss between the time a photon is absorbed and when it is emitted. The causes and magnitude of Stokes shift can be complex and are dependent on the fluorophore and its environment. However, there are some common causes.

It is frequently due to non-radiative decay to the lowest vibrational energy level of the excited state. Another factor is that the emission of fluorescence frequently leaves a fluorophore in the highest vibrational level of the ground state.

1.6 Applications of Fluorescence

The common fluorescent lamp relies on fluorescence. Inside the glass tube is a partial vacuum and a small amount of mercury. An electric discharge in the tube causes the mercury atoms to emit ultraviolet light. The tube is lined with a coating of a fluorescent material, called the phosphor, which absorbs the ultraviolet and re-emits visible light. Fluorescent lighting is more energy-efficient than incandescent lighting elements. However, the uneven spectrum of traditional fluorescent lamps may cause certain colors to appear different than when illuminated by incandescent light or daylight. The mercury vapor emission spectrum is dominated by a short-wave UV line at 254 nm (which provides most of the energy to the phosphors), accompanied by visible light emission at 436 nm (blue), 546 nm (green) and 579 nm (yellow-orange).

These three lines can be observed superimposed on the white continuum using a hand spectroscope, for light emitted by the usual white fluorescent tubes. These same visible lines, accompanied by the emission lines of trivalent europium and trivalent terbium, and further accompanied by the emission continuum of divalent europium in the blue region, comprise the more discontinuous light emission of the modern trichromatic phosphor systems used in many compact fluorescent lamp and traditional lamps where better color rendition is a goal [19].

Fluorescent lights were first available to the public at the 1939 New York World's Fair. The main Improvements since then have been better phosphors, longer life, and more consistent internal discharge, and easier-to-use shapes (such as compact fluorescent lamps). Some high-intensity discharge (HID) lamps couple their even-greater electrical efficiency with phosphor enhancement for better color rendition

White light-emitting diodes (LEDs) became available in the mid-1990s as LED lamps, in which blue light emitted from the semiconductor strikes phosphors deposited on the tiny chip. The combination of the blue light that continues through the phosphor and the green to red fluorescence from the phosphors

produces a net emission of white light. Glow sticks sometimes utilize fluorescent materials to absorb light from the chemiluminescent reaction and emit light of a different color [19].

1.6.1 Analytical chemistry

Many analytical procedures involve the use of a fluorometer, usually with a single exciting wavelength and single detection wavelength. Because of the sensitivity that the method affords, fluorescent molecule concentrations as low as 1 part per trillion can be measured.

Fluorescence in several wavelengths can be detected by an array detector, to detect compounds from HPLC flow. Also, TLC plates can be visualized if the compounds or a coloring reagent is fluorescent. Fluorescence is most effective when there is a larger ratio of atoms at lower energy levels in a Boltzmann distribution. There is, then, a higher probability of excitement and release of photons by lower-energy atoms, making analysis more efficient.

1.6.2 Spectroscopy

Usually the setup of a Fluorescence array involves a light source, which may emit an array of different light wavelengths. In general, a single wavelength is required for proper analysis, so, in order to selectively filter the light, it is passed through an excitation monochromator, and then the chosen wavelength is passed through the sample cell. After absorption and re-emission of the energy, many wavelengths may emerge due to Stokes shift and various electron transitions. To separate and analyze them, the fluorescent radiation is passed through an emission monochromator, and observed selectively by a detector [20].

1.6.3 Biochemistry and medicine

Endothelial cells under the microscope with three separate channels marking specific cellular components. Fluorescence in the life sciences is used generally as a non-destructive way of tracking or analysis of biological molecules by means of the fluorescent emission at a specific frequency where there is no background from the excitation light, as relatively few cellular components are naturally fluorescent (called intrinsic or autofluorescence). In fact, a protein or other component can be "labelled" with an extrinsic fluorophore, a fluorescent dye that can be a small molecule, protein, or quantum dot, finding a large use in many biological applications [21].

1.6.4 Microscopy

- When scanning the fluorescent intensity across a plane one has fluorescence microscopy of tissues, cells, or subcellular structures, which is accomplished by labeling an antibody with a fluorophore and allowing the antibody to find its target antigen within the sample. Labelling multiple antibodies with different fluorophores allows visualization of multiple targets within a single image (multiple channels). DNA microarrays are a variant of this.
- Immunology: An antibody is first prepared by having a fluorescent chemical group attached, and the sites (e.g., on a microscopic specimen) where the antibody has bound can be seen, and even quantified, by the fluorescence.
- FLIM (Fluorescence Lifetime Imaging Microscopy) can be used to detect certain bio-molecular interactions that manifest themselves by influencing fluorescence lifetimes.
- Cell and molecular biology: detection of colocalization using fluorescence-labelled antibodies for selective detection of the antigens of interest using specialized software, such as CoLocalizer Pro.

Observing *Fig 1.2* below, it is possible to notice different kind of fluorophores in different areas of the cells. Each fluorophores can dye a particular cell area.
In Blue *(DAPI)* is indicated the *nucleus*, in Red *(Rhodamine)* is indicated *Actin*, and in Green *(Alexa-488)* is indicated *Integrin*

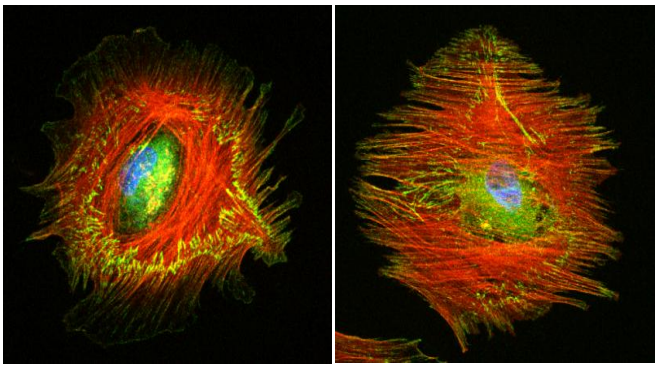

Fig. 1.2. Images of Human Mesenchymal Stem Cells (hMSCs) that have been taken at I.N.Ri.M laboratory (by G.Intermite and L. Mortati) with Two Photons Excitation (TPE) technique.

1.6.5 Other techniques

- FRET [2] Fluorescence resonance energy transfer is used to study protein interactions, detect specific nucleic acid sequences and used as biosensors, while fluorescence lifetime (FLIM) can give an additional layer of information.

- Biotechnology: biosensors using fluorescence are being studied as possible Fluorescent glucose biosensors.
- Automated sequencing of DNA by the chain termination method; each of four different chain terminating bases has its own specific fluorescent tag. As the labelled DNA molecules are separated, the fluorescent label is excited by a UV source, and the identity of the base terminating the molecule is identified by the wavelength of the emitted light.
- FACS [2] (fluorescence-activated cell sorting). One of several important cell sorting techniques used in the separation of different cell lines (especially those isolated from animal tissues).
- DNA detection [2]: the compound ethidium bromide, in aqueous solution, has very little fluorescence, as it is quenched by water. Ethidium bromide's fluorescence is greatly enhanced after it binds to DNA, so this compound is very useful in visualising the location of DNA fragments in agarose gel electrophoresis. Intercalated ethidium is in a hydrophobic environment when it is between the base pairs of the DNA, protected from quenching by water which is excluded from the local environment of the intercalated ethidium. Ethidium bromide may be carcinogenic – an arguably safer alternative is the dye SYBR Green.

1.6.6 Forensics

Fingerprints can be visualized with fluorescent compounds such as ninhydrin. Blood and other substances are sometimes detected by fluorescent reagents, like fluorescein. Fibers, and other materials that may be encountered in forensics or with a relationship to various collectibles, are sometimes fluorescent.

1.7 Fluorescence measurements in biology field

The number of measurement techniques in biology and medicine using fluorescence to quantify the measured data is increasing and then, the most techniques of medical and biology are based on measurement of fluorescence intensity.

In biology, it is often useful to cause the fluorescence of an object ("secondary fluorescence" or "induced") by treating biological components with appropriate fluorescent substances ("fluorophores"). The advantage is that, for reasons of physical or chemical affinity, the fluorophore tends to bind selectively with certain parts of the object more than others, e.g. on certain cellular organelles, nucleus etc… In this way the fluorophore behaves as a dye that selectively helps to reveal some chemical components and structural the object. The use of fluorophores is particularly interesting when searching for antigens, as they tend to bird with antibodies with a specific chemical structure.

The antibody is concentrated if there is an antigen (a specific antigen and only one), and it becomes fluorescent. The antigen-antibody reaction is very specific and allows fast and safe diagnosis. The use of

fluorescent antibodies is a technique of research, and it has become routine in many branches of medicine. Some examples used in medical and biology fields are showed below:

- high throughput tests, fundamental to study the toxicity of new discovered drugs;

- Flow cytometry allows to diagnose venereal diseases such as AIDS, cancers of blood (e.g. leukemia);

- The PCR technique is the basis for genetic research: genetic mutations, CSI.

Some examples of Fluorescence measurements, in the biology field, are FISH and FISNA [22]. FISH is a cytogenetic technique developed by biomedical researchers in the early 1980s [23] It is used to detect and localize the presence or absence of specific DNA sequences on chromosomes *(Fig 1.3)*.
FISNA is a technique to detect HIV virus. Cells containing HIV-1 DNA appear yellow-blue with a peak in fluorescence intensity of 2815 A.U. *(Fig. 1.4)*.

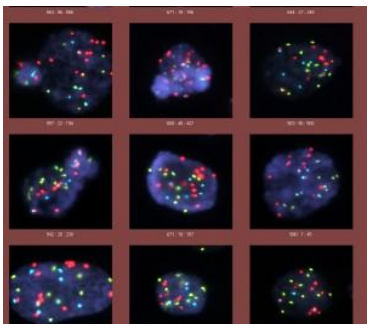

Fig. 1.3FISH (fluorescence in situ hybridization)

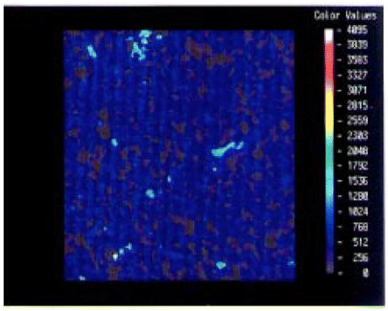

Fig. 1.4 Detection of HIV-1 DNA by FISNA

1.8 Necessity of a common measurement unit of fluorescence quantity.

The most common fluorescence instruments today don't have referability. Infact previously, the only available studies about flow cytometry. The importance of the work is described here lies in the fact a common measurement unit, would allow to compare different instruments for fluorescence measurements. Indeed, it is not always possible to use the same instruments while taking measurements, because each instrument has different properties, and each instrument is used for different measurements. Also, it could be that a particular instrument doesn't work when we need it, and then we need to use another instrument that measures the same physical quantities.

Therefore, it is not possible to make any statement about the compatibility of two different measurement sets, as the two instruments used in the two sets use different arbitrary units. It is thus necessary to define a single fluorescence unit which can be used by all instruments that we would like to use. This allows to have the same unit scale for different measurements taken with different instruments. In the present work, a measurement unit aimed at these purposes is described alongside with its properties; this unit is called MESF: Molecules of Equivalent Soluble Fluorochrome, and it is defined as follows: it is the fluorescence value that we would have if each molecule of used fluorocrome would have the same brightness of the soluble fluorochrome of reference.
It will be shown that this unit allows to relate all arbitrary units in an equivalent scale, and therefore to facilitate comparisons between different measures.

CHAPTER 2

Proposed Method

In this chapter the proposed method used for the measurements of fluorescence quantity, "*FQ*", will be described.
This method allows to measure the fluorescence quantity of unknown sample in Molecules of Equivalent Soluble Fluorochrome, *MESF* [1]. This method is divided in two phases:

- The first phase includes the *experimental work* which is divided in 3 steps:

- calibration solutions preparation of fluorescence reference material;
- fluorescence measurements of the solution with analytical instruments;
- analysis of the obtained data.

- The second phase consists of in measuring the unknown fluorescence sample with calibrated instruments.

The measured fluorescence intensity (I) is related to the fluorescence quantity *(FQ)* through the calibration function of the calibrated instruments in the first phase.
The *FQ* will be expressed in equivalent number of molecules or *MESF*.
This result will be the *FQ* of unknown sample.

2.1 Aim of the thesis

The first thesis aim is to develop a standard method to measure the fluorescence quantity with known.uncertainty.
We usually have a lot of problems when we want to compare different data that has been taken before using different instruments, because, each instrument has a different measurement system (s.a. pmt voltage, arbitrary unit, ect..)

Another problem of fluorescence instruments is that the most them don't provide satisfactory uncertainty measures, or , don't provide any at all. In this way, we can compare instruments of fluorescence, to avoid a lot of problems while taking measures with different instruments.
The second thesis aim is to identify, between all kind of instruments of fluorescence used, the most reproducible. This allows us, when possible, to make more reproducible measurements.

