Protocols

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A- Microanalysis of individual cells

1) **Culture protocol:**

Culture on thin formvar films 200-400 nm

Cryomethods (buffer rinsing, cryofixation, freeze-drying…)

2) **Microanalysis :**

PIXE-RBS-STIM microbeam analysis

Data reduction for quantitative analysis

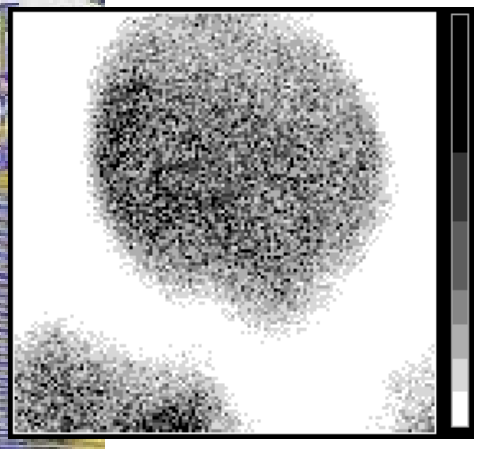
Mineral ions (K, Ca,…)

Trace elements (Mn, Fe, Cu, Zn)

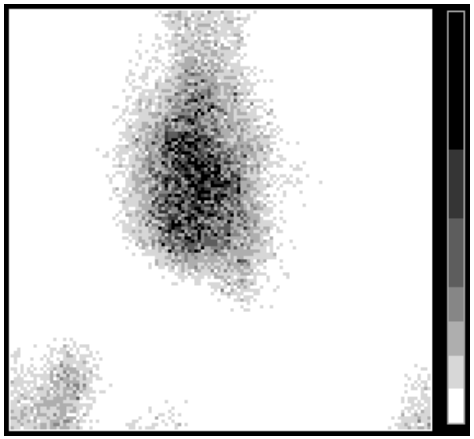
Cross-checking of results for the same cell line …

PIXE & RBS & STIM microanalysis of human cells

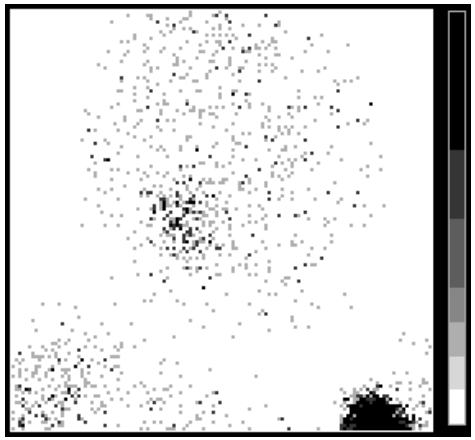
Potassium



Iodine

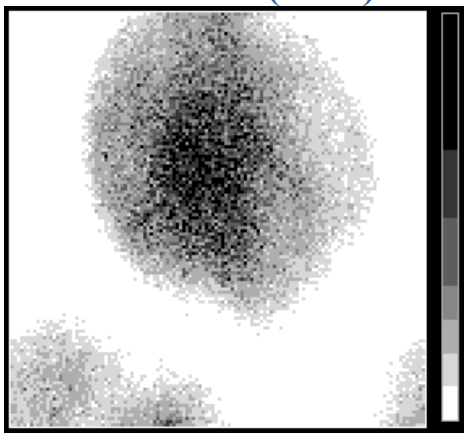


Iron



60 µm

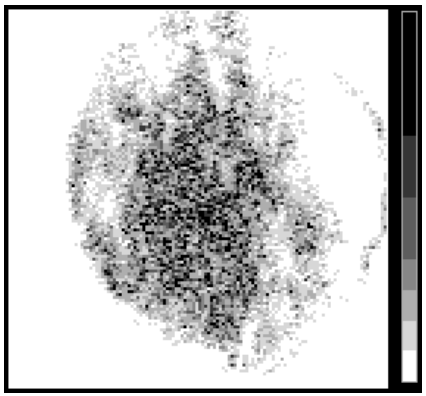
Carbon (RBS)



60 µm

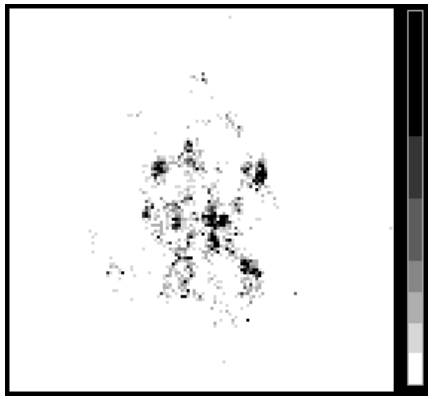
Elemental maps of an individual human ovarian carcinoma cell exposed to 12 µg/ml IDX during 2 hours. Scan size: 60 x 60 µm^2.

