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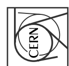

Near Infrared Microspectroscopy, Fluorescence Microspectroscopy, Infrared Chemical Imaging and High-Resolution Nuclear Magnetic Resonance Analysis of Soybean Seeds, Somatic Embryos and Single Cells

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Abstract

Novel methodologies are currently being evaluated for the chemical analysis of soybean seeds, embryos and single cells by Fourier Transform Infrared (FT-IR), Fourier Transform Near Infrared (FT-NIR) Microspectroscopy, Fluorescence and High-Resolution NMR (HR-NMR). The first FT-NIR chemical images of biological systems approaching 1micron (1μ) resolution are presented here. Chemical images obtained by FT-NIR and FT-IR Microspectroscopy are presented for oil in soybean seeds and somatic embryos under physiological conditions. FT-NIR spectra of oil and proteins were obtained for volumes as small as $2\mu^3$. Related, HR-NMR analyses of oil contents in somatic embryos are also presented here with nanoliter precision. Such 400 MHz ^{1}H NMR analyses allowed the selection of mutagenized embryos with higher oil content (e.g. ~20%) compared to non-mutagenized control embryos. Moreover, developmental changes in single soybean seeds and/or somatic embryos may be monitored by FT-NIR with a precision approaching the *picogram* level. Indeed, detailed chemical analyses of oils and phytochemicals are now becoming possible by FT-NIR Chemical Imaging/ Microspectroscopy of single cells. The cost, speed and analytical requirements of plant breeding and genetic selection programs are fully satisfied by FT-NIR spectroscopy and Microspectroscopy for soybeans and soybean embryos. FT-NIR Microspectroscopy and Chemical Imaging are also shown to be potentially important in functional Genomics and Proteomics research through the rapid and accurate detection of high-content microarrays (HCMA). Multi-photon (MP), pulsed femtosecond laser NIR Fluorescence Excitation techniques were shown to be capable of Single Molecule Detection (SMD). Therefore, such powerful techniques allow for the most sensitive and reliable quantitative analyses to be carried out both *in vitro* and *in vivo*. Thus, MP NIR excitation for Fluorescence Correlation Spectroscopy (FCS) allows not only *single molecule detection*, but also molecular dynamics and high resolution, *submicron* imaging of *femtoliter* volumes inside living cells and tissues. These novel, ultra-sensitive and rapid NIR/FCS analyses have

numerous applications in important research areas, such as: agricultural biotechnology, food safety, pharmacology, medical research and clinical diagnosis of viral diseases and cancers.

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KEYWORDS:

FT-NIR and FT-IR Instruments, applications of FCS/NIR, Agricultural biotechnology, IR Chemical Imaging and NMR, Microspectroscopy, DNA/RNA Micro-array analysis by NIR, High resolution and super-resolution FT-NIR/IR, IR Chemical Imaging by FPAW, Spotlight 300 Microspectrometer, Two photon NIR excitation for FCS, Single Cell and sister 1-molcule dynamics, FCS of molecules, single cells, Soybean oil, protein and moisture analysis, FT-NIR and FT-IR, high-resolution NMR of soybean oil in seeds and somatic embryos, chemical mutagenesis of soybean embryos, picomole FT-NIR and femtomole FCS-NIR analysis of single cells, phytochemicals detection in soybean seeds and cells by FT-NIR, high-power, femtosecond Ti:Sapphire NIR excitation for FCS, FCS/PCR, Nucleic acid hybridization, FT-IR and FT-NIR Images of Soybeans and Embryos, FT-IR and NIR Chemical Imaging Tests, Spatial Resolution Test, FT-NIR Micro-Imaging, FT-NIR Images of Soybeans and Embryos, FT-IR Reflectance Chemical Images, Somatic Embryo, NIR Reflectance Chemical Image of a Red Coat Azuki Red Bean, FT-NIR Chemical Imaging by Difference Spectroscopy (CIDS), High Resolution NMR Analysis of Soybean Oil in Somatic Embryos, HR NMR, ^{1}H NMR Spectrum of the somatic embryogenic culture of a soybean sample, TEM Micrograph of a Suspension of Soybean Somatic Embryos in Culture, Single Molecule Detection, two-photon excitation, one-photon excitation, three-photon-excitation, Fluorescence Correlation Spectroscopy (FCS), Fluorescence Resonance Energy Transfer (FRET), Fluorescence Lifetime Imaging Microscopy (FLIM), Fluorescence Recovery After Photobleaching (FRAP), Single Photon Confocal Fluorescence Correlation Spectroscopy, Inverted Epifluorescence Microscope, FCS auto-correlation, Fluorescence Fluctuations, Fluorescence Intensity, Fluorescence Correlation Spectroscopy and Imaging Experiments in Solutions and Plant Cell Suspensions, Pulsed, Two-Photon NIR Laser Excitation, Multi-photon (MPE) NIR excitation, FCS Alba Spectrometer Microspectrometer System, FCCS Cross-Correlation with Two Fluorescent Labels, FCCS Applications to DNA Hybridization.

