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Final Version

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Abbreviations and Acronyms

AC	Alternating Current
AMF	Alternating Magnetic Fields
CMOS	Complementary metal oxide semiconductor
CSIC	Agencia Estatal Consejo Superior de Investigaciones Cientificas
Eu	Europium
LMP	Lanthanide Molecular Probe(s)
Ln	Lanthanide
MIH	Magnetic Induction Heating
MNPs	Magnetic Nanoparticles
NP	Nanoparticle(s)
RF	Radiofrequencies
Sm	Samarium
TMNP	Thermometric Magnetic Nanoparticle(s) (MNPs loaded with LMPs)
UAVR	Universidade de Aveiro
WP	Work package(s)

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Table of Contents

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INSTRUCTION MANUALS: FIRST EDITION

Foreword

Work package 5 (WP5) of the project is titled:" Magnetothermal microscope for 2D thermal cellular images and its use for the development of localized intracellular Hyperthermia therapy".

The main objective of WP5 is the construction of a new device for simultaneous ac magnetic field application, luminescent thermal imaging and optical microscopy imaging of cell cultures. The device will be used for the assessment of punctual intracellular magnetic hyperthermia therapies for cancer in combination with chemotherapy and immunotherapy.

The first task (Task 5.1) in WP5 is "Instrument development". This task is described in the project as the construction of an instrument for temperature and luminescence imaging of cells cultures under application of an ac magnetic field. The instrument includes an optical microscope equipped with: i) a temperature system to obtain pixel-to-pixel temperature images and temperature time profiles; ii) a magnetic induction system.

This report is mainly given by a lead beneficiary of the WP5: Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), along with Universidade de Aveiro (UAVR), and it consist of the first edition of two manuals of the above mentioned instruments: i) magnetic- and optically-induced heating optical microscope imaging and ii) magnetic-induced heating of cell cultures under controlled conditions.

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1. Introduction

The temperature imaging system is based on the use of lanthanide luminescent temperature probes that are embedded in the hybrid heater/thermometer nanoplatforms (TMNPs), and a detection system that captures the luminescence signal of the probes and transform it into a temperature image. The TMNPs are internalized in cancer cells and heated by an external alternating magnetic field (AMF). The purpose of the project is to measure the temperature reached in the TMNP and that of the bulk of the cells with the temperature imaging system to establish the temperature gradient and to model the heat transfer process.

Therefore, the first objective of WP5 is the construction of a new device for simultaneous ac magnetic field application, luminescent thermal imaging and optical microscopy imaging of cell cultures.

Two types of instruments will be built. One of them will be referred as Instrument I and it should be able to apply alternating magnetic fields to cell cultures, and at the same time to perform temperature imaging of cells in the culture. The other one will be referred as Instrument II, and it is meant for the application of AMF to cell cultures in such a way as to permit the evaluation of the impact of AMF on cell physiology by cell cytometry. This deliverable consists on a first edition of the instruction manuals for both instruments.

Obviously, requirements for the operation of such instruments are: i) to dispose of MNPs loaded with thermometric probes (TMNPs), and ii) to internalize the TMNPs in cells. Moreover, the thermometric probes should be stable in the conditions of cell cultures in order to observe the temperature changes in life cells.

Thus, this manual will address these issues as well as an instrument description and guidelines to operate the instrument.

1.1. Hybrid heater/thermometer nanoplatforms (TMNPs)

Two types of hybrid nanoplatforms are contemplated in this project:

i) In the first type of TMNP, the magnetic heating nucleus is located at the core of the nanostructure while the temperature sensing probe will be constituted by molecular lanthanide temperature complexes embedded is a polymer shell;

ii) In the second type of TMNPs, the temperature probe is a lanthanide solid material that is linked to the magnetic heather as a dual nanoparticle (NP) system or ideally as a core-shell NP structure. In both cases, the NPs are embedded in polymer shell that provides the necessary conditions for interaction with the cells.

Moreover, another type of non-magnetic NPs will be used as temperature probes for the measurement of the cell bulk temperature. These NPs will consist on either molecular lanthanide complexes embedded in polymer NPs, or in lanthanide NPs coated with a polymer.

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In any case, the thermometric NPs should accomplish certain optical requirements to be used for temperature imaging: i) they should hold a luminescent emission intensity unaffected in a cell culture medium; ii) their emission under available excitation source should be sufficiently intense to be detected by the CMOS camera implemented in the instrument.

1.2. First edition of Instrument I

In this first stage of development of the instrument, TMNPs of type i have been used as test samples, and the instrument has been tuned to work with them. These TMNPs are in a preliminary stage of development and their performance will be optimized along the project. In the tests of the instrument the TMNPs have been included in dry gels and fixed cells samples. AMF applicator and temperature measurements have been tested separately.

1.3. First edition of Instrument II

The AMF applicator of Instrument I is actuating only on cells situated in a small area of the cell culture in such a way that is not feasible to separate them from the non-treated cells in order to study the effect of the TMNPs on cell physiology in cytometer. Therefore, a second instrument has been designed for this purpose. In This second instrument provides a uniform magnetic field in an area of 2.5x2.5 cm that allows a simultaneous application of AMF on several cell wells representing the treated cells and the control cells, and therefore enabling a comparative study of the effect of magnetic field on cells by cytometry.

The instrument also provides a fair control of the cell cultures temperature during the experiment by means of a Peltier temperature controller installed in the cell culture compartment, and an air-cooling system of the coils and ferrite nucleus.

In this first stage of development, the construction has been practically completed, and the instrument is ready to start functioning tests.

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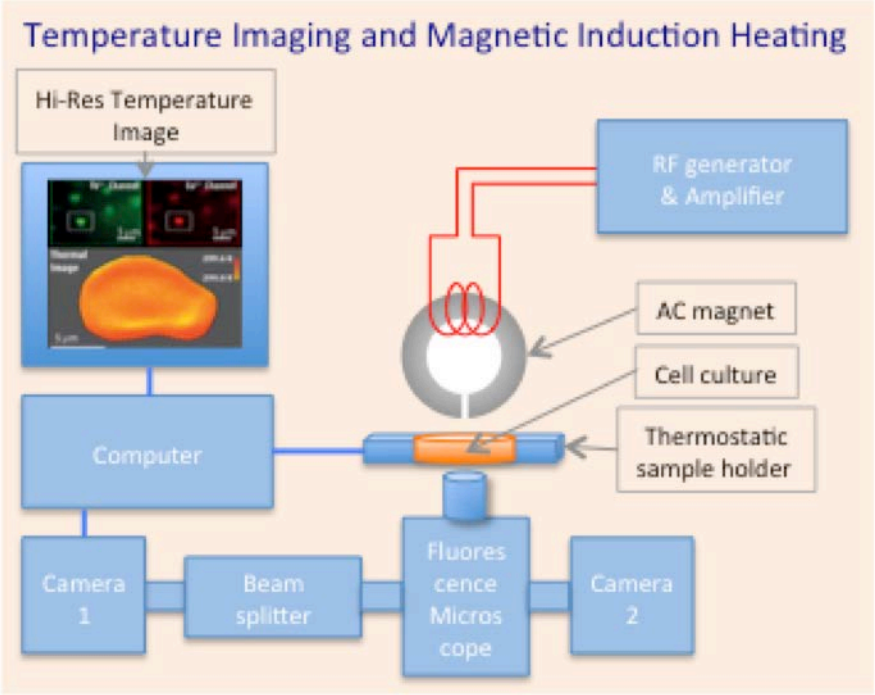
2. Instrument I. Magnetic-induced-heating & Optical Microscope Temperature Imaging