STIM (medium density)



50 µm

STIM (high density)



50 µm

Mean concentrations (µg/g dry mass) in human ovarian adenocarcinoma cells (IGROV1) with/without 2 hrs. exposure to 12 µg/ml anthracycline 4'-iodo-4'-deoxydoxorubicin (IDX)

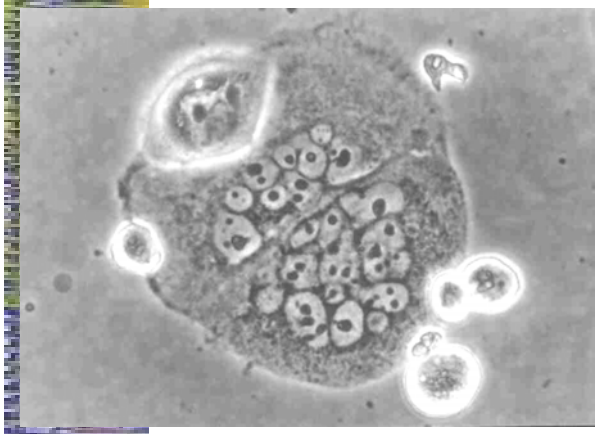
Cellular content (µg/g)	IGROV1	IGROV1 12 µg/ml IDX
Mn	9.5 ±1.6	10.2 ± 3.3
Fe	143 ± 19	138 ± 14
Cu	11.7 ± 3.8	11.4 ± 2.6
Zn	212 ± 23	192 ± 15
I		3046 ± 318

n = 5, mean ± SD

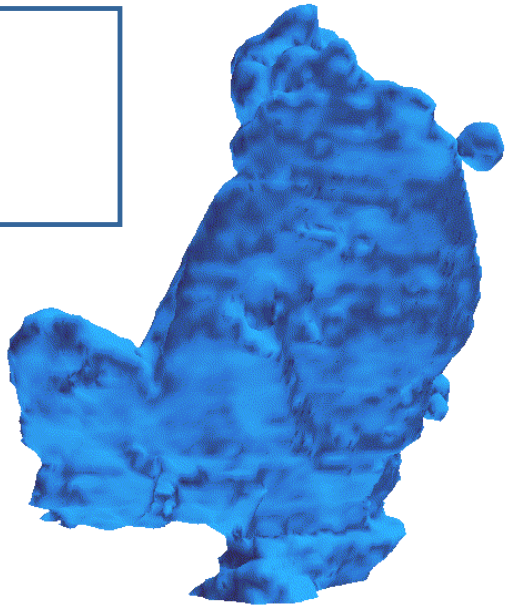
Reference : Nuclear microprobe analysis of iodine and iron distributions in tumor cells exposed to the anthracycline 4'-iodo-4'-deoxydoxorubicin. R. Ortega, Ph. Moretto, Y. Llabador, M. Simonoff, Nucl. Instr. & Meth. B130 (1997) 426-430

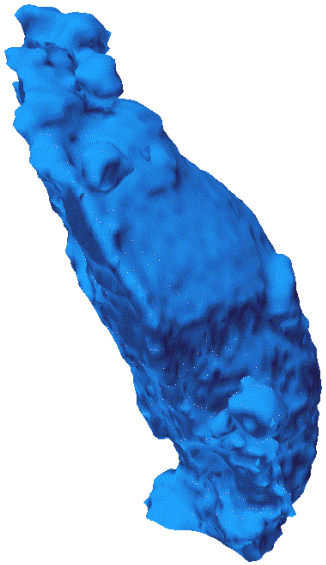
Three-dimensional mapping of individual cells using a proton microbeam.

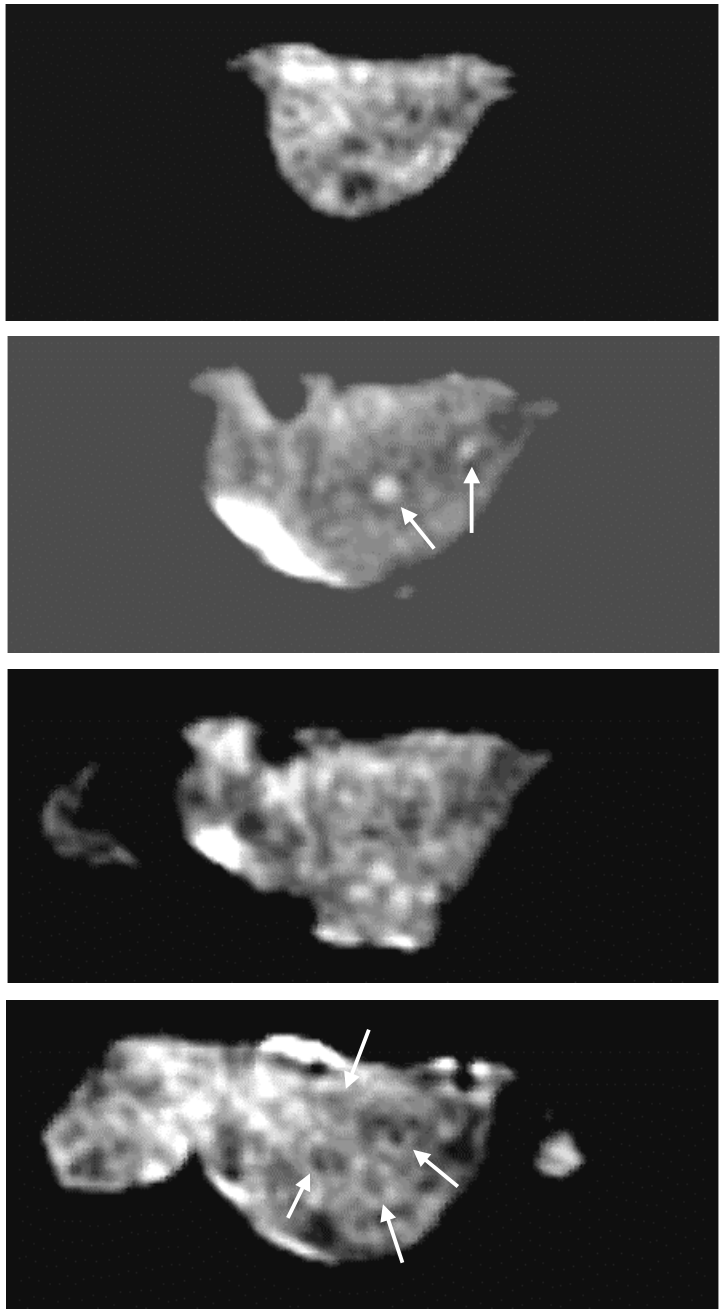
STIM tomography: 2.8 MeV proton beam - 166×166 μm^2 scan - 100 projections over 180°. (a) and (b) are views of the external envelop consisting of 128×128×64 voxels (64 stacked horizontal slices).