2.2 Some problems during the first experiment

The first taken measurements were designed to measure the amount of dye absorbed by the cells and the stability of the fluorescence signal over time [24].
An experiment of characterization in fluorescence was prepared with various fluorophores, at different colors. *NuclearMask Blue* [25] measurements of comparison taken with *Hitachi F-4500* [26] and *Tecan Infinite F-200* [27] will be show.

2.2.1 Measurements procedure.

- Firstly the dyed samples with *NuclearMask Blue* were prepared in solutions at work concentration that were indicated by producer. These samples have been used to measure short time stability and then as control fluorescence measurements.
- Secondly cells culture dyed with the same dyes of the previous point have been grown.
- Samples in solution have been analyzed with I *Hitachi F-4500* e *Tecan Infinite F-200*
- Samples in cells have been analyzed only with *Tecan Infinite F-200*.

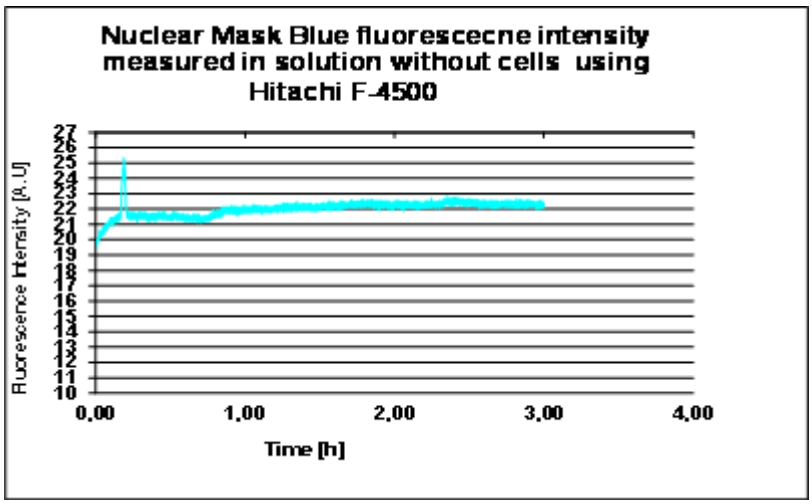

Fig. 2.1 Fluorescence intensity in relation with time scan, expressed in hours, measured with Hitachi F-4500 using solution without cells.

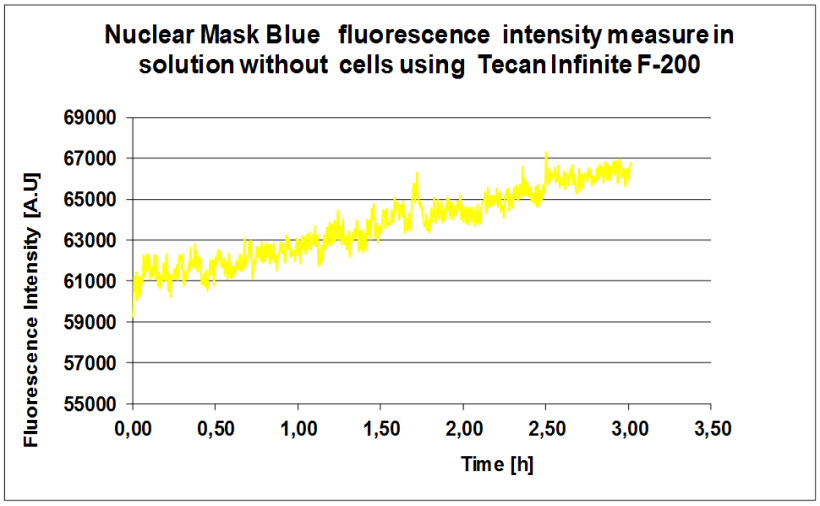

Fig. 2.2 Fluorescence intensity in relation with time scan, expressed in hours, measured with Tecan Infinite F-200 using solution without cells.

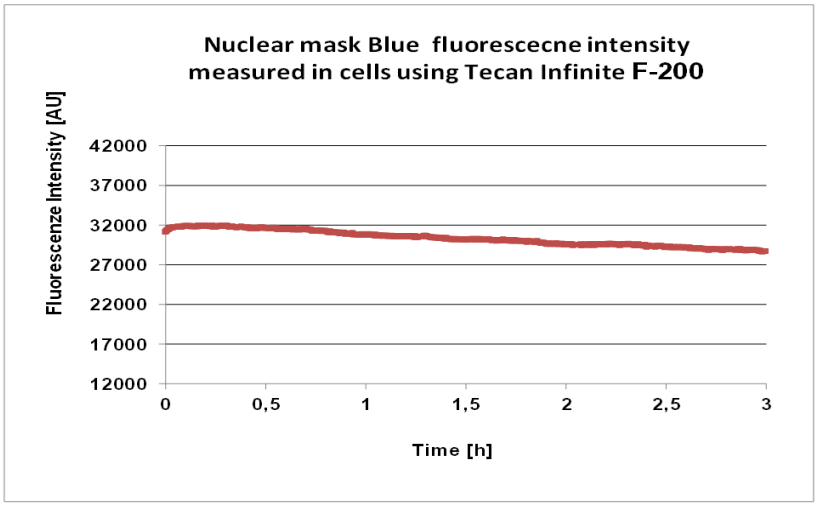

Fig. 2.3 Fluorescence intensity in relation with time scan expressed in hours measured with Tecan Infinite F-200 using solution with cells.

Seeing the *Fig. 2.1, 2.2, 2.3* above, it is not possible to compare taken measurements with *Hitachi F-4500* with taken measurements by *Tecan Infinite F-200*, because fluorescence intensity scales are different.

To solve this problem a protocol that allows a comparison between *Hitachi* and *Tecan* has been created.

2.3 Analytical instruments used

In this paragraph are shown Analytical instruments with their performances and parameters used during the measurements. *Hitachi F-4500 spectrofluorimeter* and *Tecan multplate reader Infinite F-200* have been used to compare the measurements.

The reason *why Hitachi F-4500* and *Tecan multplate reader Infinite F-200* have been chosen is because they were used during the first experiments, as showed in *paragraph 2.2*. Starting on the comparisons shown in paragraph 2.2 a method has been studied.

2.3.1 Hitachi F-4500 Spectrofluorimeter

Hitachi F-4500 is a spectrofluorimeter that allows acquisition of spectra in less than one second and the acquisition of 3-dimensional plots in less than one minute.

Photometry: in addition to fluorescence, both phosphorescence and luminescence are included as standard items. The high energy rough put and excellent signal to noise ratio allows measurements of chemiluminescent and bioluminescent compounds.
Wavelength Scan: can speeds up to 30,000 nm per min and wavelength drive rates of 60,000 nm per min provide rapid scans for both excitation and emission monochromators, so that analyses are completed faster than ever before. Photosensitive compounds can be scanned quickly with minimum damage.
Pre-Scan: Our patented pre-scan function ensures automatic and fast selection of the optimum exitation and emission wavelengths for your unknown samples .
Geometry: *Hitachi*`s unique horizontal beam geometry increases sensitivity and reduces the sample volume required for a standard 10mm cuvette.
Operation: The operating system is based on Microsoft Windows.F-4500 Product Specifications:

- Monochromator: Mechanically ruled concave diffraction gratings Blaze;
- Wavelength: Excitation 300 nm/Emission 400 nm;
- Detector: R3788 Photomultiplier tube;
- Wavelength range: 200 ~ 730 nm and 0-order (200 ~ 900 nm with optional photomultiplier);
- Resolution: 1.0 nm Band - pass: EX: 1.0, 2.5, 5.0, 10.0 nm, EM: 1.0, 2.5, 5.0, 10.0, 20.0 nm;
- Wavelength accuracy: 2.0 nm Wavelength scan speed: 15, 60, 240, 1,200, 2,400, 12,000, 30,000nm/min (500 nm/s);
- Wavelength drive speed: 60,000 nm/min (1,000 nm/s);
- Response: Response from 0 to 98%: 0.004, 0.01, 0.05, 0.1, 0.5, 2.0, 8.0 s;
- Light source: 150 watt Xe lamp;

- Minimum sample volume: 0.6 mL (with use of standard 10 mm cell)Sensitivity: 100:1 using Raman band of water;
- EX wavelength: 350 nm;
- Bandpass: 5.0 nm Response: 2.0 s;
- Analog output: 0 to 1 V full scale (20 bit D/A);
- Communication: Between instrument and computer via GPIB;
- Working temperature: 15 to 35 C Working humidity: 45 to 80% (condensation not allowed, 70% or less at 35 C or more);
- Power Consumption: 100, 115, 220, 230, 240 V AC, 50/60 Hz, 400 VA;
- Dimensions: 680 W x 660 D x 340 H mm Weight: 58 kg

Fig 2.1 Hithachi F-4500 spectrofluorimeter [26]

2.3.2 Tecan Infinite F-200 multiplate reader

Tecan Infinite F200 is a multifunctional microplate reader based filter with injection option, that provides high performance for the vast majority of microplate applications and research. It has been designed as a general purpose laboratory instrument for professional use, supporting common 6 to 384-well microplates.

This instrument allows long incubation of the microplate, the chance to set shaking movements, duration of all operations, including cycles, and dispensation of liquid material in wells. These actions are managed by a user interface operated by i-Control software, ranging from scheduling experiments to the cleaning of injectors.

Light Source	UV Xenon flashlamp
Wavelength selection	
Bandwidth	Ex: < 5 nm for $\lambda \leq$ 315 nm and < 9 nm for $\lambda >$ 315 Em: < 20 nm
Wavelength range	

Fluorescence intensity	Standard: Ex 230 - 600 nm, Em 330 - 600 nm Optional: Ex 230 - 850 nm, Em 280 - 850 nm
Fluorescence polarization	Standard: Ex 300 - 600 nm, Em 330 - 600 nm Optional: Em 330 - 850 nm
Absorbance	230 - 1000 nm
Detectors	Fluorescence - PMT, optionally UV and red-sensitive; Absorbance –Uv silicon photodiode; Luminescence - photon counting system with low dark ; Current PMT;
Plate formats	6- to 384- well plates, cuvettes, NanoQuant Plate™
Temperature control	Ambient +5°C up to 42°C
Shaking	Linear, orbital
Luminescence sensitivity values	Standard
Glow luminescence* **	225 amol ATP / well (9 pM; low volume 384-well plate)
Flash luminescence** **	12 amol ATP / well (218 fM; 384-well plate)
Absorbance	
Ratio accuracy 260 / 280 nm	± 0,07
Precision @ 260 nm	< 0,2 %
Accuracy @ 260 nm	< 0,5 %
Measurement range	0 - 4 OD
AlphaScreen	
Detection Limit	≤ 50 ng/ml Omnibeads
Uniformity	≤ 5% CV
Z´value	≥ 0.8
Typical reading time	< 11 min (384-well plate)
Injectors	
Pump speed	100 - 300 µl/s
Injection volume	selectable in 1 µl increments; max. volume: 800 µl per stroke
Dead volume	100 µl including pump back
Fastest Read Times:	
- 96 well plate	20 sec
- 384 well plate	30 sec
- Wavelength Ex / Em-scan, 96 well plate 450 – 550 nm 5 nm step	150 sec

Fig 2.2 Tecan Infinite F-200 Multiplate reader [27].

2.4 Experimental Work

For experimental work a Standard Reference Material (STM) has been used to calibrate our fluorescent system. The STM chosen was Fluoresceine STM 1932. It has been chosen because it had photostability and it was available at I.N.Ri.M laboratories. In this chapter all procedures to obtain a protocol measurement will be shown. Instruments configuration, when possible, has been set with the most similar configuration between fluorescent instruments used.

2.4.1 Protocol of the Preparation of Calibration Solution

- ***FLUORESCIEN Standard Reference Material 1932***

During the preparation of the calibration solution FLUORESCIEN Standard Reference Material 1932 at 60.97 µmol·kg–1 ± 0.40 µmol·kg–1 [28] has been used. In the certificate of Fluorescein SRM 1932 has been indicated the value of Molar Absorption Coefficient:

Wavelength (nm)	Molar Absorption Coefficient ($kg \cdot cm^{-1} \cdot mol^{-1}$)	Uncertainty ($kg \cdot cm^{-1} \cdot mol^{-1}$)
488.0	8.50×10^4	0.07×10^4
490.0	8.70×10^4	0.07×10^4
490.5	8.71×10^4	0.07×10^4
491.0	8.70×10^4	0.07×10^4

Tab 2.1 Reference Values for Molar Absorption Coefficient of SRM 1932 at 22.4 °C - 0.5 °C

- ***Preparation of Calibration Solutions***

The calibration solution have been prepared starting from stock solution, and after, different quantity of PBS has been added. Two different kinds of concentrations have been prepared. They have been called "**at one dilution"** and "**at two dilutions".**

➢ *Concentrations at one dilution method.*

Nominal quantities of fluorescein stock solution and PBS were established before, by precise calculations, to have specific concentration values, and they have been used to prepare solutions.
This method has been used for the following concentration values:
1:10, 1:11, 1:12, 1:15, 1:20, 1:30, 1:50, 1:70, 1:100.