2.1. Instrument description

A scheme of the instrument is depicted below



The instrument is formed by 4 parts:

Part 1) Fluorescence Microscope

Part 2) Temperature Imaging System

Part 3) AC Magnetic Field Applicator

Part 4) Sample Holder

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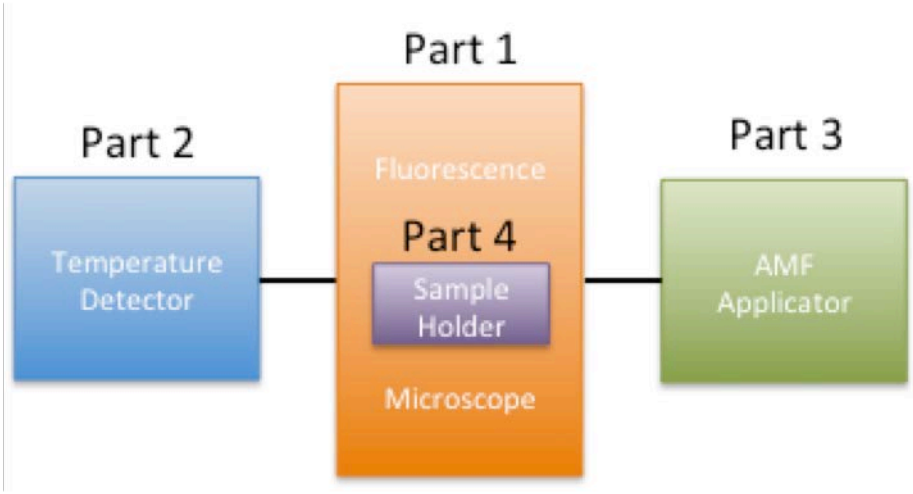
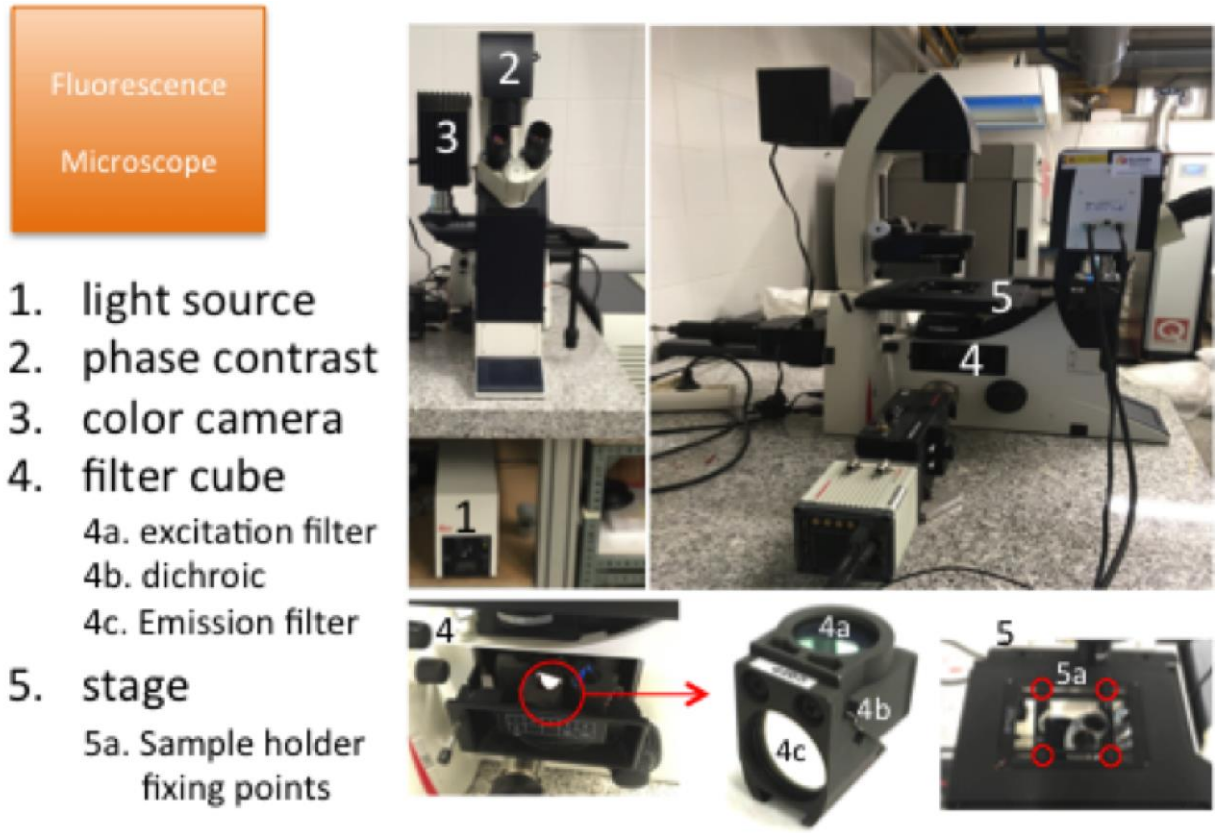


2.1.1. Part 1



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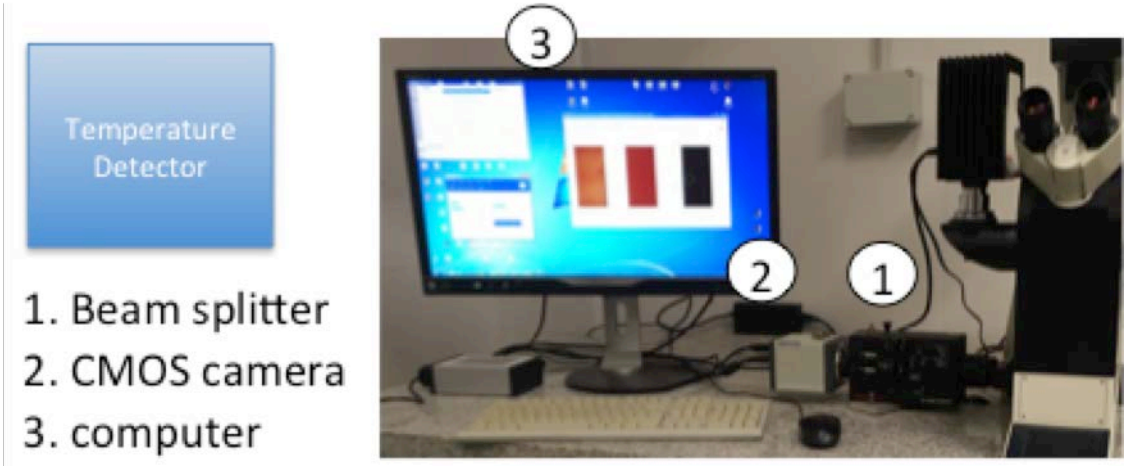
The main components of the fluorescence microscope used in temperature imaging are a halide light source or alternatively a LED light source for fluorescence excitation, a transmitted light halogen light source 2 for phase contrast, a color camera 3 for fluorescence imaging, a wheel of filter sets 4 with 5 cubes composed of excitation band filter 4a, dichroic mirror 4b and cut-off emission filter 4c with the adequate wavelengths for lanthanide probes excitation and emission, and a stage to which a thermostatic sample holder can be coupled.