IGROV1-DDP human cell

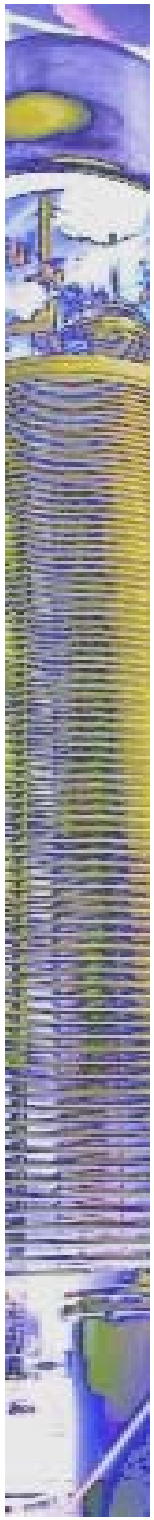






Slices number 24, 33, 37 and 44, from the top to the bottom of the specimen. Density ranging from 0.054 to 0.154 g/cm^3 and from 0.069 to 0.231 g/cm^3 (last slice).

Reference: Three-dimensional mapping of individual cells using a proton microbeam. C. Michelet, Ph. Moretto, Nucl. Instr. & Meth. B150 (1999)173-178



B- Validation of procedures and quality control (new facilities)

1) Technique

To validate the overall irradiation process (from calibration to cell recognition and targeting)

- **The single ions network (in air) on CR39 (or glass)**
- **The CGI test**

2) Cell environment

Stressing effect of ?

cell dish (assays for material cytotoxicity, volume of medium …)

culture membrane

adhesion factor (cell Tak, gelatine …)

