ELETTRA PROPOSAL DESCRIPTION
Proposal number: 2008232
Title: Detection of drug-induced signatures in normal and in tumour cells by Fourier Transform InfraRed Microspectroscopy (FTIRM).

Proposer: Giuseppe BELLISOLA
Beamline(s): SISSI
Required shifts: 18
Objectives: To identify early biochemical and morphological effects of anticancer drugs targeting intracellular signalling networks by FTIRM at single cell and sub-cellular levels. To develop procedures and optimize instrumental setup for the potential application of FTIRM to pre-clinical drug screening assays in cell models and to identify drug-resistant cancer cell subpopulations in leukaemic patients.

_____ do not change anything above this line _____

Previous Proposal Number 2007486

1.Background
New candidate drug molecules, as well as anticancer molecules designed to target components of intracellular signalling networks, need pre-clinical testing and validation in cellular and animal models before to undergo evaluation in clinical trials. The cellular models assays that evaluate drug efficacy are based on the measurement of tumor cell proliferation or death, that remains a timeconsuming and in some cases difficult to standardize process. *In vitro* cell-based assays are critical for the selection of the most effective active agents, for instance, against chronic myeloid leukemia (CML) [1] and cost-effective platforms and infrastructures have been developed to screen drugs. Therefore, there is a great interest to improve high throughput assays that can efficiently recognize the effect of a compound before the measurement of a biological endpoint like proliferation, cell death or angiogenesis as well as to recognize early chemotherapy resistant cancer cell populations selected in the patient [2].
In Fourier Transform InfraRed absorption Spectroscopy (FTIR), the specific absorption frequencies can be remarkably informative. The resulting IR spectrum comprises a series of peaks as a function of the wavenumber that depict the state of chemical bonding in the sample allowing a rapid multicomponent analysis to be carried out with no or minimal sample manipulation. By Fourier Transform InfraRed Microspectroscopy (FTIRM) this information can be spatially resolved at single cell level [3] allowing to discriminate between normal and cancerous cells in tissues [4], to characterize structural and functional changes during cell differentiation processes [5] or induced by a drug treatment [6,7] and to predict drug resistance in cancer cells [8].

2.Motivation for the present proposal
We have recently applied FTIRM to identify early signatures induced in the IR spectrum of human leukocytes and in human K562 erythro-leukaemic cells by short (a few minutes) *in vitro* incubations of cells with drug molecules that specifically activate or inhibit intracellular phosphonetworks. Working with a Globar source and a large aperture (e.g., for typically size > 70 μm), reproducible IR absorption mean spectra (from roughly 100 cells) could be acquired by a single element MCT detector at an acceptable Signal to Noise (S/N) ratio and compared. Significant IR changes were observed in the phosphate region 1300-940 cm^{-1} spectral interval of stimulated with respect to unstimulated human blood polimorphonuclear neutrophils (PMNs) that could be also imaged by a Focal Plane Array (FPA) detector (see Figure 1, A, C, and D). The short term effects of Imatinib, a selective inhibitor of BCR-ABL tyrosine kinase activity, and of 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), a potent and selective inhibitor of the Src-family tyrosine kinase were tested in two distinct clones of K562 cells respectively with low and high expression of protein tyrosin phosphatase gamma (PTPγ) [9]. Early spectral changes induced by drugs were identified at 1740 cm^{-1} (ester band) and in the 1300- 800 cm^{-1} frequency interval (Figure 1, B). However, it is fundamental to identify biochemical information also at cellular and sub-cellular levels (e.g. the nucleus) for a more detailed biochemical explanation of identified variation.
Two complementary approaches might be applied to reach the objectives of present proposal. With a conventional thermal source and a FPA detector faster data collection at a spatial resolution roughly two times the diffraction limit can be obtained in a high number of single cells within larger sample area in a few minutes [10] while to obtain good quality IR spectra at the highest possible spatial resolution and S/N ratio in the IR frequency interval from 800 to 4000 cm^{-1} the use of Synchrotron Radiation (SR) IR microscope equipped with a single-point MCT detector seems more appropriate. Both instrumental settings are currently available at the beamline SISSI of ELETTRA. Therefore, we aim at applying FTIRM to identify early signatures of drugs in cell models and to identify drug resistant cancer cell sub-populations in the blood of leukaemic patients. The final goal intends to develop procedures and optimize instrumental setup to extend the repertoire of FTIRM applications to the High Throughput Screening (HTS) of drugs in cell models and to the early identification of drug resistant cancer cell populations in patients.