➢ *Concentrations at two dilutions method.*

The same method has been used like at one dilution, but 1:100 concentration has been used as stock solution. This is the reason why it has been called at two dilutions method.
This method has been used for the following concentration values:
1:250, 1:500, 1:750, 1:1000, 1:5000, 1:10000

In this way, different values of concentration in 1 ml of volumes have been obtained. When possible, the samples containing fluorescein solution were prepared immediately before the use, otherwise after preparation, they were stocked in a fridge and covered from light.

- ***Calculation of real concentration of the fluorescein solutions:***

Starting from the nominal concentrations calculated before with Microsoft Excel ®, during the preparation of solutions, quantity of fluorescein and PBS have been weighed.
Real concentration were calculated by using masses ratio, as follows:

$$R_1 = \frac{m_{stock\_sol}}{m_{stock\_sol} + m_{PBS}} \qquad \textit{Eq 2.1}$$

$$R_2 = \frac{m_{stock\_sol_{100}}}{m_{stock\_sol_{100}} + m_{PBS}} \qquad \textit{Eq 2.2}$$

Where it is possible to find:

m_{stock_sol} = mass of stock fluorescein solution
m_{PBS} = PBS mass
$m_{stock\_sol_{100}}$ = mass of stock fluorescein solution at 1:100 concentration
R_1 = mass ratio of one dilution method
R_2 = mass ratio of two dilution method

After mass ratio calculation, the real concentration was calculated as follows:

$$C_1 = R_1 * C_{stock\ fluorescein\ solution} \qquad Eq\ 2.3$$

$$C_2 = R_2 * C_{stock\ fluorescein\ solution} \qquad Eq\ 2.4$$

Where:

C_1= concentration of one dilution method

C_2= concentration of two dilution method

The values of real concentration have been obtained using an analytical balance Sartorius CPA225D model [29].

In the following table will be report Sartorius CPA225D specifications [30].

Max Measurement [g]	Sensibility [mg]	Ripetibility [mg]	Linearity [mg]	Response Time [sec]
220	0.1	±0.1	±0.1	6

Tab 2.2 Analytical balance Sartorius CPA225D model specifications

Fig 2.3 Analytical balance Sartorius CPA225D model

2.4.2 Measuring of Fluorescence Intensity of the Calibration Solutions

- ***Hitachi F-4500 spectrofluorimeter method.***

Firstly, fluorescein solution at a specific chosen concentration has been put inside a cuvette of 700 µl. 700 µl was the smallest volume available.

The instrument parameters then have been set to measure as follows:

Ex. 488 nm;
Emission range: 500-700 nm;
Scan Speed: 15 nm/min;
PMT Voltage: 700v

Tab 2.3 Hitachi F-4500 set parameters

- Scan speed at 15/nm/min has been chosen because it was the slowest speed available;
- In this way, less noise contribution has been possible to have, so that the measurement had more accuracy;
- PMT voltage at 700v has been chosen, because it was the best value that allowed to make all measurements without going out of scale;
- Temperature of laboratory has been set at 23 °C with external temperature control;

After setting of *Hitachi F-4500 spectrofluorimeter*, the measurements have been made as follows:

1. the instrument turned on;
2. pre-heating of the lamp for 5 minutes;
3. cuvette of 700 µl has been filled using micropipette;
4. The sample, composed by a cuvette of 700 µl containing fluorescein solution, has been put inside the instrument;
5. Measurement start;
6. 5 cycles have been effected to obtain a statistical average of spectra;
7. 3 measurements have been effected for each cycles;
8. Between one cycle and another the sample was pulled out, and the fluorescein solution was mixed with a syringe to have an homogeneous solution during measurements;
9. Before measurements, longer side of the cuvette was exposed to the lamp light flow;
10. After measurement, the used solution was stoked in the fridge protected from light.

- ***Tecan Infinite F-200 multiplate reader method.***

A 96 well plate has been used. At the first time fluorescein solution at a specific chosen concentration has been put into one of the 96 wells of the plate. The instrument parameters were then set to measure as follows:

Kinetic Measurement		
Temperature System	23° C	
Kinetic Cycles	5	
Interval Time	Minimal	
Mode	Fluorescence Bottom Reading	
Multiple Reads per Well (Circle)	2 x 2	
Multiple Reads per Well (Border)	2550	µm
Excitation Wavelength	485	nm
Emission Wavelength	535	nm
Excitation Bandwidth	20	nm
Emission Bandwidth	25	nm
Gain	60	Manual
Number of Flashes	25	
Integration Time	20	µs
Lag Time	0	µs
Settle Time	0	ms
Mode	Absorbance	
Multiple Reads per Well (Circle)	2 x 2	
Multiple Reads per Well (Border)	3300	µm
Wavelength	560	nm
Bandwidth	10	nm
Number of Flashes	25	

Tab 2.4 Tecan Infinite F-200 set parameters

- Fluorescence in Bottom Reading, has been chosen because, the solution was in contact with well surface, so that there was less uncertainty in reading.
- 2x2 reading per well in different point of well has been chosen. In this way uncertainty due to non-homogeneous solution was reduced
- 25 flashes have been chosen because they were the maximum available value. In this way measurement had high number of values to have good statistical average.
- Gain has been chosen at 60 in manual mode, because it was the best value that allowed to effect all measurements without going out of scale.

After setting of *Tecan Infinite F-200 multiplate reader*, the measurements have been made as follows:

1. the instrument turned on;
2. pre-heating of the lamp for 5 minutes;
3. *Tecan Infinite F-200* parameters have been set before measurement;

4. the chosen well of plate of 100 µl has been filled using micropipette;
5. The sample composed by a well of 100 µl containing fluorescein solution has been put inside the instrument;
6. Measurement start;
7. 5 cycles have been done to obtain a statistical average of spectra;
8. 25 Number of Flashes have been done for each cycles;
9. Fluorescence Bottom Reading mode has been used;
10. After measurement, the used solution was stoked in the fridge protected from light.

2.4.3 Data Analysis and Calculation of the Calibration Curve

Calibration curves have been obtained for each analyzed instrument. Any way, the measurand is always fluorescence intensity.

- ***Hitachi F-4500 spectrofluorimeter method.***

1. The *Hitachi F-4500 spectrofluorimeter* measure a fluorescence spectra;
2. The fluorescence values were taken from peak of fluorescence spectra *(Fig 2.4)* , that is a maximum value at specific wavelength. It has been chosen because it was easier to identify, and to take from all spectra points. It was also the work region where there was the best linearity response, as shown in the following chapter. In this way it is always possible to take the same position, and then to compare between different taken values at different measures;
3. For each concentration of fluorescein solution the data average has been calculated;
4. Calibration curve has been obtained by interpolating data, that were expressed in arbitrary units *A.U.*; with the number of molecules of fluorescein *NF* (molecules number of fluorescein);
5. For each AU value there is a specific *NF* value.

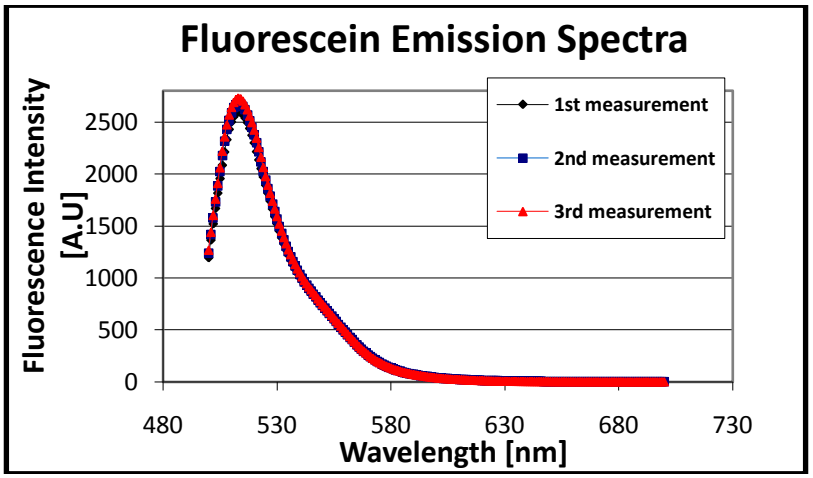

Fig. 2.4 Peak of the fluorescein spectra emission at 511 nm, excited at 488 nm.

- ***Tecan Infinite F-200 multiplate reader method***

The *Tecan Infinite F-200* is a multiplate reader that measures fluorescence intensity only. Measurements data can be taken easier than *Hitachi F-4500*, because *Tecan infinite F-200* issued directly fluorescence intensity data.

1. The average fluorescence values were taken directly from data instrument for each point of well
2. The average fluorescence values have been obtained by using 4 different points (2x2 Multiple Reads per well).
3. Calibration curve has been obtained by interpolating data, that were expressed in arbitrary units AU with the number of molecules of fluorescein *NF* (molecules number of fluorescein);
4. For each AU value there is a specific *NF* value.

Interpolating data has been possible to obtain the following best fit equation.

$$I = m * FQ + q \qquad \textit{Eq 2.5}$$

Where we can find:

I = fluorescence intensity of calibration sample;

M = angular coefficient of the curve;

FQ = fluorescence quantity expressed in *MESF* (Molecules of Equivalent Soluble Fluorochrome);

q = intercept of the curve.

2.5 Results of the Proposed Method

Before obtaining results of the proposed method, some testing has been done. It has been possible to find the best method in this way. Following are show some results of preliminary studies.

2.5.1 Preliminary studies using Hitachi spectrofluorimeter F-4500

Before starting measurements, the linearity characterization of *Hitachi spectrofluorimeter* was necessary. At first it was important to study gain curve of *Hitachi F-4500 instrument*. It was necessary because we needed to identify the best work gain of PMT . The best work gain resulted to be *700v*.

In the *Fig 2.5* is shown gain curve of *Hitachi F-4500*. It is possible to look fluorescence intensity in relation with gain system, using the same sample of fluorescein from fitting equation curve has been obtained *y = 3E-24x8,9447*.

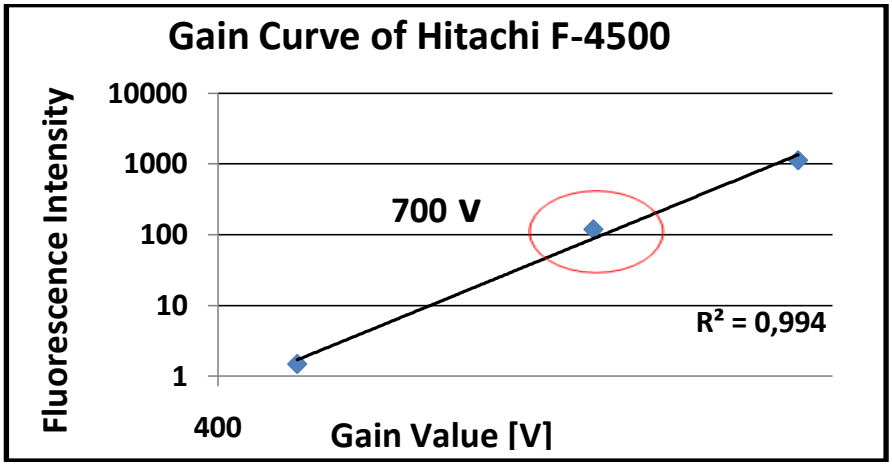

Fig. 2.5 The graph shows gian curve of Hitachi F-4500.

After gain study a linearity study of *Hitachi F-4500* has been made. It was important, because there was the necessity to know the best work region, where the instrument has the best linearity at all concentrations.

In the peak region, the best linear response has been obtained, because ratios between peak value and other position value of spectra are similar near the peak region, while moving towards the tail, of the spectrum, relationships change *(Fig 2.6)*. It is due to a greater contribution of the noise where the signal is lower than where the signal is stronger.

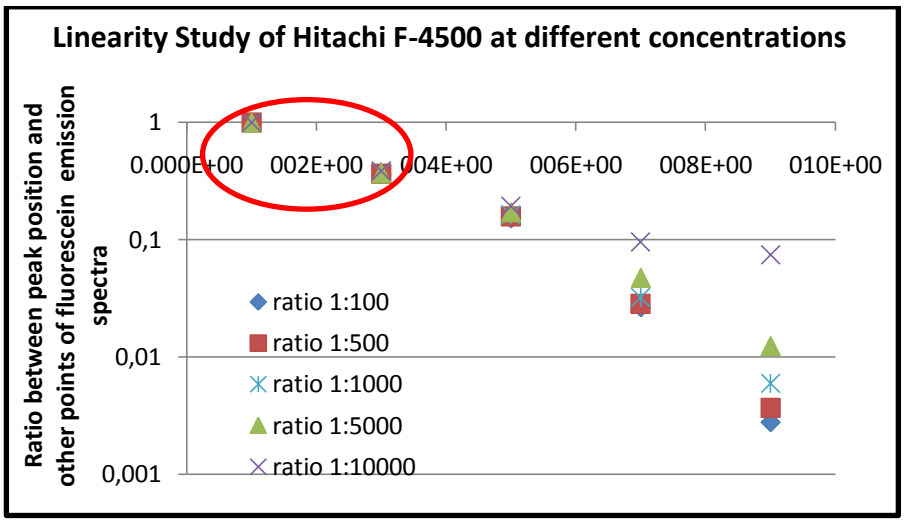

Fig. 2.6 Fluorescence signal rates between different concentrations

- ***The first method*** involved the smallest side exposed to light. In this way there was a longer optical path exposed to light, and the molecules, that were on the bottom of the path, received less light than the first molecules. It was caused by absorption, having than a non-uniform emission, in particular at the highest concentration, having more density, absorbed more light than the lowest concentration.