UV light

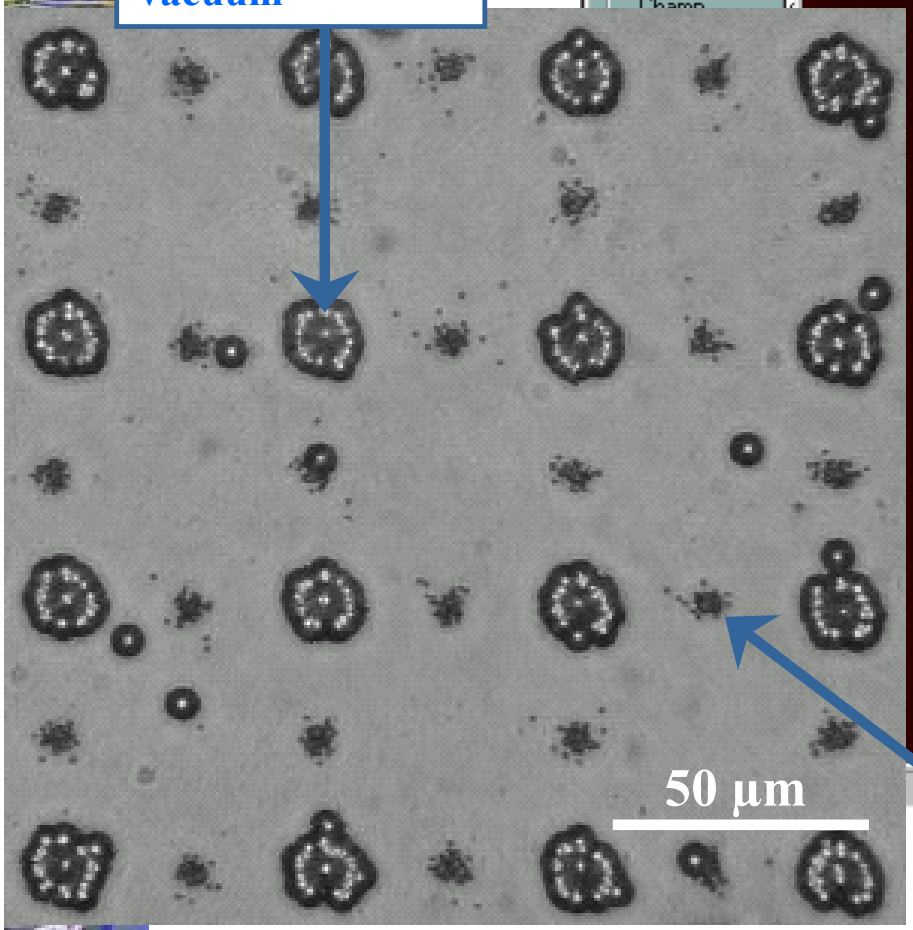
temperature

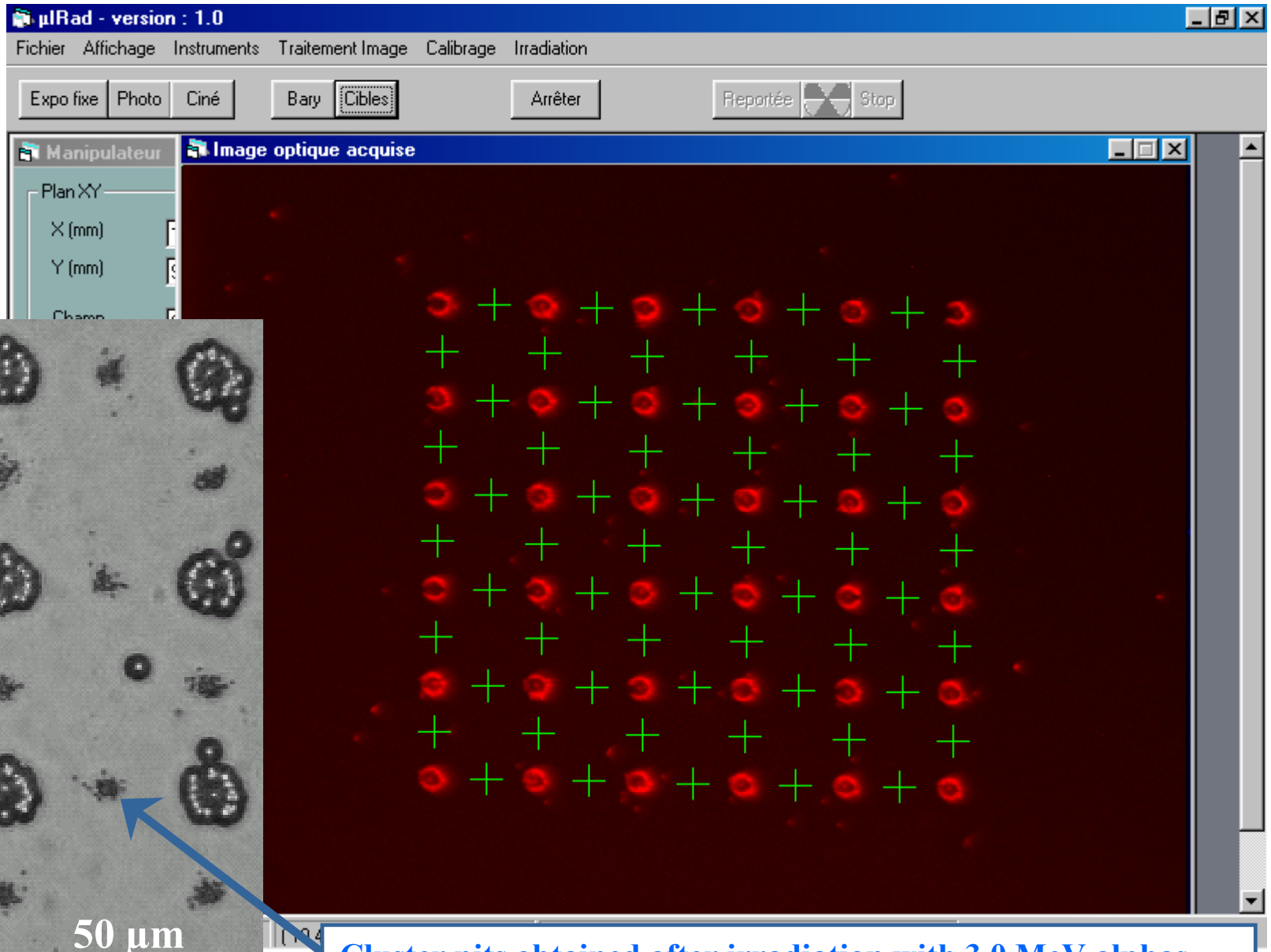
CO2 ?