3.Experimental plan
Human leukocyte subpopulations will be separated in blood samples from donor volunteers (a transfer agreement approved by Local Ethical Committee has been already obtained) and from leukaemic patients. As concerns HTS procedures, we plan to use K562 cells, a widespread accepted and validated cell model for drug testing and screening [11]. Cells will be stimulated/inhibited in time-course and in dose-response experiments by specific intracellular phosphonetworks agonists/antagonists. To identify key spectral signatures and correlate them to the activation state of a specific signalling molecule or a molecules family, cells will be pre-treated with specific and selective inhibitors of signal transduction pathways by three distinct approaches based on: (i) commercial specific inhibitory drugs; (ii) two different Trojan peptide technologies, based on Penetratin-1 [12] and on TAT; (iii) siRNA technology. All these technologies are set-up and running in our laboratories together with appropriate analytical instrumentation to achieve complementary biochemical and functional information in samples. A range of compound sets will be used in a hierarchical order in K562 cells (e.g.:

Gold Standard: Imatinib mesylate for CML; Standards Compound Set: selected compounds known to have an effect against tyrosine kinases; Target Class/Pathway Set: set of compounds selected as representative of areas of interest).
Spectra will be collected in transmission mode using ZnSe optical windows (already tested and validated for cell lines in use) in the IR frequency interval from 800 to 4000 cm^{-1} by conventional thermal source and FPA detector. Selected cells from each samples will be then mapped at sub-cellular level exploiting the high brightness of SR and the major sensitivity of MCT-single point detector in order to gain major details on intracellular events. Statistical analysis and a detailed comparison between differently treated and untreated samples will be performed in order to establish, if existing, a correlation between early and late events induced by drugs on cells. To identify key spectral signatures we will apply different methods for data analysis on spectra. By the so-called "group frequency approach" we will monitor vibrations due to pre-assigned specific functional groups and we will explore changes in these functional groups as potential markers. Deconvolution analysis in selected peak areas and comparison of different conditions applied to cells will allow the extraction of spectral sub-components. Unsupervised (e.g., cluster analysis or principal component analysis) and supervised (e.g. linear discriminant analysis and partial least squares analysis) classification methods will be applied to discriminate between sensitive and resistant cancer cells based on differences in IR spectral profiles.

4. Explain why this work calls for access to ELETTRA

The complexity of the planned experiment requires a FTIR microscope, with fast acquisition capabilities and high S/N ratio at high spatial resolution. The quality of FTIR spectra obtained using SR or Globar sources is roughly comparable up to a 20µm x 20µm aperture, but S/N ratio is very poor below this spatial resolution for Globar while it should remain suitable up to 3µm x 3µm with a SR source. Thus, SR FTIR coupled with an MCT detector have an advantage over a thermal source for single cell analysis and mapping at the subcellular level. A VERTEX 70 interferometer coupled with a Bruker Hyperion 3000 IR/Vis microscope equipped with a single MCT detector and working with SR IR is already available and optimized for the mapping of biological samples at the beamline SISSI of ELETTRA. Moreover, the same instrument equipped with a 64x64 pixels FPA detector and working with a Globar source can also ensure the high throughput measurements and fast imaging of different cells in many samples for statistics.