Only 3 measures per sample have been done to have a reliable statistics and to avoid sample degradation during measurement.

The *Fig 2.7* shows that with high concentration there is more absorption by molecules of solution, and there is not a linear increase of fluorescence intensity.

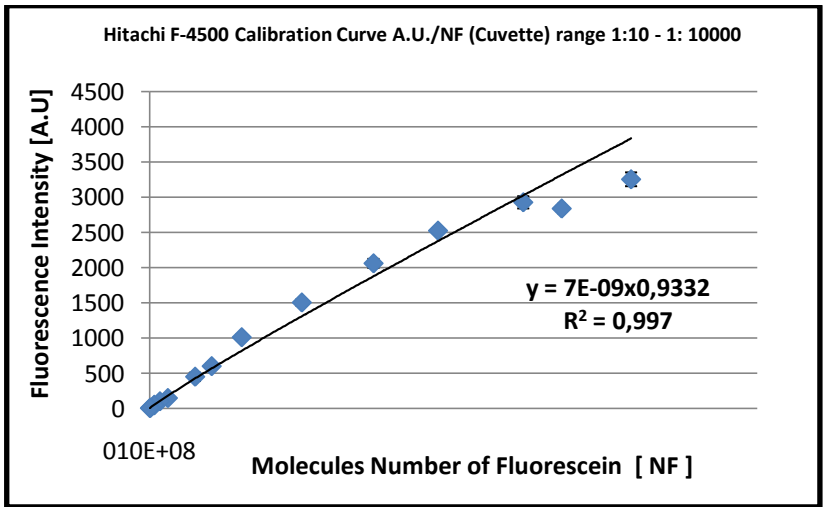

Fig. 2.7 Hitachi F-4500 Calibration Curve A.U./NF (Cuvette) range 1:10 - 1: 10000

- ***The second method*** involved the biggest side exposed to light.

In this way there was a smaller optical path exposed to light, and the molecules that were on the bottom of the path received about same light than the first molecules, having than a uniform emission light. In this way, the difference in number of molecules, between concentrations along the path is lower than first method.

3 measurement by 10 cycles per sample have been done, obtaining a total of 30 measurements.

Fig. 2.8 The figure shows the cuvette position during first and second methods. It is possible look a longer optical path in position 1 than position 2.

Unfortunately, in this way another problem occured. It had uncertainty too height. There was a too high uncertainty. It has been caused by making 10 measurements (for cycle 3 cycles): there was a deposit of fluorescein at the bottom during the measurement, which caused a non-homogeneity of the solution, and also a photo- degradation due to a longer exposure to light.

The *Fig 2.9* shows as in high concentration there is less absorption by molecules of solution at the highest concentration, and there is a linear increase of fluorescence intensity, but with uncertainty too high.

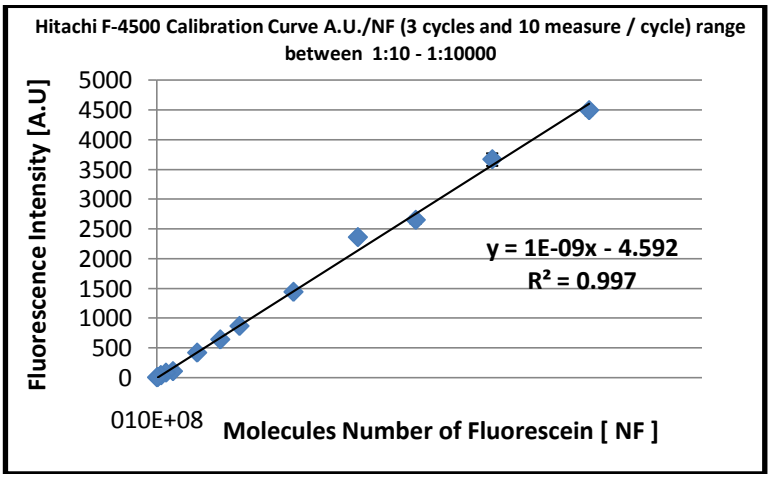

Fig. 2.9 Hitachi F-4500 Calibration Curve A.U./NF (3 cycles and 10 measure / cycle). Range between 1:10 - 1:10000.

- ***The third method*** has been effected to avoid deposit of fluorescein at the bottom of cuvette, between one measurement and another. The sample was pulled out, and the fluorescein solution was mixed with a syringe to have an homogeneous solution during measurements. For increase number of mix during tests measurements has been increased from 3 until 5 and number of cycle decreased from 10 until 3, with a total of 15 measurements per concentration.

In this way it always had high number of measurements but with more break to mix the solution.

The *Fig 2.10* shows as mixing solution between one measurement and another. The fit is more linear than *Fig.2.8*.

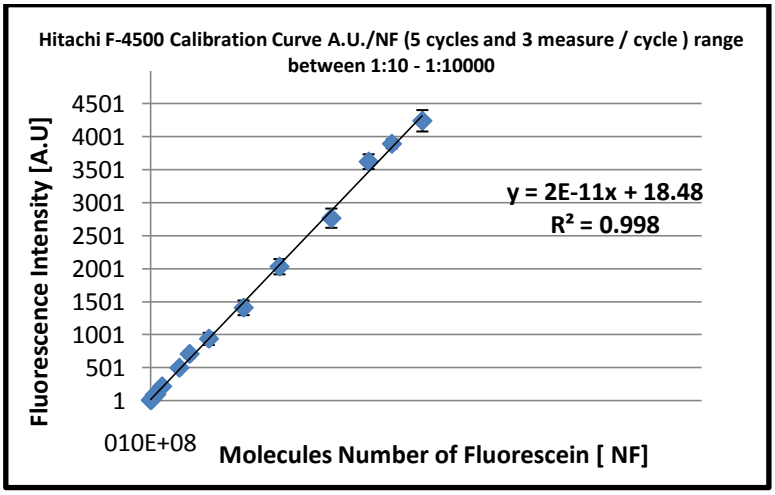

Fig. 2.10 Hitachi F-4500 Calibration Curve A.U./NF (5 cycles and 3 measure / cycle). Range between 1:10 - 1:10000.

- ***The fourth method*** was necessary because having one calibration curve, for both region of concentrations, caused still too much uncertainty during comparison between different instruments for this purpose the single curve was divided in two different region of calibration In this way uncertainty has been reduced again.

The *Fig.2.11* shows fit of concentration regions between *1:10 and 1:1000* for *Hitachi F-4500.*

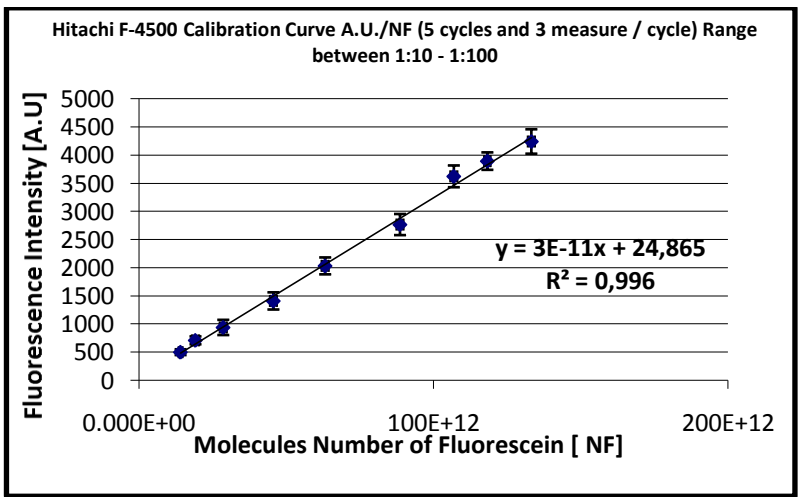

Fig. 2.11 Hitachi F-4500 Calibration Curve A.U./NF (5 cycles and 3 measure / cycle). Range between 1:10 - 1:100.

The *Fig.2.12* shows fit of concentration regions between 1*:250 and 1:10000* for *Hitachi F-4500*

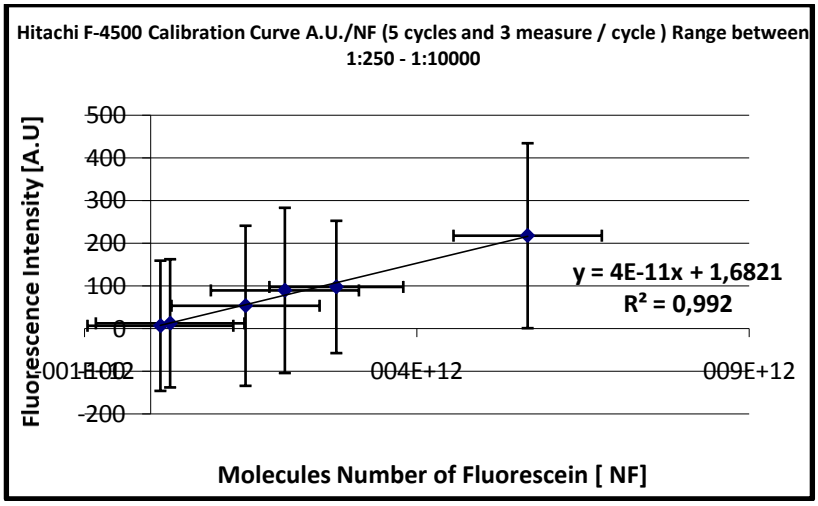

Fig. 2.12 Hitachi F-4500 Calibration Curve A.U./NF (5 cycles and 3 measure / cycle) . Range between 1:250 - 1:10000.

2.5.2 Preliminary studies using Tecan Infinite F-200

Before starting measurements, it was very important to aquire *Tecan Infinite F-200* gain curve. In this way it was possible to set the best gain for measurements.

After a lot of studies, the best gain to use for all measurements was found at 60. This value allowed to effect different measures at different concentrations without going out of scale. Starting from gain 60, following gain curve, is possible to convert gain as required, (e.g. 60 to 100).

Gain characterization was studied before starting any measurement on *Tecan Infinite F-200*, fitting the gain curve equation curve has been obtained.

The *Fig.2.13* shows fit of Gain value. The same solution has been used for each point.

After fitting *y = 9,2149e0,0693x* equation has been obtained.

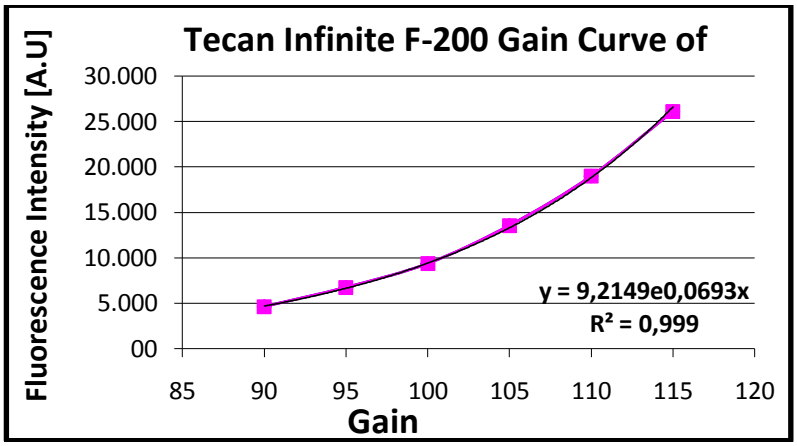

Fig. 2.13 Tecan Infinite F-200Gain Curve

Using the experience from *Hitachi F-4500* measurements, fourth method has been used directly. The Fig.2.14 shows fit of concentration in range between *1:10 and 1:100* dilutions for *Tecan Infinite F-200.*