Clonogenic surviving fraction of controls (UV exposed), plating efficiency control…

Targeting accuracy test : etching CR39 track detectors

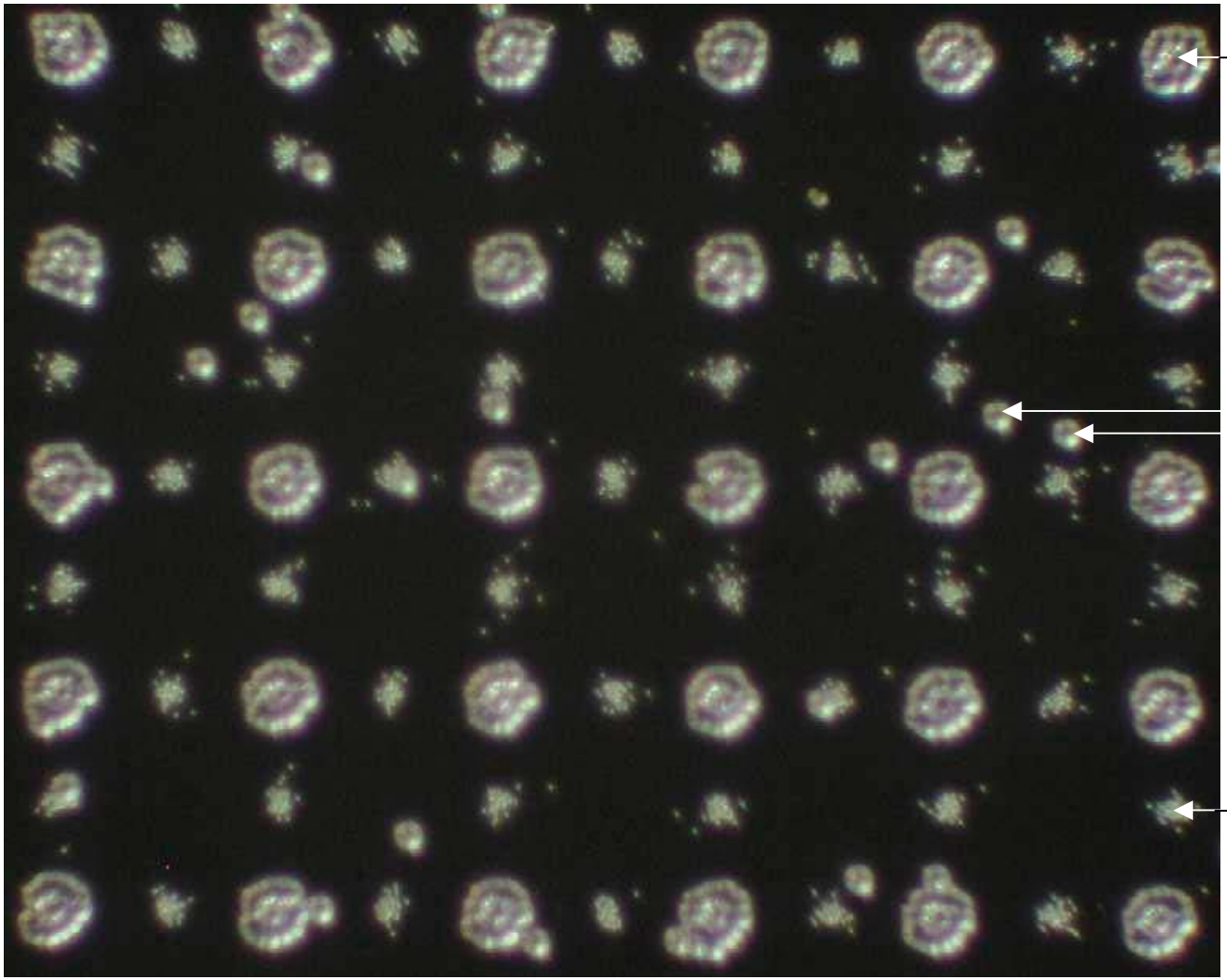
Circular targets obtained under vacuum





Cluster pits obtained after irradiation with 3.0 MeV alphas (50 ions per pit) in air after automatic targeting

Targeting accuracy test: results

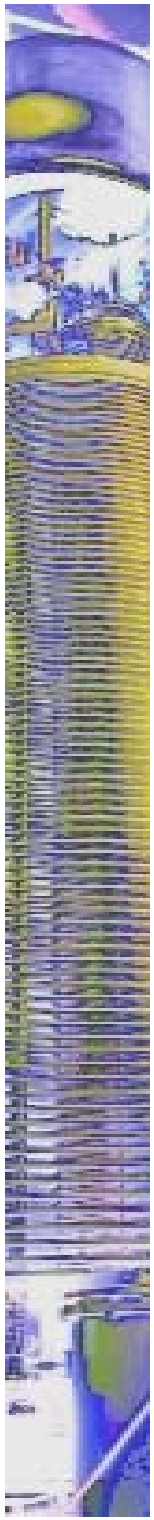


Circular targets obtained after irradiation under vacuum with 3 MeV alphas and KOH etching

Particle tracks resulting from slit scattering under vacuum (etched twice…)

Cluster pits after irradiation with 50 alpha particles in air

20 µm



B- Validation of procedures and quality control (continuation)

2) **Reliability for high statistics irradiation (large number of irradiated cells):**

Standardize a test using the same cell line (fibroblasts, keratinocytes…) :

A simple dose-response curve (range 1-10 alpha particles per nucleus)

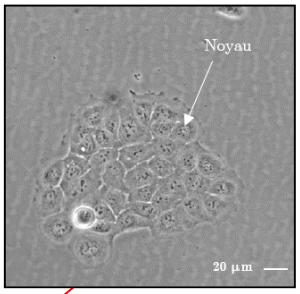
3) **Reliability for low statistics irradiation : Particle track detection in nuclei**

Re-visiting of individual cells immediately after irradiation

Fluorescent distribution of proteins aggregates along the particle trajectories

- Immunostained H2AX (DSBs) ?
- CDKN1A ?
- 53BP1 ?
- Others ?