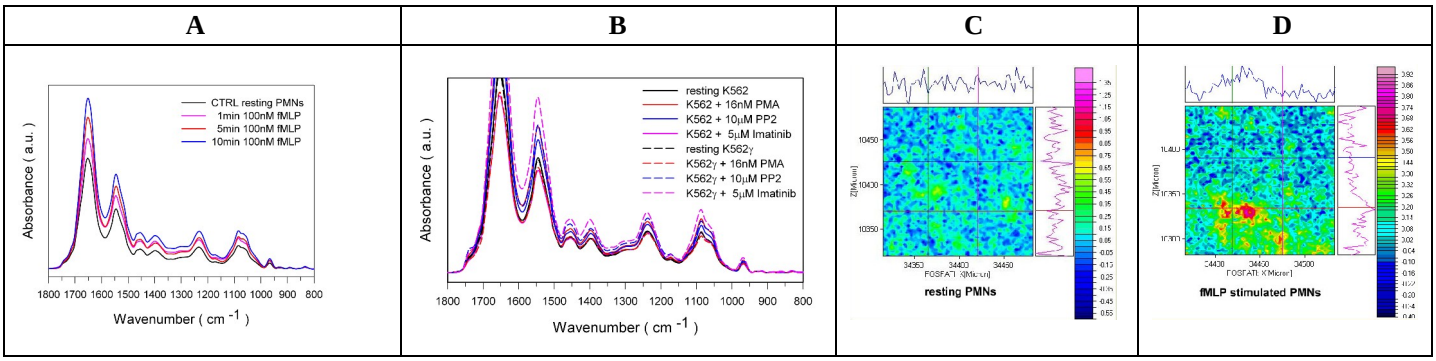

Figure 1. Mean IR spectra of resting and fMLP (N-formyl Met-Leu-Pro) stimulated human PMNs (**A**) and of K562 leukaemic cells (**B**) with low (K562, solid) and high (K562γ, dash) expression of PTPγ incubated for 10 minutes with 16nM PMA, 10µM PP2, or 5µM Imatinib mesylate, respectively. Formalin fixed cells were deposited in monolayer on a ZnSe IR transparent window (roughly 100 cell in each 170x170 μm^2 illuminated sample area). Spectra were acquired in transmission modality by an FTIR Bruker Hyperion 3000 IR/Vis microscope equipped with a Globar source and single element MCT detector at SINBAD - Frascati. A number of 128 scans were co-added in the 4000-700 cm^{-1} spectral interval. In **C** and **D**: chemical imaging of phosphates (ν_s and ν_{as} PO_2^- stretching vibrations at 1086 cm^{-1} and 1240 cm^{-1}, respectively) in unstimulated (**C**) and fMLP stimulated (**D**) PMNs obtained at the beamline SISSI of ELETTRA by a similar instrumentation but equipped with an 64x64 pixels FPA detector.

5) References

[1] B.M. Mow et al., *Blood*, **99**, 664-671, (2002).

[2] R.D. Blumenthal & D.M. Goldenberg, *Mol Biotechnol*, **35**, 185-198 (2007).

[3] N. Jamin et al., *Proc Natl Acad Sci USA*, **95**, 4837-4840, (1998).

[4] P. Lasch et al., *Technol Cancer Res Treat*, **1**, 1-7 (2002).

[5] KZ. Liu et al., *Br J Haematol*, **136**, 713-722 (2007).

[6] J.M. Le Gal et al, *Cancer Research*, **53**, 3681–3686 (1993).

[7] V. Erukhimovitch et al., *J Biomed Opt*, **11**, 064009 (2006).

[8] A. Gaigneaux et al., *Eur J Biochem*, **269**, 1968–1973 (2002).

[9] A. Mafficini, et al. *Biomarker Insights*, **2**: 217–224 (2007).

[10] L.M. Miller & R.J. Smith, *Vibrational Spectroscopy*, **38**, 237-240 (2005).

[11] C.B. Lozzio & B.B. Lozzio. *Blood* **45**, 751-758, (1975).

[12] C. Giagulli et al., *Immunity*, **20**, 25-34 (2004).