For temperature imaging the adequate filter cube must be selected. For instance, for Sm/Eu temperature probe, the cube is equipped with a 340-380 nm excitation filter and a 400 nm dichroic.

The sample holder is coupled to the pins marked with red circles in the stage 5 image.

2.1.2. Part 2



The temperature detection system is composed of:

1. A beam splitter 1 that divides the emission beam into two beams of low and high frequency, then each of the beams pass through selected narrow band filters that collect the main emission peaks of the lanthanide pair used in the NP probe. In the case of Sm/Eu temperature probes, the high wavelength filter is centered at 640 nm corresponding to the main peak of Sm, and the low frequency filter is centered at 610 nm corresponding to the main peak of Eu.
2. A CMOS camera that captures separately the images of each of the lanthanides in the temperature probe.
3. A computer equipped with software that transforms the emission images in a color code temperature image by collecting the intensity at every pixel of both images and then applying the calibration equation to obtain a temperature value for every pixel and transformed in a color image by using a predetermined color code.

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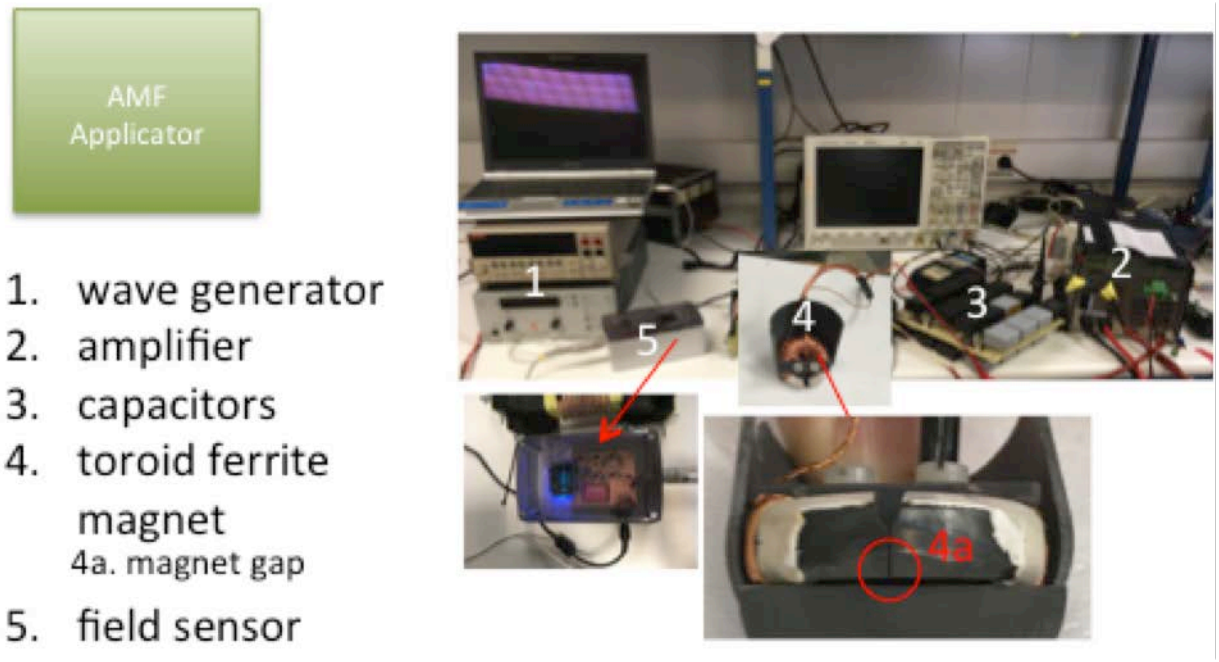
2.1.3. Part 3



The magnetic field applicator consists of: 1) wave generator; 2) amplifier; 3) a set of capacitors for the LC circuit; 4) an electromagnet that forms part of the LC circuit, which is mounted in a plastic structure that fits into a 5) sample holder that holds the 6) cell well, which is placed in 7) a thermostatic holder that can be coupled to the microscope stage; and 8) a magnetic field sensor.

The magnet has been designed and fabricated by our co-operator in the University of Toulouse, Julian Carrey, in order to avoid the heating of the metal parts of the microscope by Eddy currents generated by the magnetic field created by the magnet. Therefore, the gap is very narrow so the field flux does not reach the objective.

2.1.4. Part 4

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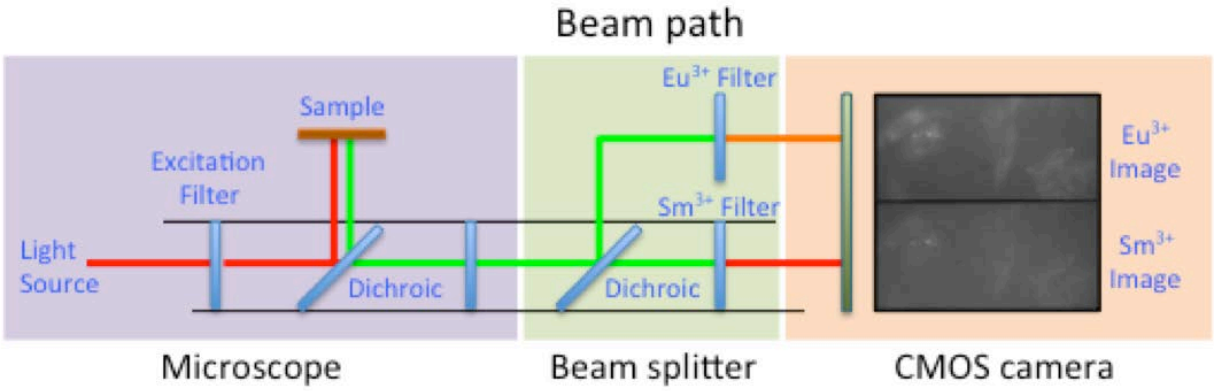
2.2. Set-up for temperature imaging



In order to operate the system the set up should be adjusted to the type of molecular Ln probes used in cell cultures.

In the microscope cube, excitation band pass filter, dichroic mirror and emission edge filter should be placed according with the molecular sensor probe chosen in the experiment.

Band pass filters in the beam splitter will also be placed accordingly with the emission spectrum of lanthanide complexes.