Protocol: clonogenic survival

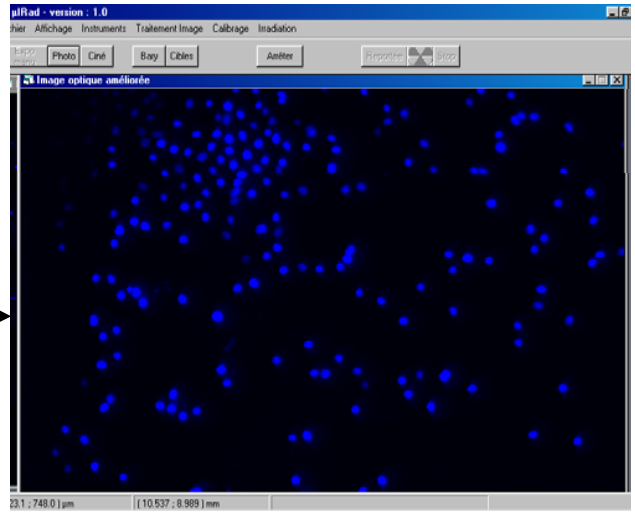


Cellules en culture ~ 2 millions

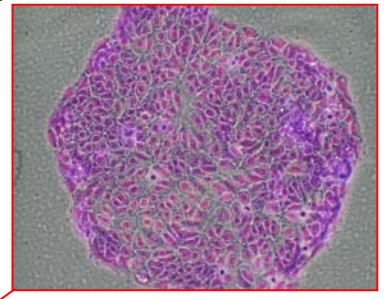
Trypsination, réensemencement

Cellules en culture ~ 1500 cellules

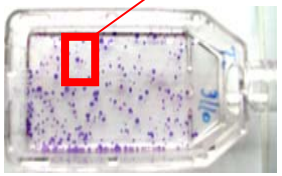
Irradiation



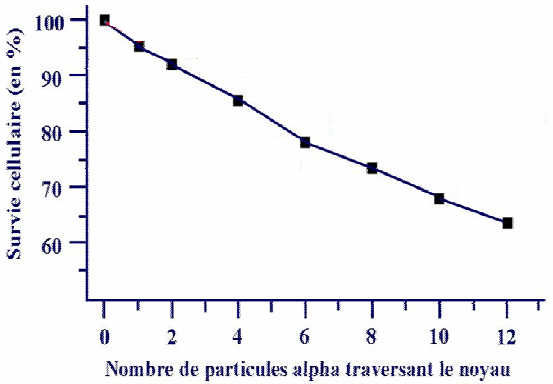
Trypsination, réensemencement

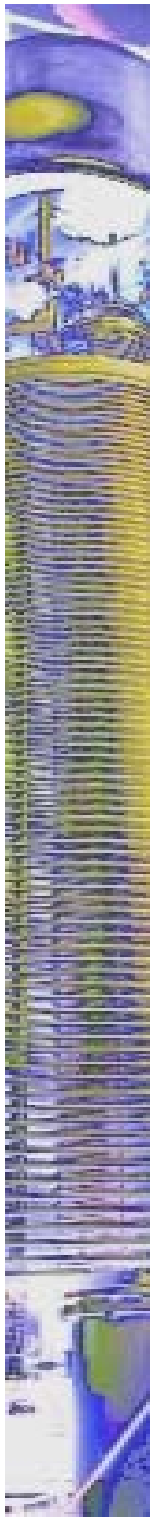


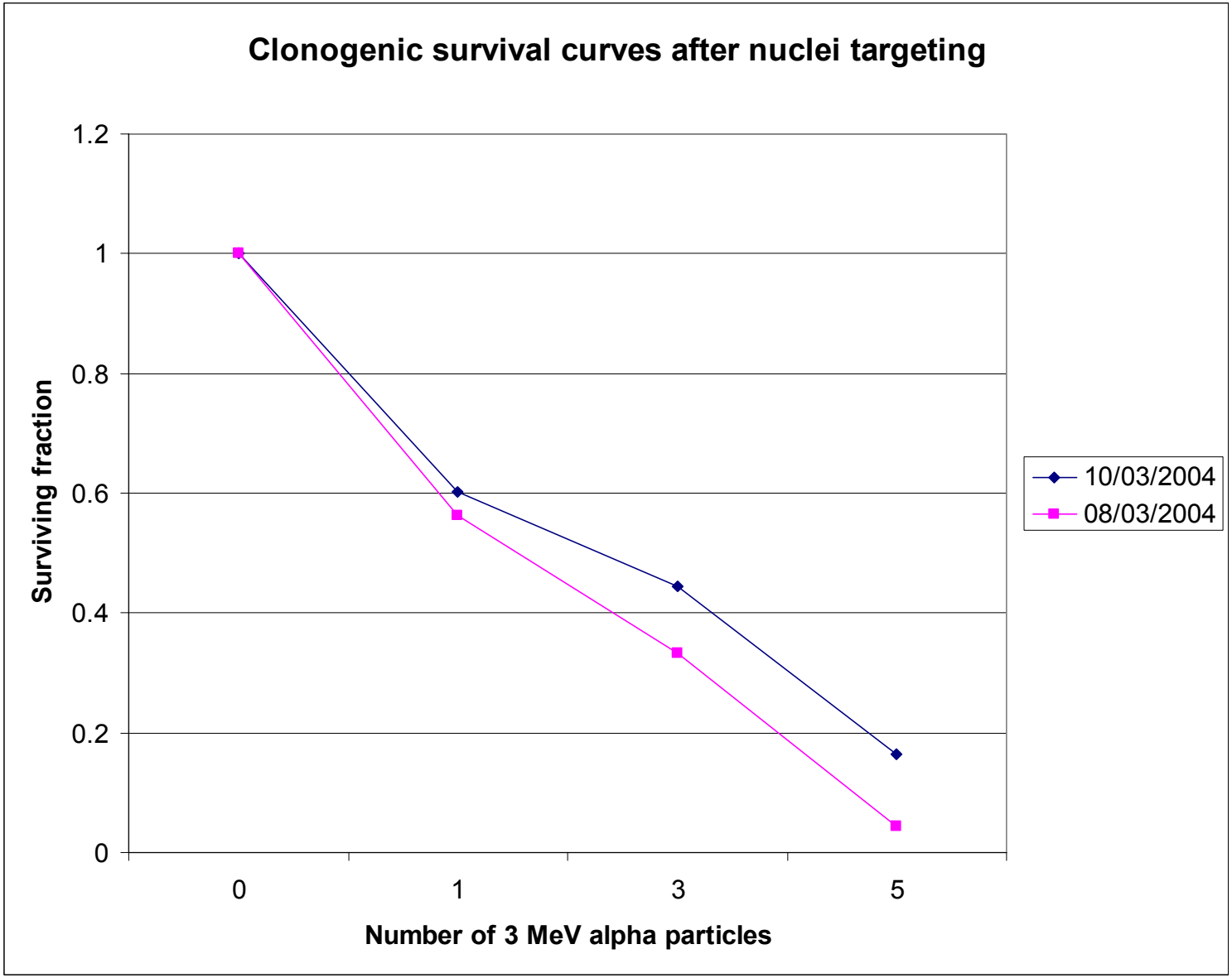