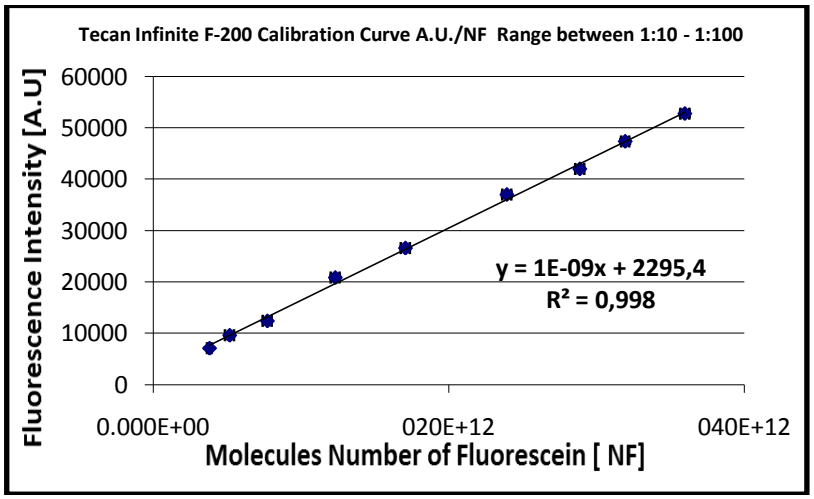

Fig. 2.14 Tecan Infinite F-200 Calibration Curve A.U./NF Range between 1:10 - 1:100

The *Fig.2.15* shows fit of concentration regions between *1:250 and 1:10000* for *Tecan Infinite F-200*

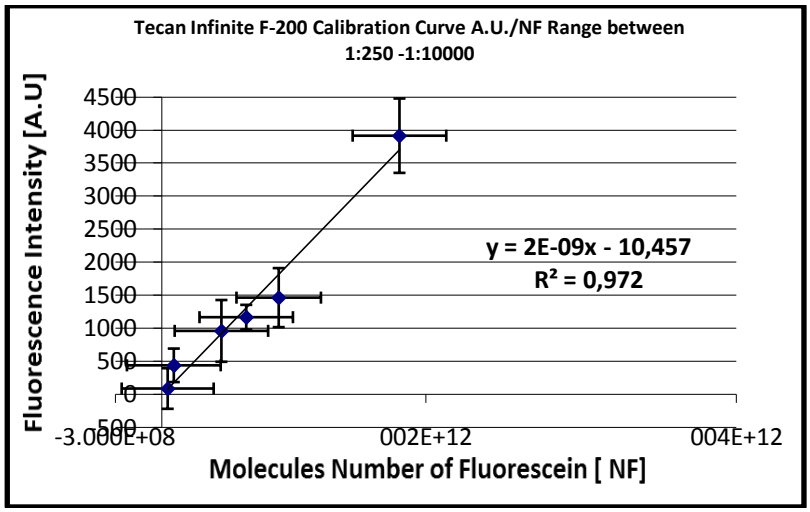

Fig. 2.15 Tecan Infinite F-200 Calibration Curve A.U./NF. Range between 1:250 -1:10000

CHAPTER 3

Equation of the Measurand

In this chapter the *MESF* model is discussed. The theoretical model is described with the explanation of the fluorescence intensity and mass of calibrateion sample. The analysis of the experimental model is then performed in order to define, *NF* (number of fluorescein molecules) of calibrateion sample and then *FQ* (fluorescence Quantity) of Unknown sample expressed in *MESF*.
NF (molecules number of fluorescein) has been distinguished by *FQ* expressed in *MESF* (Molecules of Equivalent Soluble Fluorochrome) of an unknown sample, but they are basically the same thing.

3.1 The experimental model

As said in chapter 2, the fluorescence intensity increases linearly with the amount of molecules present in the solution. This is possible to look Eq. 2.5:

$$I = m * FQ + q$$

Where we remember:
I = measured fluorescence intensity
m = angular coefficient of the curve
FQ = fluorescence quantity in *MESF* (of Unknown fluorophore
q= intercept of the curve

In this equation, q (intercept) has been explained as:

$$q = \overline{I_t} + m * \overline{NF_t} \qquad \textit{Eq. 3.1}$$

Where:

$\overline{I_t}$ = average of the values of fluorescence intensity of samples calibration

$\overline{NF_t}$ = average of number of molecules *"NF"* of Fluorescein of calibration
NF (number of fluorescein molecules) has been distinguished from *MESF* (Molecules of Equivalent Soluble Fluorochrome of an unknown, but they are basically the same thing.

By equations *Eq 2.5* and *Eq 3.1* is obtained the following equations of the measurand:

$$FQ = \frac{(I_u - \overline{I_t} + m * \overline{NF_t})}{m} \qquad \textit{Eq. 3.2}$$

For each concentration range, 2 different *FQ* equations are considered, and than *Eq 3.2* became:

$$FQ_{1,2} = \frac{(I_u - \overline{I_{t1,2}} + m * \overline{NF_{t1,2}})}{m} \qquad \textit{Eq. 3.3}$$

Once obtained equation of measurand, as shown in the following chapter, some possible influence quantities have been considered, and their uncertainties have been estimated.

CHAPTER 4

Characterization of the influence quantities of Fluorescence Quantity

Some influence quantities, like light noise, uncertainty of instruments, uncertainty in solutions preparation and uncertainty in volume measurements have been identified. Any considered uncertainty has been estimated and they will be show in the following paragraph.

As shown in chapter 3 measurand equation is:

$$FQ_{1,2} = \frac{(I_u - \overline{I_{t1,2}} + m * \overline{NF_{t1,2}})}{m} \qquad \textit{Eq. 4.1}$$

Starting from this equation, all influence quantities of fluorescence will be shown.

4.1 Average of Fluorescence Intensity of calibration samples $\overline{I_t}$

As said before, uncertainty sources have been identified, but unfortunately it has not been estimated for any component yet.

To estimate uncertainty has been calculated standard deviation of all measure, where any component could not to be estimated in other way.

Repeatability and reproducibility standard deviations have been considered to estimate I_t uncertainty.

- ***Standard deviation on Repeatability:***

The maximum value between all *standard deviation on repeatability* calculated, for any cycle, at specific concentration, has been considered, and it has been estimated as follows:

$$\sigma_{repeatibility} = \sigma_{MAX\_std\_cycle} = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \langle x \rangle)^2}{N-1}} \qquad \textit{Eq. 4.2}$$

Where we can find:

x_i = peak value of i-th measurement

$\langle x \rangle =$ average peaks value of the considered cycle

N = number of measurements of considered cycle

- ***Standard deviation on Reproducibility:***

To estimate reproducibility standard deviation, the average between all *standard deviation* on *repetibility* for any concentration has been calculated.

$$\sigma_{reproducibility} = \frac{\sum_{i=1}^{N} \sigma_i}{N} \qquad \textit{Eq. 4.3}$$

Here, we can find:

σ_i = $\sigma_{repeatibility}$ of i-th cycle

N = number of peak cycle for any concentration

The total uncertainty estimated is shown in the following equation:

$$\mu(I_{it}) = \sqrt{\sigma^2{}_{ripet} + \sigma^2{}_{ripro}} \qquad \textit{Eq. 4.4}$$

$\overline{I_t}$ Has been defined as average of the sum of the all fluorescence intensity values at specific, range dilution, at different concentrations. For both ranges dilution $\overline{I_t}$ has been calculated as follows:

$$\overline{I_{t1,2}} = \sum_{i=min\_range}^{max\_range} I_{it} \qquad \textit{Eq. 4.5}$$

Where:

I_{it} = calibration value at a concentration included in a specific range dilution and have been measured by fluorescence instrument as said before in chapter 2.

In *Eq.4.5* is shown $\overline{I_{t1,2}}$ of fluorescence value for each dilution method.

Uncertainty $\mu(I_t)$ has been estimated as follows:

$$\mu\left(\overline{I_{t1,2}}\right) = \frac{\sqrt{n}}{n}\mu\left(I_{it1,2}\right)_{max\_range\_1,2} \qquad \textit{Eq. 4.6}$$

Uncertainty $\mu(\overline{I_{t1,2}})$ has been estimated considering the maximum value between all values of $\mu(I_{it1,2})$.
In this way, the largest uncertainty range has been considered.

4.1.1 Values obtained of $\mu(\overline{I_{t1,2}})$

To estimate $\mu(\overline{I_{t1,2}})$ has been used *Eq 4.1*. In this equation is included the highest value of uncertainty of fluorescence intensity, $\mu(I_{it1,2})_{max\_range\_1,2}$ for each range.

Below is shown estimated value of $\mu(\overline{I_{t1,2}})$ for each instrument considered.

- ***Hitachi F-4500***

  In range at one dilution has been considered the uncertainty value corresponding to 1:50 ration concentration:

$$\mu(\overline{I_{it1}})_{1:50}\% = 14\ \%$$

While in range at two dilutions has been considered the uncertainty value corresponding to 1:750 ration concentration:

$$\mu(\overline{I_{it2}})_{1:750}\% = 12\ \%$$

- ***Tecan Infinite F-200***

  As for *Hitachi F-4500*, to estimate $\mu(\overline{I_{t1,2}})$ the same method has been used.
  In range at one dilution has been considered the uncertainty value corresponding to 1:30 ration concentration:

$$\mu(\overline{I_{it1}})_{1:30}\% = 1{,}5\ \%$$

While in range at two dilutions has been considered the uncertainty value corresponding to 1:5000 ration concentration:

$$\mu(\overline{I_{it2}})_{1:5000}\% = 1{,}4\ \%$$

4.2 Fluorescence Intensity of Unknown sample I_u

I_u is fluorescence intensity measured of Unknown sample. Uncertainty $\mu(I_u)$ has been considered the same of $\mu(\overline{I_t})$.

4.3 Angular Coefficient m

m has been estimated from the curve fit equation (see chapter 2). After fitting, a mathematical check through the following relation, has been done:

$$m = \sum \frac{[(I_i - \overline{I_t}) * (NF_i - \overline{NF_t})]}{\sum[(NF_i - \overline{NF_t})^2]} \qquad \textit{Eq. 4.7}$$

Starting from above relation, uncertainty relation of μ(m) has been estimated as follows:

$$\mu(m) = \frac{1}{n-2} \sum \frac{[(I_{it} - \overline{I_t})]}{\sum[(NF_{it})^2] - \frac{(\sum NF_{it})^2}{n}} \qquad \textit{Eq. 4.8}$$

Where:

NF_{it} = calibration value at a concentration expressed in number of molecules of fluorescein at i-th for each concentration. In *chapter 5*, NF_{it} uncertainty will be show.

4.4 Average of number of molecules of fluorescein $\overline{NF_t}$

As $\overline{I_t}$, $\overline{NF_t}$ has been defined as average of the sum of the all fluorescence intensity values at different concentrations, as follows:

$$\overline{NF_{t1,2}} = \frac{\sum_{i=min\_range}^{max\_range} NF_{it1,2}}{n} \qquad \textit{Eq. 4.9}$$

In Eq. 4.9 is shown $\overline{NF_{t1,2}}$ for each dilution method.

Uncertainty $\mu(\overline{NF_{t1,2}})$ has been estimated as follows:

$$\mu\left(\overline{NF_{t1,2}}\right) = \frac{\sqrt{n}}{n} \mu\left(NF_{it1,2}\right)_{max\_range\_1,2} \qquad \textit{Eq. 4.10}$$

Uncertainty $\mu\left(\overline{NF_{t1,2}}\right)$ has been estimated considering between all values of the $\mu\left(NF_{it1,2}\right)$ the maximum value. In this way, the largest uncertainty range has been considered.

CHAPTER 5

Characterization of the influence quantities of Number of Fluorescein molecules

In this chapter is quantified the uncertainty of each influence quantity in the *NF* measurand equation, as reported in chapter 4. Each quantification is related to the measurements carried out at INRIM.
To have a $NF_{1\ and}\ NF_2$ relation, some physical quantities have been considered. These physical quantities are the most important variables that influence *NF* calculation.

5.1 NF Equations of Hitachi F-4500

To estimated *NF*, Na, stock concentration solution and stock fluorescein mass have been considered. The masses have been measured with the analytical balance Sartorius CPA225D model, that has been shown in chapter 2.
The sample was composed by cuvette ,with shape of a parallelepiped (with depth, width, height of the incident light beam indicated respectively as L,d and H) and fluorescein solution inside it. The parallelepiped measurement has been effected with a caliber.

$$NF_{it1} = N_A * \left[C_{madre} * \left(\frac{m_f}{m_{sol}} \right) * \rho_f * [L * H * d] \right] \qquad \textit{Eq. 5.1}$$

$$NF_{it2} = N_A * \left[C_{madre} * \left(\frac{m_f}{m_{sol_{1:100}}} \right)_{1:100} * \left(\frac{m_{f_{1:100\_2}}}{m_{stock\_sol}} \right)_{R1} * \rho_f * [L * H * d] \right] \qquad \textit{Eq. 5.2}$$

Where:

N_A = Avogadro constant;

C_{Stock}= stock solution concentration;

m_f = mass of stock fluorescein;

m_{sol}= mass of solution;

m_{sol_100} = mass of solution at concentration 1:100;

m_{sol_100}= portion mass of sololution at concentration 1:100 used as stock solution for 2 dilution method;

ρ_f = fluorescein density;

L=cuvette width

d = cuvette depth

H= height of the incident light beam

5.1.1 Cuvette width $\mu(d)$, and cuvette depth $\mu(L)$ uncertainty

To estimated L and d uncertainty, standard deviation of 10 measurements have been calculated, σ_{L_ripet}, (using caliber on the same sample) and caliber sensibility, $\mu(c)$.