2.3. Operator skills

In this first version, the operator should have a short training on fluorescence microscope and cell culture handling, and on magnetic induction heating. This training will be provided in location and it will take 8h.

The imaging system utilizes a calibration equation to transform the intensity values into temperature values. This calibration equation is previously obtained for the kind of TMNP to be used in the imaging experiments. The calibration optical system is described below. The software to operate the system is implemented in the same computer used for temperature imaging.

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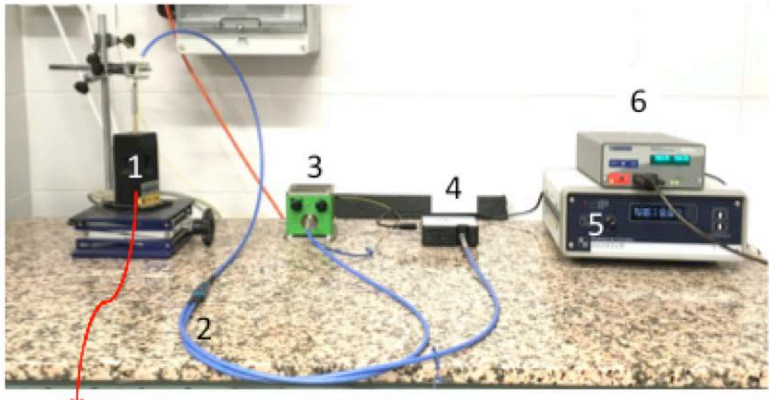
2.4. Operations instructions

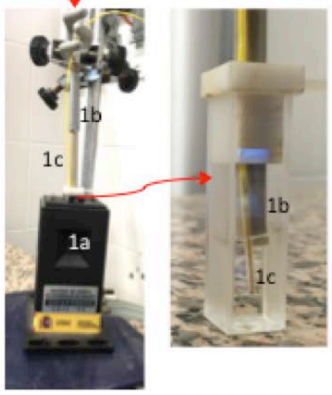
2.4.1. Calibration of the molecular thermometer probes

Thermometric response of the TMNPs will be carried out in liquid suspensions with an optical fiber system depicted below

1. cuvette Peltier holder
2. double optical fiber
3. LED light source
4. spectrophotometer
5. temperature control
6. optical fiber thermometer



1a. cuvette holder
1b. temperature probe
1c. optical thermometer probe



The program for temperature monitoring and data acquisition is implemented in the computer. The picture below shows the icon that opens the program and the windows for the control of the system.

The program yields the emission spectrum of the sample and the temperature. Once the temperature is stable, the operator has to introduce the chosen initial and final wavelengths of integration for the two peaks corresponding to each lanthanide, and the program plots the temperature parameter, Δ=I(Ln1)/I(Ln2) vs T. Then, the temperature in control 5 is set for another value.

Two runs of measurements are performed heating and cooling. The calibration curve is fitted to a second order equation that will be used as input for the temperature imaging system.

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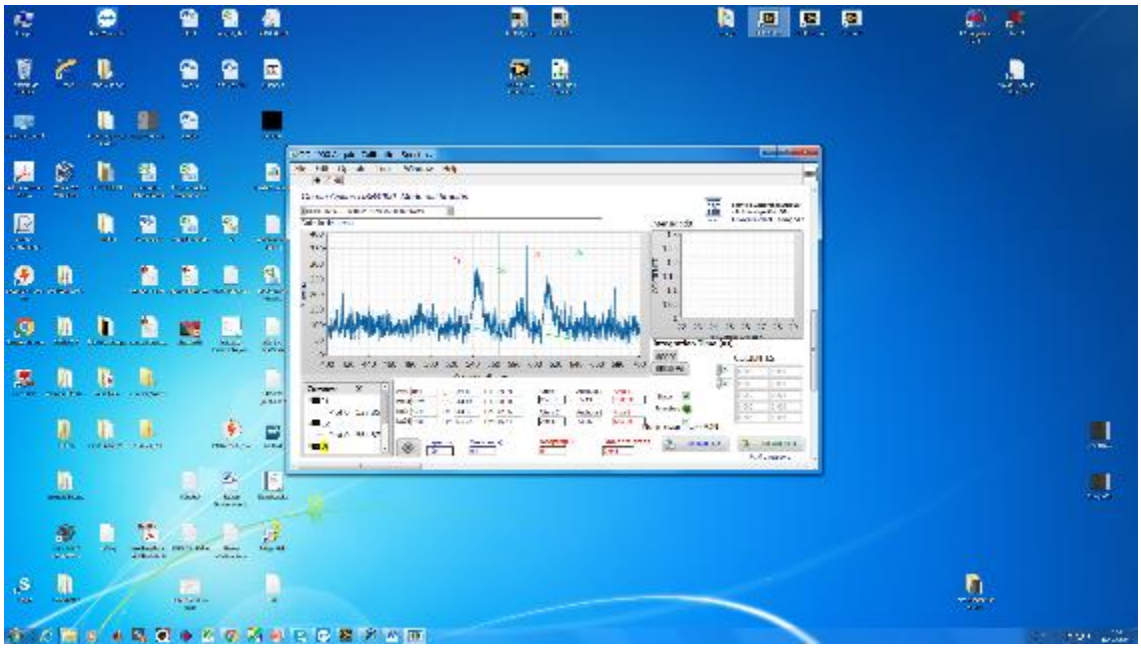

2.4.2. Cell sampling and MNP internalization

Cell wells with glass bottom the appropriate optical quality for microscope observation will be used for cell cultures.

After achieving an optimal cell density, the culture will be seeded with TMNPs for a time enough to be internalized in the cells.

The well will be immediately placed in the thermalized sample holder.

2.4.3. Microscope optical and temperature imaging

Once the cells are placed in the holder, phase contrast and fluorescence images of the cells will be taken with the color camera (part1.3).

Then, the microscope beam will be directed to the temperature detection system (part 2 of the instrument).

2.4.4. Running the software for ratiometric thermometry

This software has been designed for capturing the images of luminescence emission of each lanthanide and transforms them, pixel by pixel in a color-coded temperature image. The system also allows the selection of an area of observation and plots the variation of T in that area with the time.

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1) To open the program click the icon shown below.

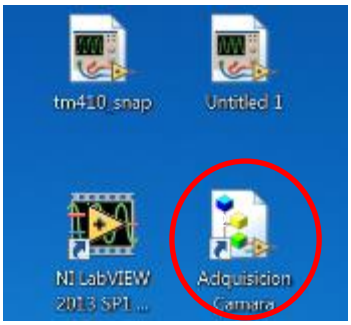

Figure 1. The icon of the software.

2) Introduce the specific calibration equation of **TMNPs** previously obtained
   (a) Go to ***window***, select the ***Show block diagram***, and double click the "calculus" marked by red square in Fig. 2.
   (b) In the windows of Fig. 3, the functions of different media can be input in to the system.

