Proliferative stem(/MSC)cell number per unit area (P197)

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CAWG CCQM, Nov 2023

Scope of the project

Successful participation in this pilot study will demonstrate the competencies of participating laboratories in:

a) differential counting of (stem) cells seeded on planar 2D scaffolds

b) deriving proliferated stem cell number per unit area and proliferated stem cell fraction on planar 2D scaffolds

c) documenting the uncertainties of their cell-count measurement results

Confirmed participation:
INRIM, NIST, NRC, BAM, NIBSC, KRISS, INMETRO, VNIM, NIM-C and Tubitak

Measurand: To be Discussed

Proliferative stem cell number per unit area on each substrate will be assessed by:

- counting number of dual (**Hoechst** and **Edu Alexa Fluor 488**) stained cells ($\boldsymbol{X}$) in mm^2

Proliferative stem cell fraction on each substrate will be derived from:

- Counting number of dual (Hoeschst and Edu Alexa Fluor 488) stained cells ($\boldsymbol{X}$) in mm^2
- Counting number of Hoeschst stained cells ($\boldsymbol{Y}$) in mm^2
- Proliferative stem cell fraction = ($\boldsymbol{X}/\boldsymbol{Y}$)

Measurement claim: To Be discussed

- **Claim:**

The number of proliferated stem cells per unit area (in the range 0 to 5000 cell/mm^2)

and

The proliferated stem cell fraction in the range 0 to 1 on 2D substrates (synthetic vs. native)

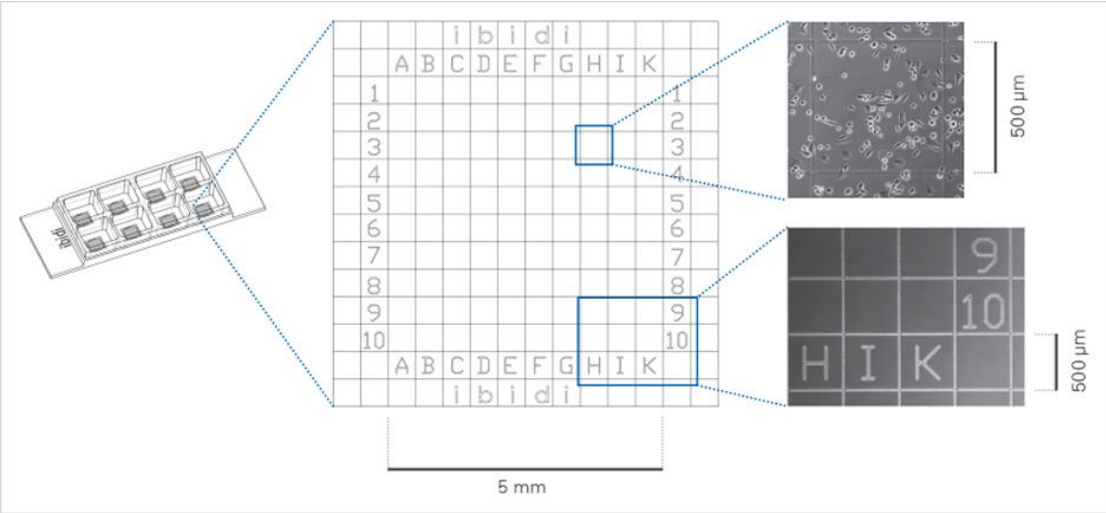
Participating labs would:

- Image the prepared cells on substrates (native and synthetic) using fluorescence microscope able to discern (Edu, green; ex. at 488 nm and Hoechst, blue; ex. at 405 nm)
- Microscope in inverted setup should be capable of collecting images in phase contrast or brightfield to identify each defined quadrats for counting the cells within it (20x objectives are suitable to resolve cellular details and a unit grid square for the study)
- A camera to acquire the digital images of the stained cells
- A computer system for image analysis to obtain the ratio of proliferated cells and total number of cells
- Data (count) can be extracted either manually or by using algorithms of automated image analysis software
- Calculating uncertainty budget:
  based on P123 discussion all participating laboratories should do it

Study material:

Participants will receive two 8-well chambered dishes – 1 chamber with seeded cells and 1 chamber with beads for calibration