Uncertainties have been estimated as follows:

$$\mu(L) = \sqrt{{\sigma_{L\_ripet}}^2 + \mu(c)^2} \qquad \textit{Eq. 5.3}$$

Where:

σ_{L_ripet} = standard deviation on 10 measurements using caliber on the same sample

$\mu(c)$= caliber sensitivity

$$\mu(d) = \sqrt{{\sigma_{d\_ripet}}^2 + \mu(c)^2} \qquad \textit{Eq. 5.4}$$

Where:

σ_{d_ripet} = standard deviation on 10 measurements using caliber on the same sample

$\mu(c)$ = caliber sensitivity

5.1.2 Height Uncertainty of incident light beam, $\mu(H)$

Height of the incident light beam has been measured with a caliber. To know the true height of the incident light beam a caliber has been used on a semi-transparent paper. The measurement has been effected on the paper at the same distance from the cuvette center.

To estimate H uncertainty, $\mu(H)$, standard deviation of 10 measurements has been calculated, σ_{H_ripet} , (using caliber on the same sample) and caliber sensibility, $\mu(c)$.

Uncertainties have been estimated as follows μ(L) and μ(d):

$$\mu(H) = \sqrt{\sigma_{H\_ripet}{}^2 + \mu(c)^2} \qquad \textit{Eq. 5.5}$$

Where as in the previous equation :
σ_{H_ripet} = standard deviation on 10 measurements using caliber on the same sample;
μ(c) = caliber sensitivity

5.1.3 Density of solution Uncertainty, $\mu(\rho_f)$

ρ_f uncertainty, $\mu(\rho_f)$, has been estimated as average difference value between ρ_{H2O} = 1 g/ml and $\rho_{fluorescein\ stock}$ = 1,003 g/ml, as is shown below:

$$\mu(\rho_f) = \frac{\rho fluorescein\ stock\ + \rho_{H2O}}{2} \qquad \textit{Eq. 5.6}$$

5.1.4 Mass Uncertainty, μ(m)

To estimate *m* uncertainty, $\mu(m)$, standard deviation of 10 measurements have been calculated, σ_{m_ripet}, (using balance Sartorius CPA225D on the same sample) and balance sensibility, μ(b), .
Uncertainties have been estimated as follows:

$$\mu(m) = \sqrt{\sigma_{m\_ripet}{}^2 + \mu(b)^2} \qquad \textit{Eq. 5.7}$$

Where:
σ_{L_ripet} = standard deviation on 10 measurements using the balance Sartorius CPA225D on the same sample
μ(b) = balance sensitivity

5.1.5 Stock solution concentration Uncertainty, $\mu(C_{stock})$

Uncertainty C_{stock} has been found in NIST Certificate of Analysis of standard referenc Material 1932 [28].

$$\mu(C_{stock}) = \pm 0{,}40\ \mu mol * kg^{-1} \qquad \textit{Eq. 5.8}$$

5.2 NF Equations of Tecan Infinite F-200

The same uncertainties have been considered for m, ρ_f, C_{stock}, but, the sample shape of *Tecan Infinite F-200* was different from sample shape of *Hitachi F-4500*.
The sample was composed by a cylindrical well with Height and radius measured with a caliber.

$$NF_{it1} = N_A * \left[C_{madre} * \left(\frac{m_f}{m_{sol}} \right) * \rho_f * [\pi r^2 * H] \right] \quad \textit{Eq. 5.9}$$

$$NF_{it2} = N_A * \left[C_{madre} * \left(\frac{m_f}{m_{sol_{1:100}}} \right)_{1:100} * \left(\frac{m_{f_{1:100\_2}}}{m_{stock\_sol}} \right)_{R1} * \rho_f * [\pi r^2 * H] \right] \quad \textit{Eq. 5.10}$$

To Obtain $\mu(NF_{it1})$ and $\mu(NF_{it2})$ different uncertainty causes (like balance sensibility and repeatability, solution density, caliber sensitivity to measure the solution volume that was exposed to light flow) have been considered.

5.2.1 Well height uncertainty,$\mu(H)$

To estimate H uncertainty, $\mu(H)$, standard deviation of 10 measurements has been calculated, σ_{H_ripet} , (using caliber on the same sample) and caliber sensibility, as in *Hitachi F-4500* sample.
Uncertainties have been estimated as follows:

$$\mu(H) = \sqrt{{\sigma_{H\_ripet}}^2 + \mu(c)^2} \quad \textit{Eq. 5.11}$$

Where:
σ_{H_ripet} = standard deviation on 10 measurements using caliber on the same sample
$\mu(c)$= caliber sensitivity

5.2.2 Diameter Uncertainty of the incident light beam, $\mu(\mathbf{D})$

Diameter of the incident light beam has been measured by difference between well diameter and light distance from well board (given by *Tecan* instrument) as follows:

$$D_{Light} = D_{well} - Distance_{light\ beam} \quad \textit{Eq. 5.12}$$

Where:
D_{well} = diameter of well measured using caliber;
$Distance_{light\ beam}$ = data given by *Tecan* instrument

To estimate *D* uncertainty, $\mu(D)$, standard deviation of 10 measurements has been calculated , σ_{D_ripet}, (using caliber on the same sample) and caliber sensitivity, μ(c),.
Uncertainties have been estimated as follows μ(D):

$$\mu(D) = \sqrt{\sigma_{D\_ripet}{}^2 + \mu(c)^2} \qquad \textit{Eq. 5.13}$$

Where as in the previous equation :
σ_{D_ripet} = standard deviation on 10 measurements using caliber on the same sample
$\mu(c)$= caliber sensitivity

In the next chapter, uncertainty budget of fluorescein molecules for *Hitachi F-4500* and *Tecan Infinite F-200* will be shown.

CHAPTER 6

Uncertainty Budget of Number of Fluorescein molecules

Operational parameters, measured variables and uncertainties were discussed in the previous chapters where several approaches of number of fluorescein molecules *(NF)* were proposed. In this chapter uncertainty budget of *NF* will be shown.

Any uncertainty budget regarding any fluorescence instrument and any range analyzed will be discussed in the following paragraphs.

6.1 Uncertainty Budget of NF

Uncertainty Budget has been estimated for any concentration. This method has been adopted with both instruments used, *Hitachi F-4500* and *Tecan infinite F-200*.

The highest *NF* uncertainty among all those estimated for each dilution range, for each instrument used, it shown below.

6.2 NF Uncertainty Budget of Hitachi F-4500

In the following paragraphs uncertainty budget for 1:70 rate concentration, that is the worst case in one dilution range, and *1:10000* ratio concentration, that is the worst case of two dilution range, are shown. To estimate calibration number of fluorescein molecules, *NF*, has been considered the solution volume for samples under light beam of *Hitachi F-4500*.

6.2.1 NF Uncertainty Budget of one dilution range

Uncertainty budget at one dilution, using *Hitachi F-4500* model is shown in the *Table 6.1*. The table shows the highest uncertainty of fluorescein molecules number of calibration, *NF*, for *HitachiF-4500* sample in one dilution range.

Budget NF T1 = calibration number of fluorescein molecules, NF

Uncertainty Budget of 1:70 ratio concentration (the highest uncertainty)

Measurand Equation:

$$NF_{it1} = N_A * \left[C_{stock} * \left(\frac{m_f}{m_{sol}} \right) * \rho_f * [L * H * d] \right]$$

X	[X]	x	**u(x)**	u%(x)	c(x)	$[u(x)c(x)]^2$	Absolute index	Relative index
Variable								
mf	g	0,01457	0,000845	5,80%	1E+15	1E+24	98,57%	100,00%
msol	g	1,00032	0,000845	0,08%	-2E+13	3E+20	0,02%	0,02%
ρf	g/ml	1,0030	0,0015	0,15%	2E+13	8E+20	0,07%	0%
L	dm	0,0198	0,00002	0,10%	1E+15	3E+20	0,03%	0,03%
H	dm	0,0182	0,00002	0,10%	1E+15	3E+20	0,03%	0,03%
d	dm	0,09920	0,00010	0,10%	2E+14	3E+20	0,03%	0,03%
Cstock	mol/Kg	0,0000610	0,0000004	0,66%	3E+17	2E+22	1,26%	1,28%
X	[X]	x	u(x)	u%(x)	k	$[u(x)c(x)]^2$	Absolute index	Relative index
Measurand								
NF	**n.molecules/dm^3**	1,92E+13	1,1E+12	**5,84%**	1	1,25E+24		
X	[X]	x						
Constant								
Na	mol^{-1}	6,0221415E+23						

Table 6.1 Uncertainty budget example of NF at one dilution for Hitachi F-4500

The same thing has been done for two dilutions range, as it will be shown in the following paragraph.

6.2.2 NF Uncertainty Budget of two dilution range

Uncertainty budget *"at two dilutions"*, using *Hitachi F-4500* model is shown in *Table 6.2* The table shows the highest uncertainty of calibration number of fluorescein molecules, *NF*, for *Hitachi F-4500* sample in two dilution range.

Budget NF T2 = calibration number of fluorescein molecules, NF								
Uncertainty Budget of 1:10000 ratio concentration (the highest uncertainty) ***Measurand Equation:*** $NF_{it2} = N_A * \left[C_{stock} * \left(\frac{m_f}{m_{sol_{1:100}}} \right)_{1:100} * \left(\frac{m_{f_{1:100\_2}}}{m_{stock\_sol}} \right)_{R1} * \rho_f * [L * H * d] \right]$								
X	**[X]**	**x**	**u(x)**	**u%(x)**	**c(x)**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Variable								
mf	**g**	0,0215	0,0008	3,92%	7E+12	3E+19	18,84%	23,38%
msol1:100	**g**	2,0101	0,0008	0,04%	-7E+10	4E+15	0,00%	0,00%
msol1:100_2	**g**	0,0104	0,0008	8,10%	1E+13	1E+20	80,56%	100%
msol	**g**	1,0262	0,0008	0,08%	-1E+11	1E+16	0,01%	0,01%
ρf	**g/ml**	1,0030	0,0015	0,15%	2E+13	8E+20	0,07%	0%
L	**dm**	0,0198	0,00002	0,10%	1E+15	3E+20	0,03%	0,03%
H	**dm**	0,0182	0,00002	0,10%	1E+15	3E+20	0,03%	0,03%
d	**dm**	0,09920	0,00010	0,10%	2E+14	3E+20	0,03%	0,03%
Cstock	**mol/Kg**	0,0000610	0,0000004	0,66%	3E+17	2E+22	1,26%	1,28%
X	**[X]**	**x**	**u(x)**	**u%(x)**	**k**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Measurand								
NF	**n.molecules/ dm^3**	1,44E+11	1,3E+10	9,03%	1	1,7E+20		
X	[X]	x						
Constant								
Na	**mol-¹**	6,0221415E+23						

Table 6.2 Uncertainty budget example of NF at two dilutions for Hitachi F-4500.

6.3 NF Uncertainty Budget of Tecan Infinite F-200

In the following paragraph uncertainty budget for *1:70 rate* concentration, that is the worst case in one dilution range, and *1:10000* ratio concentration, that is the worst case of two dilution range are shown. To estimate the calibration number of fluorescein molecules, *NF* , like in the previous paragraph , has been considered the solution volume for samples under light beam of *Tecan Infinite F-200* system..

6.3.1 NF Uncertainty Budget of one dilution range

Uncertainty budget at one dilution, using *Tecan Infinite F-200* model is shown in *Table 6.3*. The table shows the highest uncertainty of calibration number of fluorescein molecules, *NF*, for *Tecan Infinite F-200* sample in one dilution range.

Budget NF T1 = calibration number of fluorescein molecules, NF								
Uncertainty Budget of 1:70 ratio concentration (the highest uncertainty) ***Measurand Equation:*** $NF_{it1} = N_A * \left[C_{stock} * \left(\frac{m_f}{m_{sol}} \right) * \rho_f * [\pi r^2 * H] \right]$								
X	**[X]**	**x**	**u(x)**	**u%(x)**	**c(x)**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Variable								
mf	**g**	0,01457	0,0008	5,80%	4E+14	9E+22	98,61%	100,00%
msol	**g**	1,0003	0,0008	0,08%	-5E+12	2E+19	0,02%	0,02%
ρf	**g/ml**	1,0030	0,0015	0,15%	5E+12	6E+19	0,07%	0%
H	**dm**	0,02325	0,00002	0,10%	2E+14	2E+19	0,03%	0,03%
Radius	**dm**	0,01150	0,00001	0,10%	3E+14	1E+19	0,03%	0,03%
Cmadre	**mol/Kg**	0,0000610	0,0000004	0,66%	8E+16	1E+21	1,26%	1,28%
X	**[X]**	**x**	**u(x)**	**u%(x)**	**k**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Measurand								
NF	**n.molecules/ dm^3**	5,18E+12	3,0E+11	5,84%	1	9,16E+22		
X	[X]	x						
Constant								
Na	**mol-¹**	6,0221415E+23						
π		3,141592654						

Table 6.3 Uncertainty budget example of NF at one dilution for Tecan Infinite F-200.