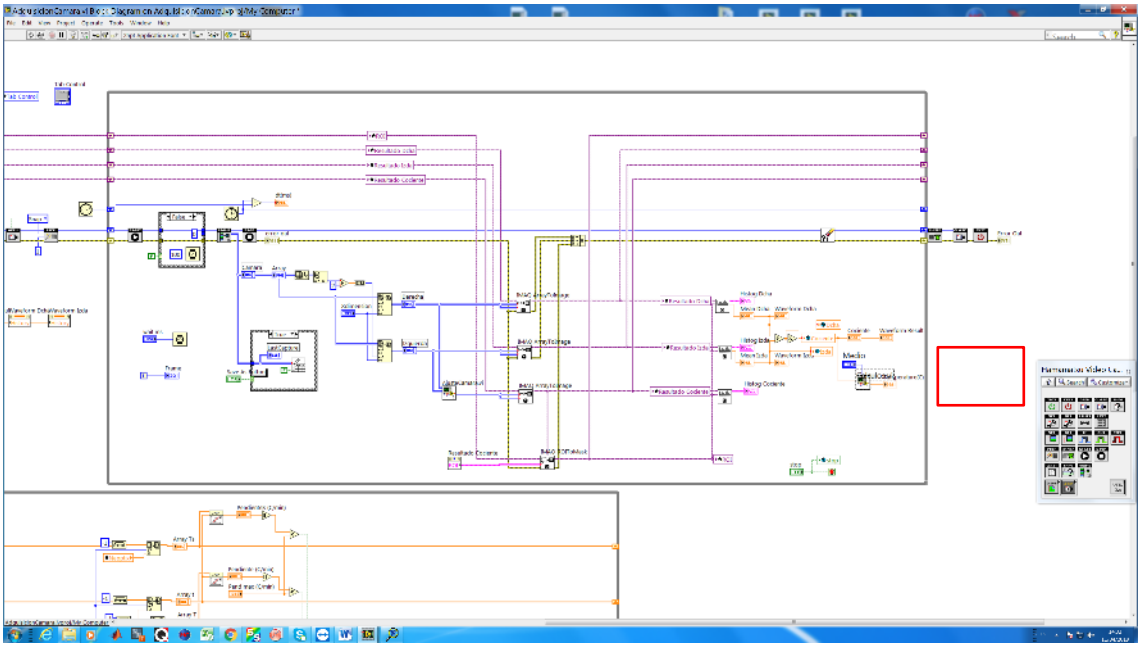

Figure 2. The block diagram of the main window.

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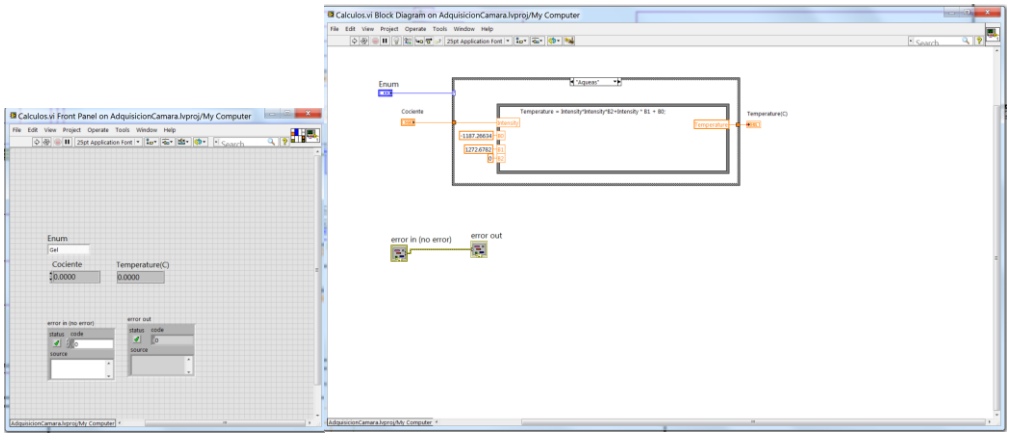

Figure 3. The windows for introducing the ratio vs temperature functions.

3) Temperature imaging and T(t) plotting on selected areas of interest. Introduce the specific calibration equation of **TMNPs** previously obtained

Then, click the icon on the desktop ***Adquisicion Camara*** and a following window will appear:

Figure 4. The second window of the software after clicking the icon.

- After turning on the CMOS camera, and clicking the *Adquisicion camara* marked by red square in Fig. 4, the main window of the software will come out as below.

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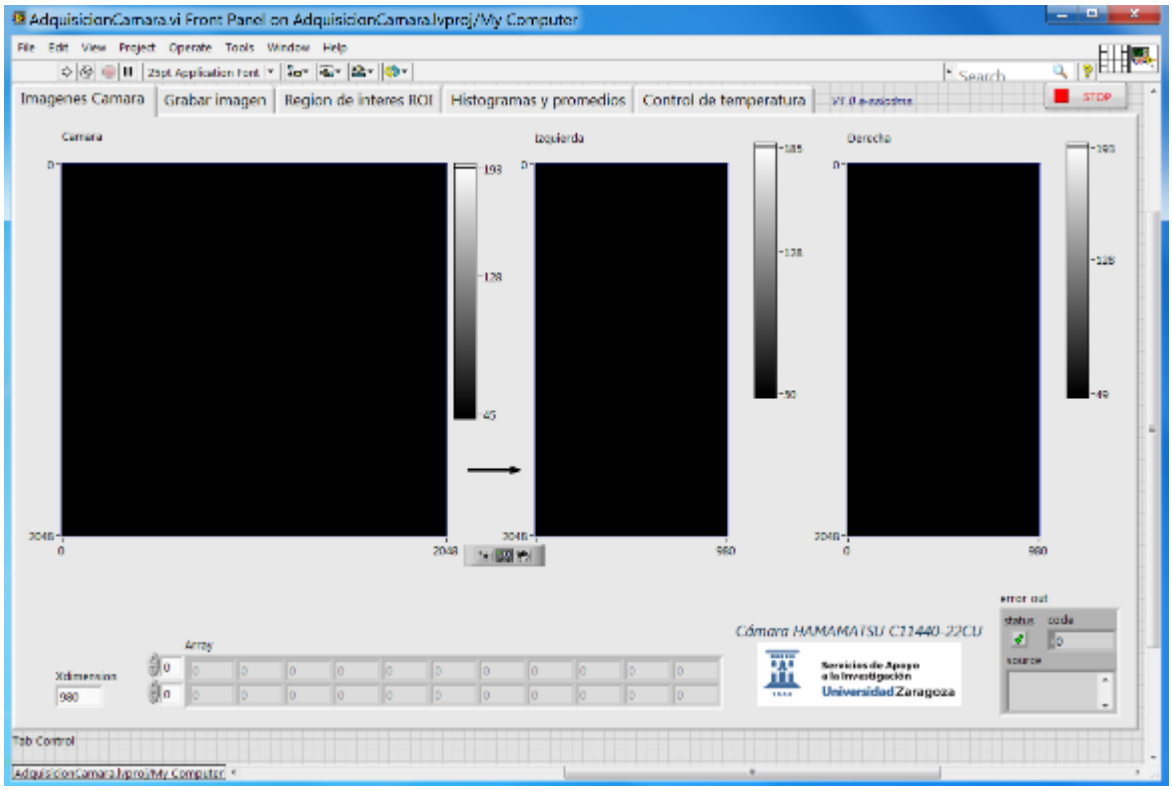

Figure 5. The main window of the software.