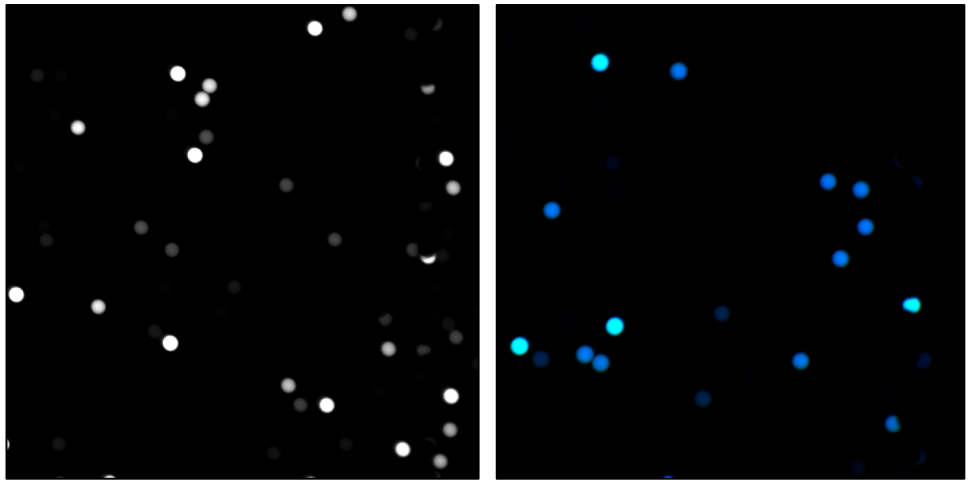
1 seeded with double stained hMSCs to be counted



- will count cells in 3 defined quadrants (of 500 µm x 500 µm) in each one of the 6 well (2x3 wells with native and synthetic material coating), for a total of **18 quadrants:** e.g. quandrants H3, A1, E9.

NOTE: Numbers and letters will be given to each participant by the pilot laboratories.

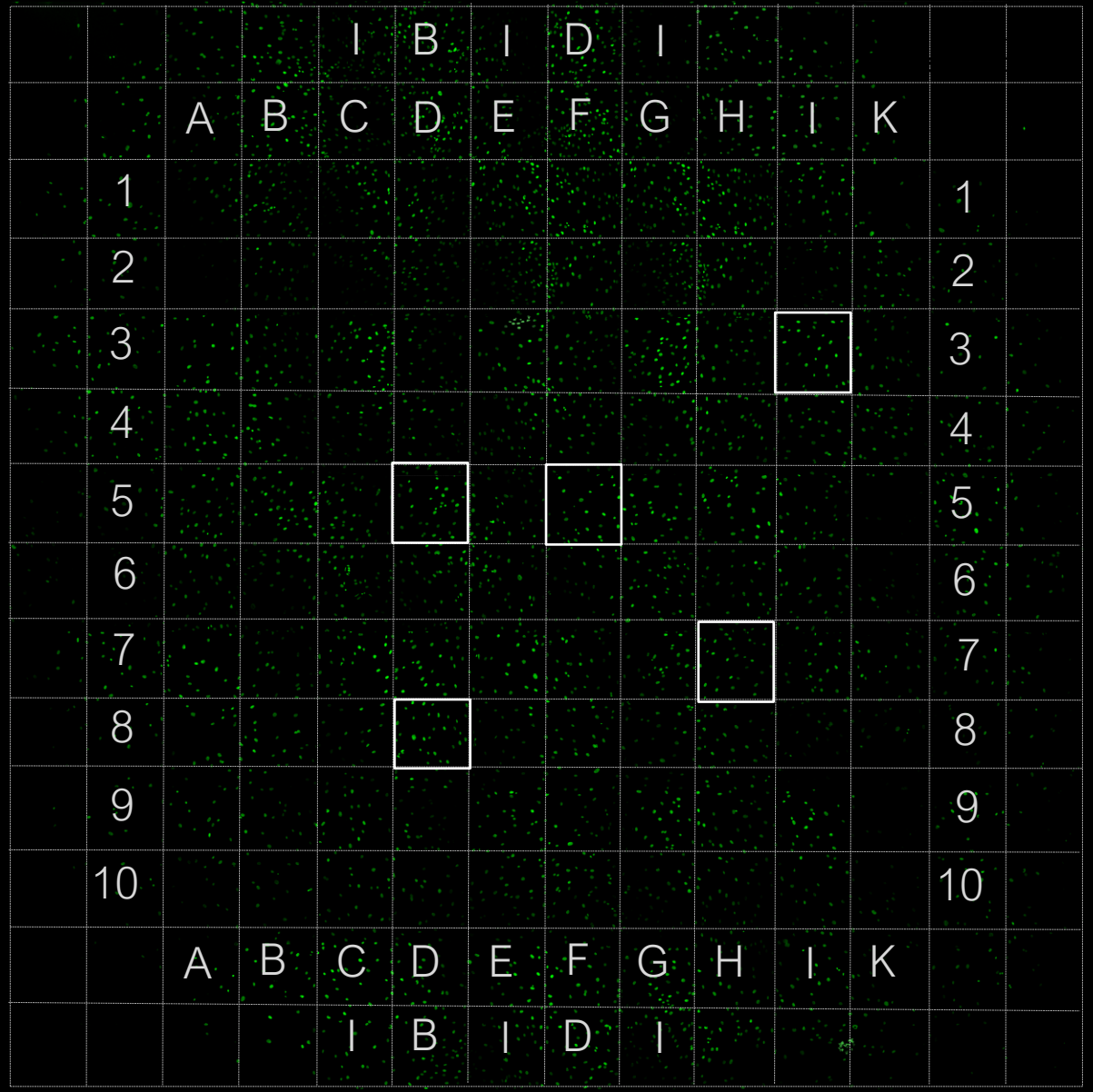
1 seeded with fluorescent beads to test the fluorescence detection