6.3.2 NF Uncertainty Budget of one dilution range

Uncertainty budget at two dilutions, using *Tecan Infinite F-200* model is shown in Table 6.4. The table shows the highest uncertainty of calibration number of *Fluorescein* molecules, *NF*, for *Tecan Infinite F-200* sample in one dilution range.

Budget NF T2 = calibration number of fluorescein molecules, NF

Uncertainty Budget of 1:10000 ratio concentration (the highest uncertainty)

Measurand Equation:

$$NF_{it2} = N_A * \left[C_{stock} * \left(\frac{m_f}{m_{sol_{1:100}}} \right)_{1:100} * \left(\frac{m_{f_{1:100\_2}}}{m_{stock\_sol}} \right)_{R1} * \rho_f * [\pi r^2 * H] \right]$$

X	[X]	x	u(x)	u%(x)	c(x)	$[u(x)c(x)]^2$	Absolute index	Relative index
Variables								
mf	**g**	0,0216	0,0008	3,92%	2E+12	2E+18	18,83%	23,38%
msol1:100	**g**	2,0101	0,0008	0,04%	-2E+10	3E+14	0,002%	0,003%
Msol1:100_2	**g**	0,0104	0,0008	8,10%	4E+12	1E+19	80,54%	100%
msol	**g**	1,0003	0,0008	0,08%	-4E+10	1E+15	0,008%	0,01%
ρf	**g/ml**	1,0030	0,0015	0,15%	4E+102	3E+15	0,03%	0,03%
H	**dm**	0,02325	0,00002	0,10%	2E+12	1E+15	0,01%	0,01%
Radius	**dm**	0,01150	0,00001	0,10%	7E+12	6E+15	0,04%	0,06%
Cstock	**mol/Kg**	0,0000610	0,0000004	0,66%	6E+14	6E+16	0, 53%	0,65%
X	**[X]**	**x**	**u(x)**	**u%(x)**	**k**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Measurand								
NF	**n.molecules/ dm^3**	3,88E+10	3,5E+09	9,03%	1	1,2E+19		
X	[X]	x						
Constant								
Na	**mol-1**	6,0221415E+23						
π		3,141592654						

Table 6.4 Uncertainty budget example of NF at two dilutions for Tecan Infinite F-200.

As possible to see, in all uncertainty budgets, the same results have been obtained as in *Hitachi F-4500* system. The calculation procedures have been effected in different time and with different equations. In this way, it has been possible to confirm the true value of uncertainty budget regarding for the preparation of solutions.

CHAPTER 7

Uncertainty Budget of Fluorescence Quantity FQ

Considering all parameters, has been possible to estimate the uncertainty budgets of *FQ*.
Uncertainty budget of *FQ* for any range has been estimated, but, here uncertainty budget of hypothetical measurement is reported as an example. An example for any instrument used will be shown.
There is a relation between *FQ* uncertainty budget and *NF* uncertainty budget, because inside FQ equation *(see Eq.4.1)* $\overline{NF_t}$ is present.

7.1 FQ Uncertainty Budgets of Hitachi F-4500

In *Table 7.1* is possible to see an example of *FQ* uncertainty budget of *Hitachi F-4500* system for a measurement done at *2550 A.U* includes in one dilution range.
A conversion of ***2550 A.U*** results to be ***7,8E+13 ± 5,4E+12 MESF.*** This means that there is an uncertainty percentage of ***6,9%***

Budget FQ1= equivalent molecules of fluorescein , MESF

Measurand Equation:

$$FQ_1 = \frac{(I_u - \overline{I_{t1}} + m * \overline{NF_{t1}})}{m}$$

Uncertainty budget:

X	[X]	x	u(x)	u%(x)	c(x)	$[u(x)c(x)]^2$	Absolute index	Relative index
Variables								
$\overline{NFt}$	number of molecules	6,86+13	1,34 E+12	1,95%	1E+00	2E+24	6%	13%
m	**A.U./ad**	3,23E-11	4,61E-12	14,29%	-3E+23	2E+24	**7%**	**13%**
I	**A.U.**	2550	122,02	4,78%	3E+10	1E+25	49%	100%
$\overline{\overline{I_t}}$	**A.U.**	2236	1,07E+02	4,78%	-3E+10	1E+25	38%	77%

X	[X]	x	u(x)	u%(x)	k	$[u(x)c(x)]^2$	Absolute index	Relative index
Measurand								
FQ	**MESF**	7,80E+13	± 5,4E+12	6,9%	1	2,9E+25		

X	[X]	x
Constants		
Na	**mol-¹**	6,02214150E+23

Table 7.1 Uncertainty of fluorescein quantity for Hitachi F-4500 sample in one dilution range at 2550 A.U.

In *Table 7.2* is possible to see an example of *FQ* uncertainty budget of *Hitachi F-4500* system of a measurement effected at 95 A.U included in two dilutions range.

A conversion of ***95 A.U*** results to be ***2,5E+12 ± 2,1E+11 MESF.*** This means that there is an uncertainty percentage of ***8,5%***

Budget FQ1= equivalent molecules of fluorescein, MESF								
Measurand Equation: $FQ_1 = \frac{(I_u - \overline{I_{t1}} + m * \overline{NF_{t1}})}{m}$								
Uncertainty budget:								
X	**[X]**	**x**	**u(x)**	**u%(x)**	**c(x)**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Variables								
$\overline{NFt}$	number of molecules	2,05+12	7,57E+10	3,69%	1E+00	6E+21	13%	36%
m	**A.U./ad**	3,80E-11	9,51E-12	25,00%	-1E+22	1E+22	24%	64%
I	**A.U.**	95	4,83	5,09%	3E+10	2E+22	37%	100%
$\overline{\overline{I_t}}$	**A.U.**	80	4,05E+00	5,09%	-3E+10	1E+22	26%	70%
X	**[X]**	**x**	**u(x)**	**u%(x)**	**k**	**$[u(x)c(x)]^2$**	**Absolute index**	**Relative index**
Measurand								
FQ	**MESF**	2,50E+12	± 2,1E+11	8,5%	1	4,4E+22		
X	**[X]**	**x**						
Constants								
Na	**mol^{-1}**	6,02214150E+23						

Table 7.2 Uncertainty of fluorescein quantity for Hitachi F-4500 sample in two dilutions range at 95 A.U.

7.2 FQ Uncertainty Budgets of Tecan Infinite F-200

In *Table 7.3* is possible to see an example of *FQ* uncertainty budget of *Tecan Infinite F-200* system for a measurement effected at 17500 A.U included in one dilution range.

A conversion of ***17500 A.U*** results to be ***1,1E+13 ± 1,2E+12 MESF.*** This means that there is an uncertainty percentage of ***10,8%.***

Budget FQ1= equivalent molecules of fluorescein, MESF

Measurand Equation:

$$FQ_1 = \frac{(I_u - \overline{I_{t1}} + m * \overline{NF_{t1}})}{m}$$

Uncertainty budget:

X	[X]	x	u(x)	u%(x)	c(x)	$[u(x)c(x)]^2$	Absolute index	Relative index
Variables								
$\overline{NFt}$	number of molecules	1,85+13	3,61 E+11	1,95%	1E+00	1E+23	10%	1%
m	**A.U./ad**	1,40E-09	2,01E-10	14,29%	5E+21	1E+24	89%	100%
I	**A.U.**	17500	85,96	0,49%	7E+08	4E+21	0%	0%
$\overline{\overline{I_t}}$	**A.U.**	24421	1,40E+02	0,49%	7E+08	1E+22	1%	0%

X	[X]	x	u(x)	u%(x)	k	$[u(x)c(x)]^2$	Absolute index	Relative index
Measurand								
FQ	**MESF**	1,10E+13	± 1,2E+12	10,8%	1	1,4E+24		

X	[X]	x
Constants		
Na	**mol-¹**	6,02214150E+23

Table 7.3 Uncertainty of fluorescein quantity for Tecan Indinite F-200 sample in one dilution range at 17500 A.U.

In Table 7.4 is possible to see an example of *FQ* uncertainty budget of *Tecan Infinite F-200* system for a measurement done at *3500 A.U* included in two dilutions range.

A conversion of ***3500 A.U*** results to be **1,*37E+12 ± 2E+11 MESF*.** This means that there is an uncertainty percentage of ***14,9%***

Budget FQ2= equivalent molecules of fluorescein, MESF

Measurand Equation:

$$FQ_2 = \frac{(I_u - \overline{I_{t2}} + m * \overline{NF_{t2}})}{m}$$

Uncertainty budget:

X	[X]	x	u(x)	u%(x)	c(x)	$[u(x)c(x)]^2$	Absolute index	Relative index
Variables								
$\overline{NFt}$	number of molecules	5,55E+11	2,05E+10	3,69%	1E+00	4E+20	1%	1%
m	**A.U./ad**	2,66E-09	6,66E-10	25,00%	-3E+20	4E+22	99%	100%
I	**A.U.**	3500	19,77	0,56%	4E+08	6E+19	0%	0%
$\overline{\overline{I_t}}$	**A.U.**	1338	7,56E+00	0,56%	-4E+08	8E+18	0%	0%

X	[X]	x	u(x)	u%(x)	k	$[u(x)c(x)]^2$	Absolute index	Relative index
Measurand								
FQ	**MESF**	1,37E+12	± 2,0E+11	14,9%	1	4,2E+22		

X	[X]	x
Constants		
Na	**mol-¹**	6,02214150E+23

Table 7.4 Uncertainty of fluorescein quantity for Tecan Infinite F-200 sample in two dilutions range at 3500 A.U.

7.3 Result: Performances FQ proposed method

After measurements, a study on sensitivity has been conducted. It has been necessary because it was important to know how uncertainty gone with *MESF*.

In general we can see two curves, one curve on the left, refering to a *ONE DILUTION METHOD* (blue curve), a second curve on the left refering to *TWO DILUTION METHOD* (pink curve).

7.4 Sensitivity study of Hitachi F 4500

As we can see, the pink curve starts from ***1E+12 MESF*** until ***171E+12 MESF***, while the blue curve starts from ***21E+12 MESF*** until ***309E+12 MESF*.** The best use to have the lowest possible uncertainty curve consist in using the pink curve in a range between ***1E+12 MESF*** and ***29E+12 MESF*** **(**coinciding with two dilution range**),** and the blue curve in a range between ***32E+12 MESF*** and ***309E+12 MESF*** **(**coinciding with one dilution range).

In the *Fig.7.1* are shown sensitivity curves of *Hitachi F-4500* about *ONE DILUTION RANGE* (blue curve) and *TWO DILUTION RANGE* (pink curve).

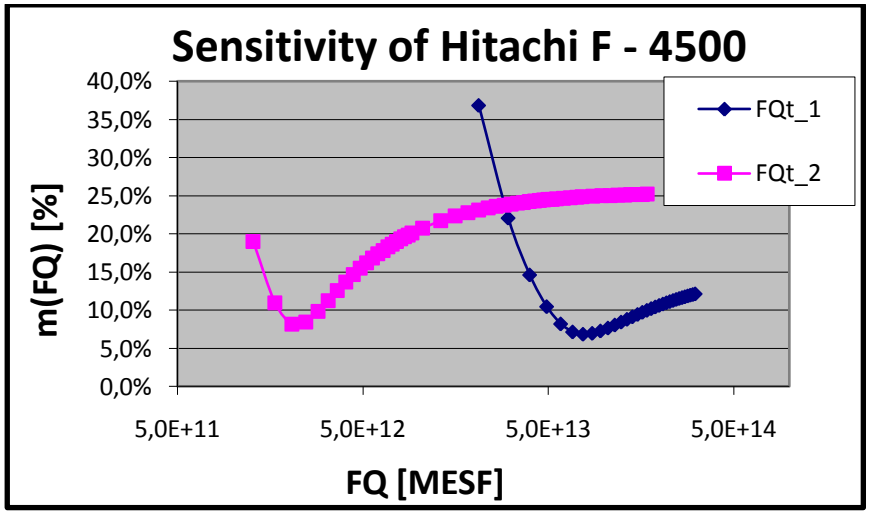

Fig. 7.1 Sensitivity curves of Hitachi F-4500.

7.5 Sensitivity study of Tecan Infinite F-200

As we can see, this case is very similar to the one seen before. The pink curve starts about ***8E+08 MESF*** until ***09E+12 MESF*** , while blue curve starts from ***04E+12 MESF*** until ***36E+12 MESF*.** The best use to have the lowest possible uncertainty curve consist of to use the pink curve in a range between ***8E+08 MESF*** and ***07E+12 MESF*** **(**coinciding with two dilution range**),** while the blues curve in a range between ***09E+12 MESF*** and ***36E+12 MESF*** **(**coinciding with one dilution range).