- Clicking the run button ⇨ live images of the emission of both lanthanides will be acquired (see below).

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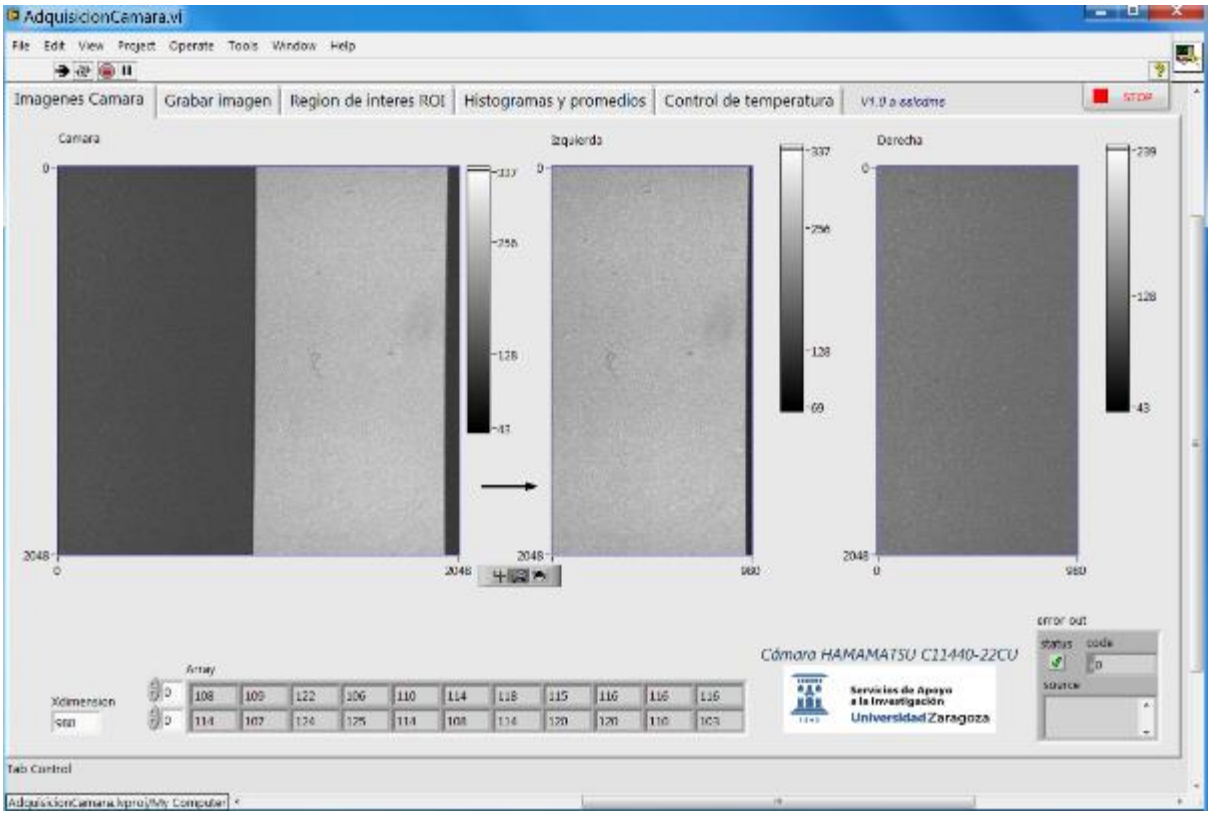

Figure 6. Image window.

The program allows several visualization options and operations: 1) Camera imaging (Imágenes cámara) (Fig. 6); 2) Save images (Grabar Imagen) (Fig. 7); 3) Select a region of interest (Región of interés) with several shape choices (Fig.8); 4) Histograms of emission images and temperature image, and Temperature vs time plots of selected area of interest (Fig. 9).

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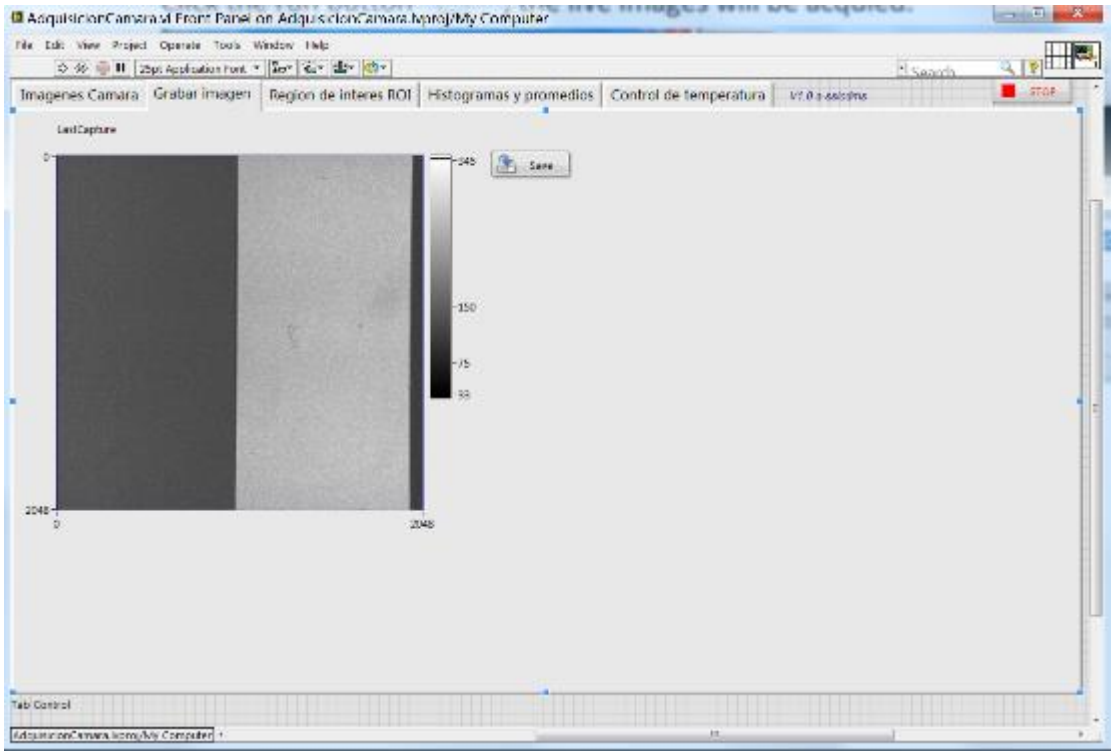

Figure 7. Image saving window.