TABLE OF CONTENTS

ABSTRACT…………………………………………………………………………………
…...1

KEYWORDS………………………………………………………………………………
….2

1. INTRODUCTION…………………………………………………………………
….4

2. PRINCIPLES……………………………………………………………………….
....5

2.1 Principles of Chemical Imaging
2.2 Principles of Fluorescence Correlation Spectroscopy
2.3 FCCS: Cross-Correlation with Two Fluorescence Labels

3. EXPERIMENTAL………………………………………………………………….
..10

3.1 Plant Material Source
3.2 FT-IR and FT-NIR Microspectrometers
3.3 High-Resolution NMR for Oil Determination
3.4 Fluorescence Correlation Spectroscopy

4. RESULTS…………………………………………………………………………
…16

4.1 FT-IR and FT-NIR Chemical Imaging Tests
4.2 FT-IR and FT-NIR Chemical Images of Soybeans and Somatic Embryos
4.3 Oil Determination in Somatic Embryos by ^{1}H High–Resolution NMR

5. LIMITATIONS AND ADVANTAGES..……………………………………………31

6. OTHER APPLICATIONS ……………………………………………………….…32

6.1 Two-Photon Observations on Plant Cells and Thylakoids
6.2 FCCS Applications to DNA Hybridization, PCR and DNA bonding

7. CONCLUSIONS AND DISCUSSION…………………………………………......39

8. REFERENCES……………………………………………………………………..
..41

1. INTRODUCTION

Infrared (IR) and Near Infrared (NIR) commercial spectrometers employ, respectively, electromagnetic radiation in the range from to ~150 to 4,000 cm^{-1}, and from 4,000 to ~14,000 cm-^{1}. The utilization of such instruments is based on the proportionality of IR and NIR specific absorption bands with the concentration of the molecular components present, such as protein, oil, sugars and/or moisture. The molecular bond stretching/vibrations, bending and or rotations cause specific absorption peaks or bands, centered at certain characteristic IR and NIR wavelengths. FT-IR/NIR spectrometers obtain spectra using an interferometer and also utilize Fourier Transformation in order to convert the interferogram from the time domain to the frequency domain. The use of interferometry in FT-IR and FT-NIR spectroscopy increases the spectral resolution, the speed of acquisition, the reproducibility of the spectra and the signal to noise ratio in comparison with dispersive instruments that utilize either prisms or diffraction gratings.

An FT-IR/NIR image is built up from hundreds, or even thousands, of FT-IR/NIR spectra and is usually presented on a monitor screen as a cross-section that represents spectral intensity as a pseudo-color for every microscopic point in the focal plane of the sample. Special, 3D surface projection algorithms can also be employed to provide more realistic representations of microscopic FT-IR/NIR images. Each pixel of such a chemical image represents an individual spectrum and the pseudo-color intensity codes regions with significantly different IR absorption intensities. In 2002, four commercial FT-IR/NIR instruments became available from PerkinElmer Co. (Shelton, CT, USA): an FT-NIR Spectrometer (*SpectrumOne-NTS*), an FT-NIR Microspectrometer (*NIR AutoImage*), an FT-IR Spectrometer (*SpectrumOne)* and an FT-IR Microspectrometer (*Spotlight300*). The results of the tests obtained using these four instruments are shown in section 3.1.

The employment of high-power, pulsed NIR lasers for visible fluorescence excitation has resulted in a remarkable increase of spatial resolution in microscopic images of live cells, well beyond that available with the best commercial FT-NIR/IR microspectrometers, allowing even for the detection of single molecules. This happens because fluorescent molecules can absorb two NIR photons simultaneously before emitting visible light, a process referred to as "*two-photon excitation*." Using two-photon NIR excitation (2PE) in a conventional microscope provides several great advantages for studying biological samples. As the excitation wavelength is typically in the NIR region, these advantages include efficient background rejection, very low light scattering and low photodamage of unfixed biological samples and *in vivo* observation. Additionally, photobleaching is greatly reduced by employing 2PE, and even more so in the case of three-photon NIR excitation (3PE). The spatial region where the 2PE process occurs is very small (of the order of 1 femtoliter, or 10^{-15} L), and it decreases even further for 3PE. Multiphoton NIR excitation allows submicron resolution to be obtained along the focusing (z) axis in epi-fluoresence images of biological samples, without the need to employ any confocal pinholes. The 2PE and 3PE systems with ~150-femtosecond (10^{-13} s) NIR pulses have several important advantages in addition to high resolution. Firstly, they offer very high sensitivity detection of nanomole to femtomole concentrations of appropriately selected fluorochromes. Secondly, these systems have very high selectivity

and the ability to detect interactions between pairs of distinctly fluorescing molecules for intermolecular distances as short as 10 nm, or less. 2PE and 3PE also allow one to rapidly detect even single molecules through Fluorescence Correlation Spectroscopy (FCS); FCS is usually combined with microscopic imaging. The principles of single photon FCS microscopy are briefly discussed next, in Section 2.2.