In *Fig.7.2* are shown sensitivity curves of *Tecan Infinite F-200* about *ONE DILUTION RANGE* (blue curve) and *TWO DILUTION RANGE* (pink curve).

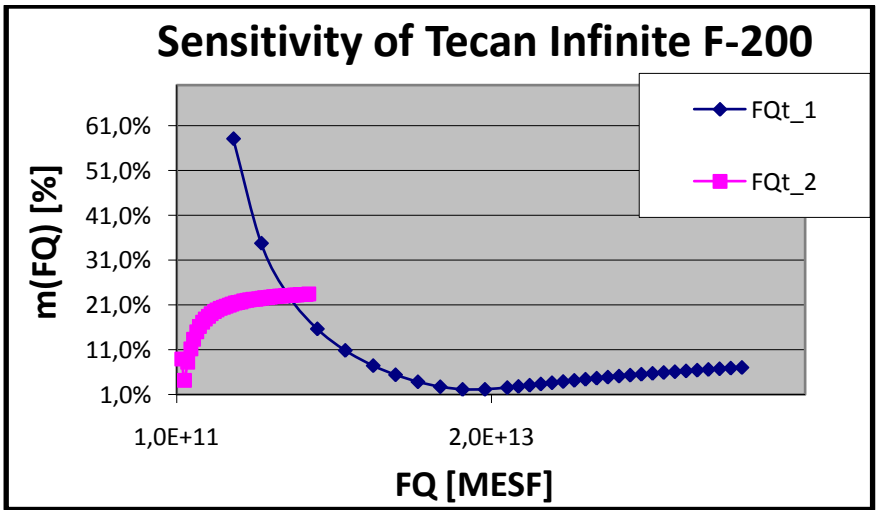

Fig. 7.2 Sensitivity curves of Tecan Infinite F-200.

In the *Fig.7.1* and *Fig.7.2* it is possible to see that in both graphs, the two dilution range has an higher uncertainty than *ONE DILUTION RANGE*. That is because are that *TWO DILUTION RANGE* requires more steps in solution preparation, and it has a lower fluorescence intensity, that is more influenced by noise, than one dilution range.

CHAPTER 8

Applications of the method

The last part of this work consists on applying the method on unknown samples using both system studied.
To have a validation for the applied method, a comparison between different fluorophores, using both fluorescence instruments, was necessary.
For this purpose comparison between *NuclearMask Blue* ®[25], *CellMask Green* ® [30] and *Fluoresceine* [28] have been effected.
In this way it has been possible to verify if measurements on each instrument were comparable to each other.
In the following paragraphs obtained measurements will be shown.

8.1 Result of comparison of measurements

The aim is to check whether the method *FQ* allows to compare the emission of fluorescence between various instruments and consequently improve the accuracy of fluorescence measurement.
As possible to see in the following figures *(Fig.8.1, Fig.8.2, Fig.8.3),* for any fluorophores and instrument, the mean values are within the error bars.
This is the reason why we can say that this method can be used and improved.
In the future other studies will be done to broaden the use and decrease the uncertainty associated with the method.

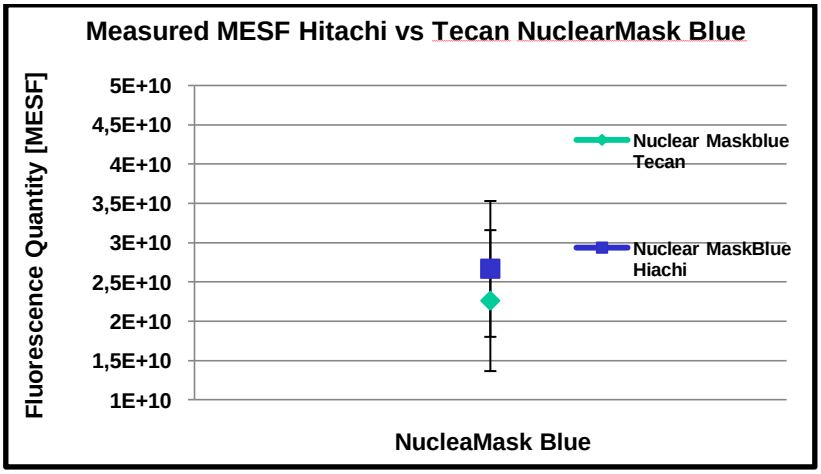

Fig. 8.1 Comparison between Hitachi F-4500 measurement and Tecan Infinite F-200 measurement of NuclearMask Blue.

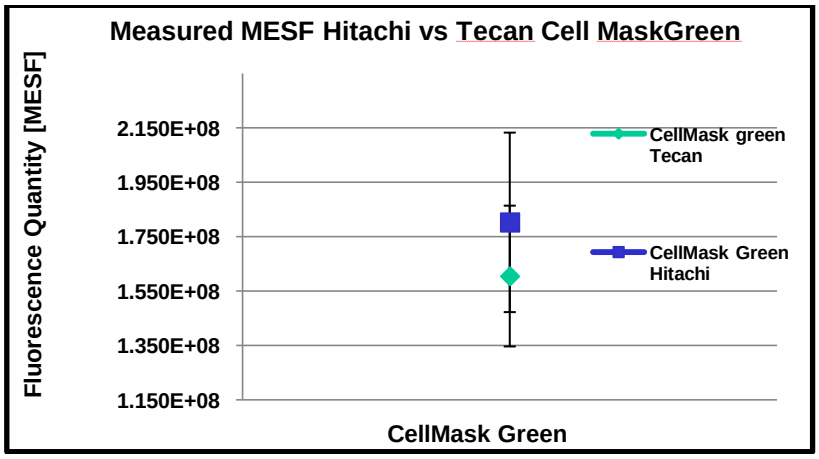

Fig. 8.2 Comparison between Hitachi F-4500 measurement and Tecan Infinite F-200 measurement of CellMask Green.

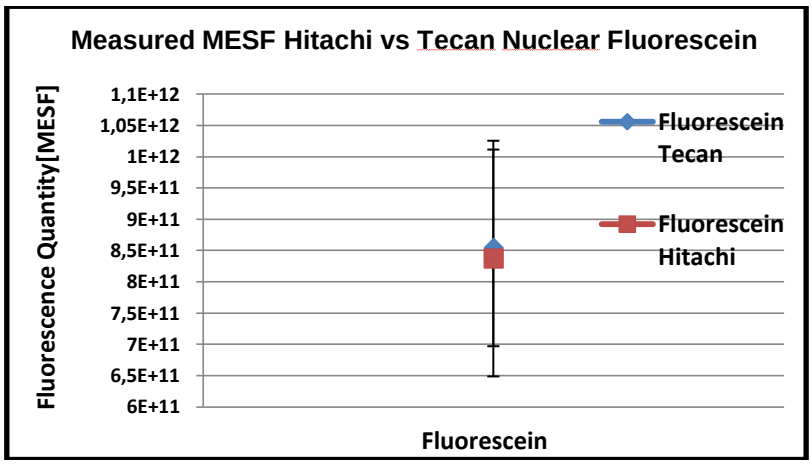

Fig. 8.3 Comparison between Hitachi F-4500 measurement and Tecan Infinite F-200 measurement of Fluorescein.

CHAPTER 9

Conclusions and future developments

In conclusion all goals obtained in this work are summarizes in the following points:

- Study of a method to prepare fluorescein solutions of calibration;
- Characterization of fluorescence instruments ad their calibration;
- Study of a measurement method of the calibration samples;
- Calculation of the uncertainty budget of the samples preparation expressed in number of molecules of fluorescein, *NF*;
- Calculation of the uncertainty budget of the measurements expressed in equivalent number of molecules of fluorescein, *MESF*, for *Hitachi F-4500*;
- Calculation of the uncertainty budget of the measurements expressed in equivalent number of molecules of fluorescein, *MESF*, for *Tecan Infinite F-200*;

In the Future it will be possible to do the same work using many more instruments that use fluorescence principle, like confocal - microscope, citofluorimeter, PCR, ect…

In this way it will be possible to compare all kind of measurements, like photostability, photobleaching or fluorescence imaging.

Below are shown examples of measurements taken in the past expressed in *A.U.*

The *Fig. 9.1* shows photobleaching study using two photons excitation microscopy (TPE), at 784 nm, on *Human Mesenchymal Stem Cell* (hMSC) stained with *NuclearMask Blue*.. The power of laser beam on the sample was 32 mW. A 20x objective was used during the experiment.

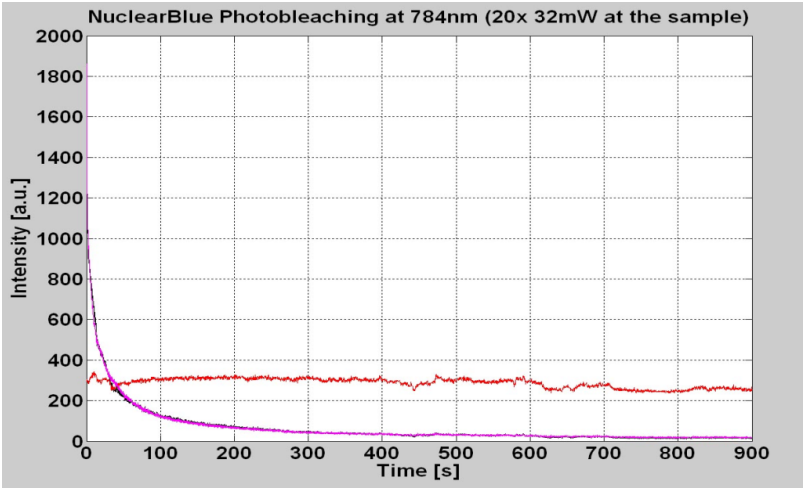

Fig. 9.1 Photobleaching study using two photons excitation microscopy (TPE) [31]

In *Fig.9.2* are shown two examples of imaging taken using two photon extitation microscopy, (TPE) [31].To the left it is possible to see a cytoplasm dyed using *CellMask Green*, while the image on the right shows a nucleus dyed using *Dapi* [32].

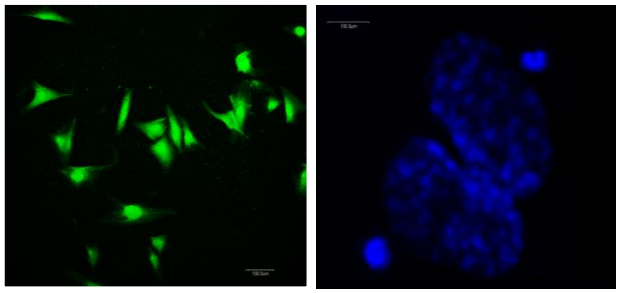

Fig. 9.2 two photon extitation microscopy, (TPE) imaging [33].

It is possible to imagine that in the future, we will have a standard protocol of measurement expressed with the same unit.

In this way, we will have the possibility to compare all parameters of studied fluorophores directly, and than to have a database expressed in *FQ* that will be independent from the instruments used to get data.

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8. Edward Daniel Clarke (1819) "Account of a newly discovered variety of green fluor spar, of very uncommon beauty, and with remarkable properties of colour and phosphorescence," *The Annals of Philosophy*, **14** : 34 - 36; from page 35: "The finer crystals are perfectly transparent. Their colour by transmitted light is an intense *emerald green*; but by reflected light, the colour is a deep *sapphire blue*; … ".
9. Haüy merely repeats Clarke's observation regarding the colors of the specimen of fluorite which he (Clarke) had examined: Haüy, *Traité de Minéralogie*, 2nd ed. (Paris, France: Bachelier and Huzard, 1822), vol. 1, page 512. Fluorite is called "chaux fluatée" by Haüy. From page 512: "... violette par réflection, et verdâtre par transparence au Derbyshire." ([the color of fluorite is] violet by reflection, and greenish by transmission in [specimens from] Derbyshire.)
10. David Brewster (1834) "On the colours of natural bodies," *Transactions of the Royal Society of Edinburgh* **12** : 538-545; on page 542, Brewster mentions that when white light passes through an alcoholic solution of chlorophyll, red light is reflected from it.
11. See:
    - Herschel, John (1845a) "On a case of superficial colour presented by a homogeneous liquid internally colourless," *Philosophical Transactions of the Royal Society of London*, **135** : 143-145; see page 145.
    - Herschel, John (1845b) "On the epipŏlic dispersion of light, being a supplement to a paper entitled, "On a case of superficial colour presented by a homogeneous liquid internally colourless" ," *Philosophical Transactions of the Royal Society of London*, **135** : 147-153.
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13. Stokes (1852), pages 472-473. In a footnote on page 473, Stokes acknowledges that in 1843, Edmond Becquerel had observed that quinine acid sulfate strongly absorbs ultraviolet radiation (i.e., solar radiation beyond Fraunhofer's H band in the solar spectrum). See: Edmond Becquerel (1843) "Des effets produits sur les corps par les rayons solaires" (On the effects produced on substances by solar rays), *Comptes rendus*, **17** : 882-884; on page 883, Becquerel cites quinine acid sulfate ("sulfate acide de quinine") as strongly absorbing ultraviolet light.

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