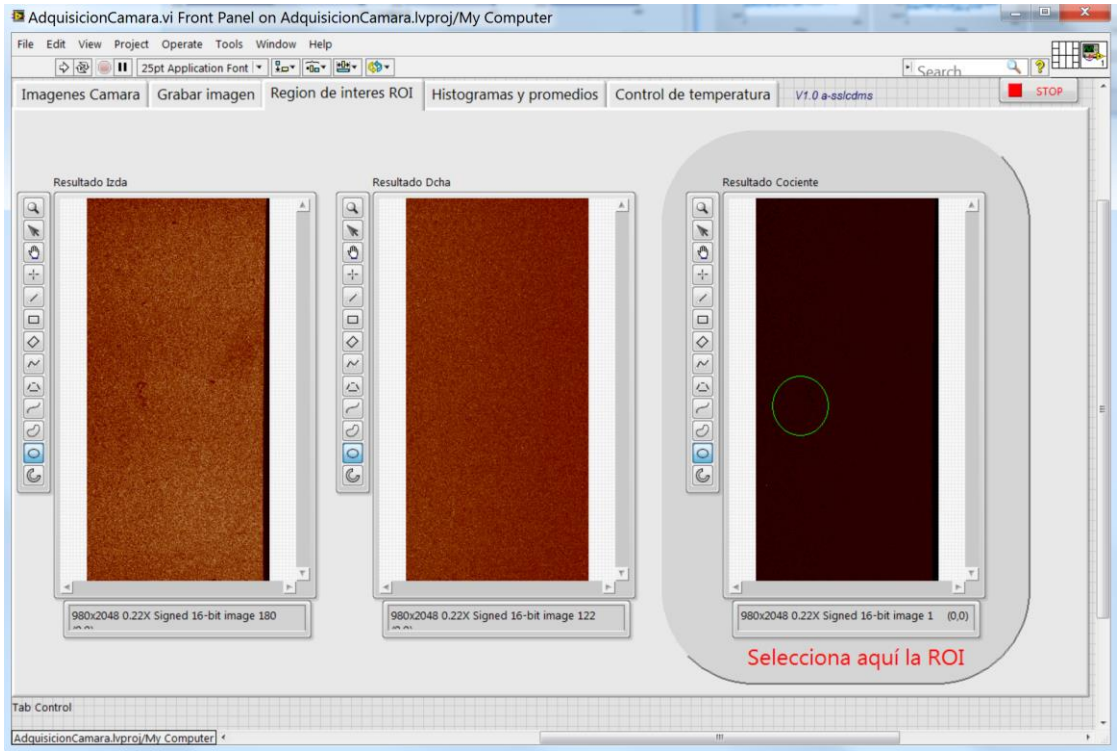

Figure 8. Select region of interest.

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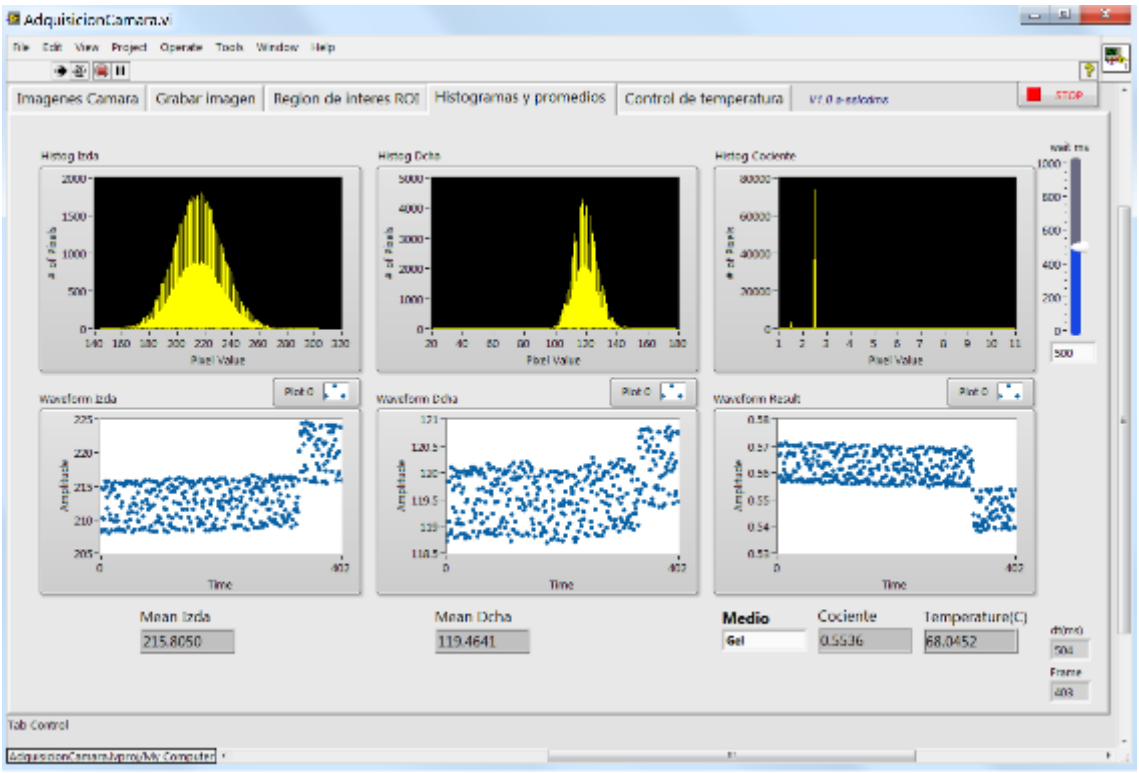

Figure 9. Histograms and mean values.

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2.4.5. Running the magnetic field application system

To operate the system, the thermostatic sample holder 5 with the cell well 6 and the magnet 4 is placed in the microscope stage, and the temperature is set to physiological value.

Then, the working frequency is set in the wave generator 1 and the intensity of the magnetic field measured by the magnetic field sensor 8 is set in the amplifier 2.

To stop the field application, turn off the wave generator.

2.4.6. Measuring the local temperature of nanoheathers in suspension

The system can also be used to measure the local temperature of nanoheaters in liquid suspensions, by using a ferrite magnet designed for that purpose, which is presented in Fig. 10

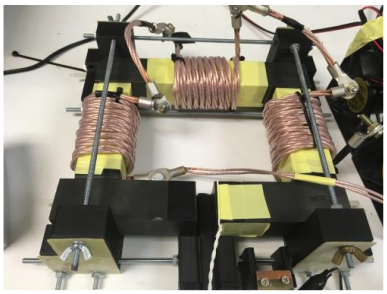

Figure 10. Magnet for AMF applicator to NPs suspensions.

This magnet has a gap wide enough to hold a cuvette containing the liquid nanoheather suspension and a second control cuvette containing water. The nanoheather temperature is measured using the same system used for the calibration of the molecular thermometer probes.