Survie clonogénique



Courbe de survie







HaCat cells : 1500 cells seeded in 10 mm dishes (polypropylene 4 µm coated with Cell-tak)

Hoechst 33342 dye at 500 nM (one hour before exposure) in KBM medium

No temperature control …

C- Evaluation of innovative method for cell or nuclei localization

Tagging of histones : (H2B-GFP proteins) ?

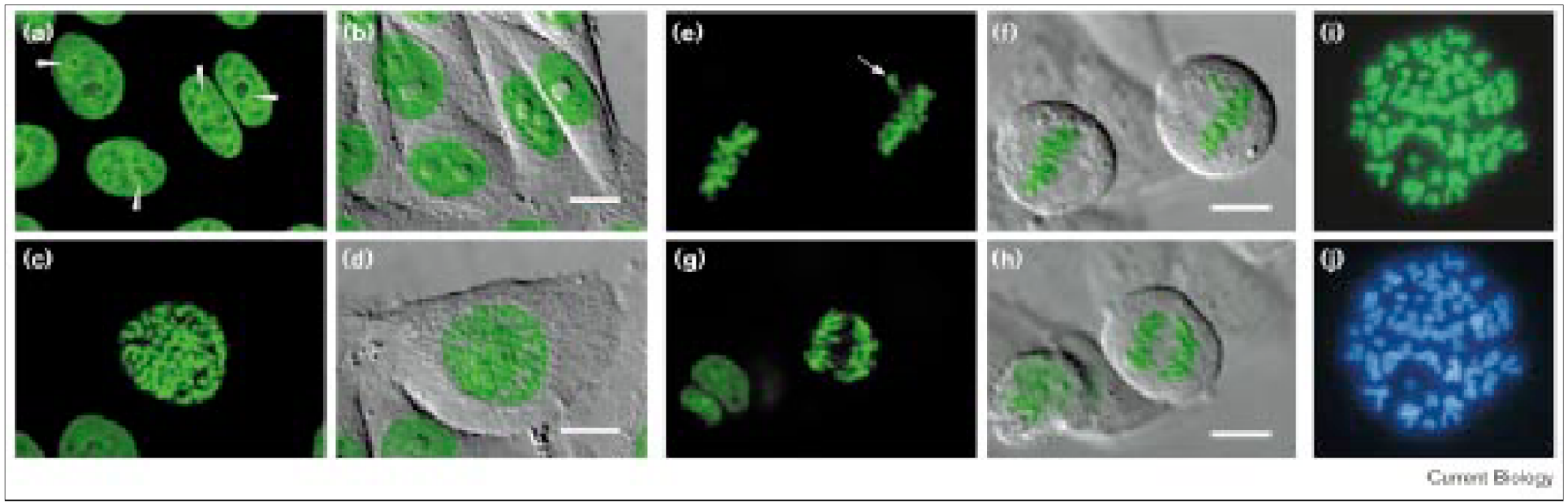
Any others ??