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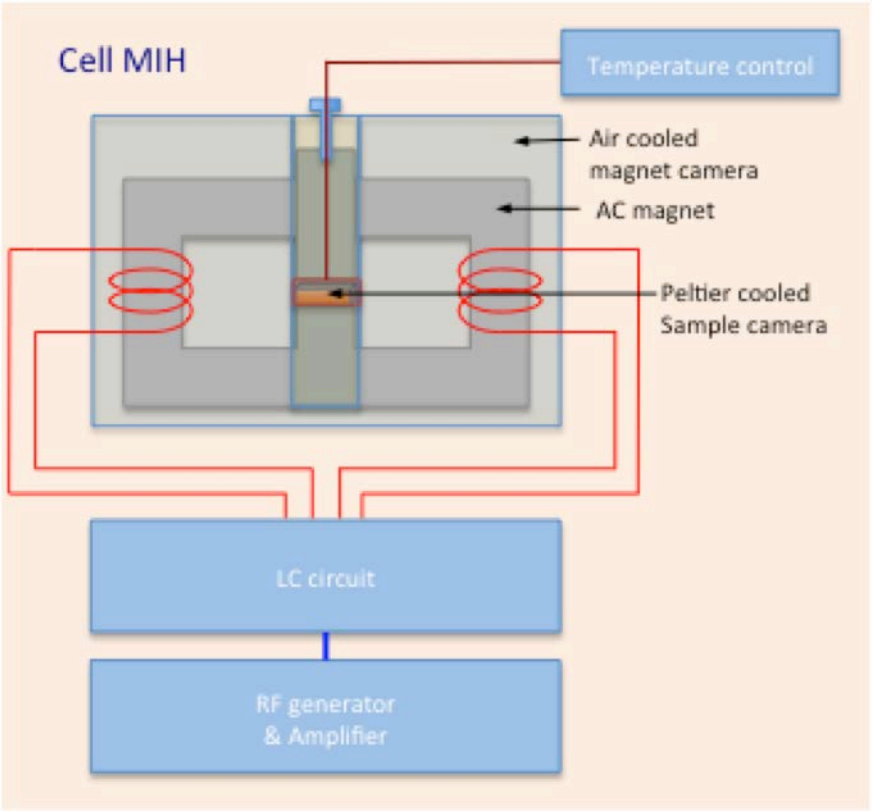
3. 3. Instrument II. Magnetic-induced heating of cell cultures under controlled conditions

3.1. Description

A scheme of the instrument is depicted below.



The instrument consists of 3 parts:

Part 1) Electromagnet

Part 2) Multi-well cell sample compartment

Part 3) AC Magnetic Field Applicator

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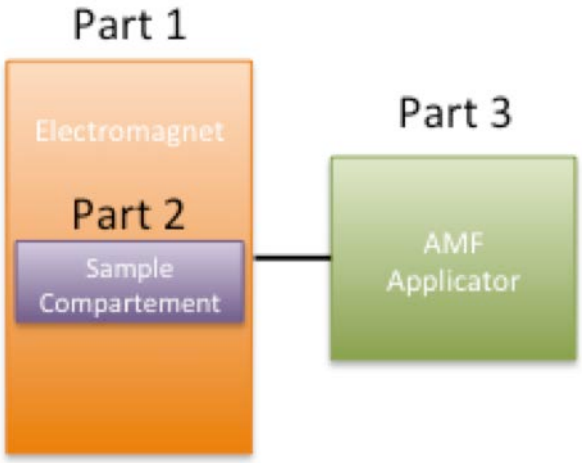
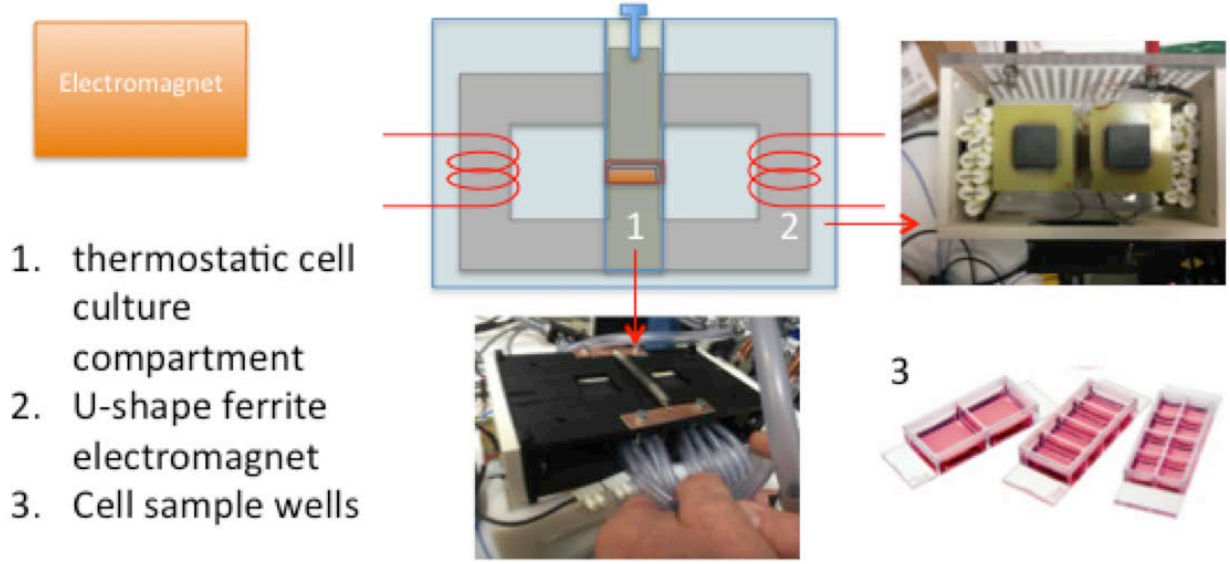


3.1.1. Parts 1&2. Electromagnet and sample compartment



3.1.2. Part 3. AMF applicator

There are two options for magnetic field applicator. In the first one, the capacitance is implemented in the air-refrigerated cage of the magnet and external wave generator and amplifier supply the current. In the second, the capacitance as well as the wave generator and amplifier are external and similar to those used in microscope system and fluid hyperthermia systems described above in sections 5 and 6.

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3.2. Operating the cell hyperthermia system

To operate the system, place the cell sample well 3 in the thermostatic sample holder compartment 1, then fix the temperature to physiological value and apply the magnetic field as described in sections 5 and 6.

Three types of cell wells can be used, allowing the application to 1, 2 or 4 cell samples simultaneously. As the magnetic field in the whole gap area is very uniform, the system permits a direct comparison of the effect of the magnetic field on a control cell culture, a cell sample with NPs inside the cells, a cell sample with NPs outside the cells, and a cell sample with NPs outside and inside the cells.

After the field application the cell samples will be examined by cell cytometry to investigate the effects of the field in cell physiology, and especially on cell necrosis, apoptosis and heat shock proteins.

4. Future developments

Instrument 1 is ready for use although several components need to be improved. One of them is the thermostatic sample holder, which is being redesigned to achieve a faster temperature control. In a near future it will be used in assays directed to enhance the performance of the temperature molecular probes, in terms of sensibility and robustness in cell culture environment.

Instrument 2 is still being tested, especially concerning the cell compartment temperature control and the magnetic field applicator.

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