Transfection of HaCat cells with the human histone H2B-GFP gene

The green fluorescent protein (GFP) is an auto-fluorescent protein isolated from the jellyfish that produces fluorescence at 509 nm under blue light illumination at 490 nm. This system is indeed less damaging than UV light employed for excitation of Hoechst 33342 dye.

The human histone H2B gene is fused to the gene encoding the green fluorescent protein (GFP) and transfected into HaCat cells to generate a stable line constitutively expressing H2B-GFP.

The H2B-GFP fusion protein is incorporated into nucleosomes without affecting cell cycle progression. Using confocal microscopy, H2B-GFP allows high resolution imaging of various chromatin condensation states in live cells.

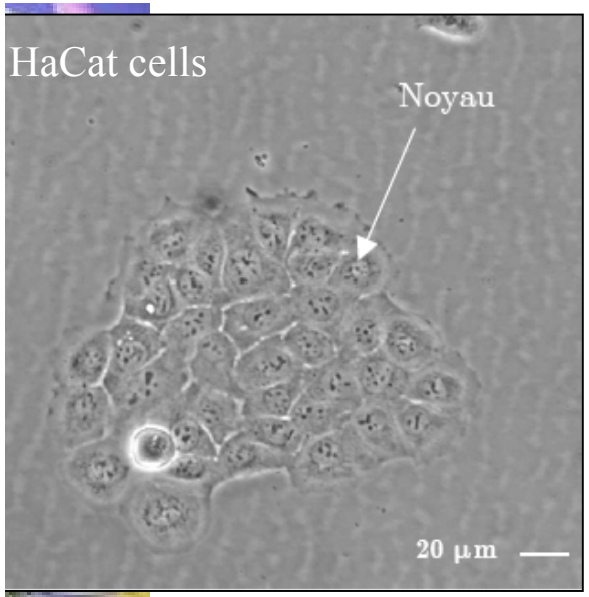


Localization of H2B–GFP protein. **(a–h)** Confocal microscopic images of live HeLa cells expressing H2B–GFP in various cell cycle phases. (a,c,e,g) The GFP fluorescence and (b,d,f,h) the corresponding differential interference contrast images are shown for (a,b) interphase, (c,d) prophase, (e,f) metaphase and (g,h) anaphase cells. Perinucleolar densely staining regions of H2B–GFP are indicated by arrowheads in (a). A pair of lagging sister chromatids with a centromeric constriction is indicated by an arrow in (e). The scale bars are 10 μm. **(i)** GFP localization and **(j)** DAPI staining of fixed chromosome spreads of HeLa cells expressing H2B–GFP.

Transduction pathways in apoptosis as a function of subcellular target

Collaborative work with F. Ichas, European Institute for Chemistry and Biology, INSERM EMI-9929, Univ. Bordeaux II

- **The approach is based on the use of fluorescent proteins (GFP) and consists in the construction of fusion proteins that once overexpressed in transfected cells can convert molecular signalling like protein-proteins interactions into fluorescent signals detectable by high resolution subcellular multiwavelength fluorescence imaging.**
- **In the field of apoptosis signalling, a role is played by the mitochondria in the transduction and the execution of the pro-apoptotic signal:**
- **In the sequence of events that led to the apoptosis, a mitochondrial step is often found that associates a depolarisation of the inner mitochondrial membrane and release of cytochrome c from the inter-membrane space. The products of the proto-oncogene Bcl-2, localised in the outer mitochondrial membrane are capable of inhibiting this mitochondrial step, while the anti-oncogenes Bad and Bax seem to promote the latter.**
- **Certain apoptotic models indicate that these early mitochondrial events may depend on the activation of a giant mitochondrial channel : the permeability transition pore – PTP.**
- **It has been shown that under conditions of oxidative stress, opening of the the mitochondrial PTP represents an early trigger and is crucial in causing cytochrome c release. Using live cell imaging and fluorescent probes based on GFP (FASEB J 2002 Feb 25).**



Infrared Microspectroscopic Characteristics of Radiation-Induced Damages

Collaborative work with JL. Lefaix and N. Gault, DRR, CEA/DSV, Fontenay-aux-Roses

▪**Fourier Transform Infrared Micro-spectroscopy (FT-IR) : Absorption spectrum sensitive to the biochemical changes in the various subcellular compartments (DNA, lipids, carbohydrates and proteins). Those spectra provide pertinent information on molecular composition and conformational structure.**

▪**It has been recently shown that FT-IR may be used for assessing the process of apoptosis at the molecular level**

Ref: Infrared microspectroscopic characteristics of radiation-induced apoptosis in human lymphocytes, N. Gailt, J.L. Lefaix, Radiat Res. 2003 Aug; 160(2): 238-50

Objectives : FT-IR study of radio-induced alterations in individual apoptotic cells after selective irradiation of cellular compartments with MeV Alphas (nuclei).

Cells : HaCaT, permanent epithelial cell line obtained by spontaneous transformation of human adult keratinocytes.

Different steps:

- Validation of the clonogenic survival curves by alpha irradiation of HaCat cell nuclei - Comparison of microbeam irradiation with classical alpha irradiation.

- Selective irradiation of nuclear compartments and FT-IR analysis of individual cells for different dose levels.