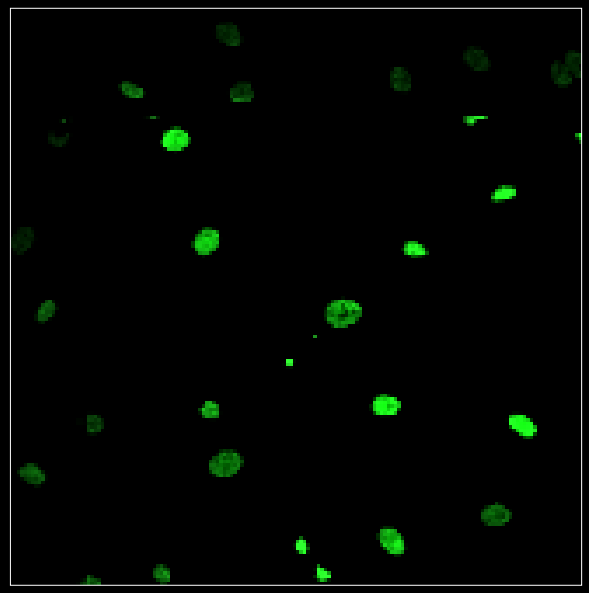
- will use the beads to set the microscope for minimum fluorescence detection, simulating the lowest fluorescence for proliferated cells showing very low Edu fluorescence.

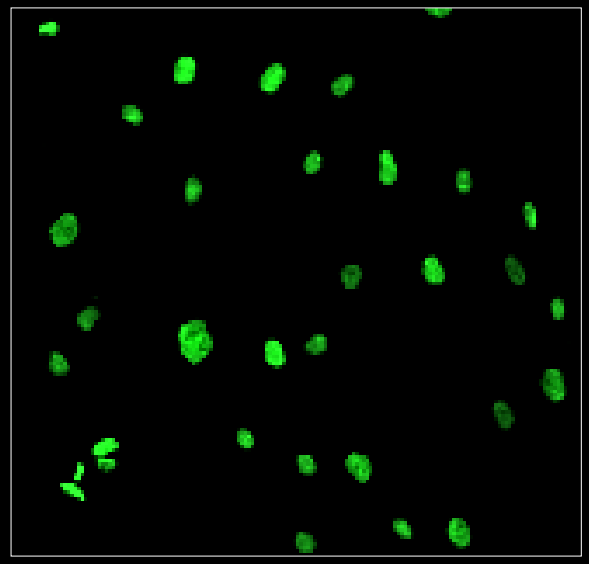
Representative micrographs: MSC with EdU on native protein substrate - full scan

An individual well – area of 7 mm x 7 mm:



1 grid square: FOV 500 µm x 500 µm





Olympus IX81 inverted
UPLSAPO 10x (air) NA 0.40
800 pixels x 800 pixels,
integration time 8 µs/pixel
in 7x7 mosaic covering area 7 mm x 7 mm

Reporting results:

Quadrant ID	Measurand X	Measurand Y	Measurand X/Y	note
For e.g B12				

INSTITUTE	
Contact person	
Email address	

- **Measurands:**

1. Number of stem cells in proliferated state: X
2. Total number of stem cells: Y
3. Proliferated stem cell fraction X/Y

Lab ID	Microscope settings	note
Microscope Objective	model, manufacturer Magnification, NA, immersion (oil, air, water etc.)	
Camera	model, manufacturer, pixel size	
Software	Name, version, etc..	
Filters	ex/em wavelengths, any other relevant information	

Uncertainty: each participant will give a brief explanation on uncertainty evaluation, including sources and reference guidelines.

Examples of uncertainty sources

- Fluorescence: minimum detectable fluorescence intensity
- Colocalization shifts
- Counting: exclusion or inclusion of cells on the edge of the quadrant
- Counting: false positive (i.e. dots of the grid)
- Manual or automatic counting

Proposed time-line: updated

Time	Event
Dec 2023	Sample distribution
April 2024	Data return and analysis
Oct 2024	Data presentation
Dec 2023	Report draft A

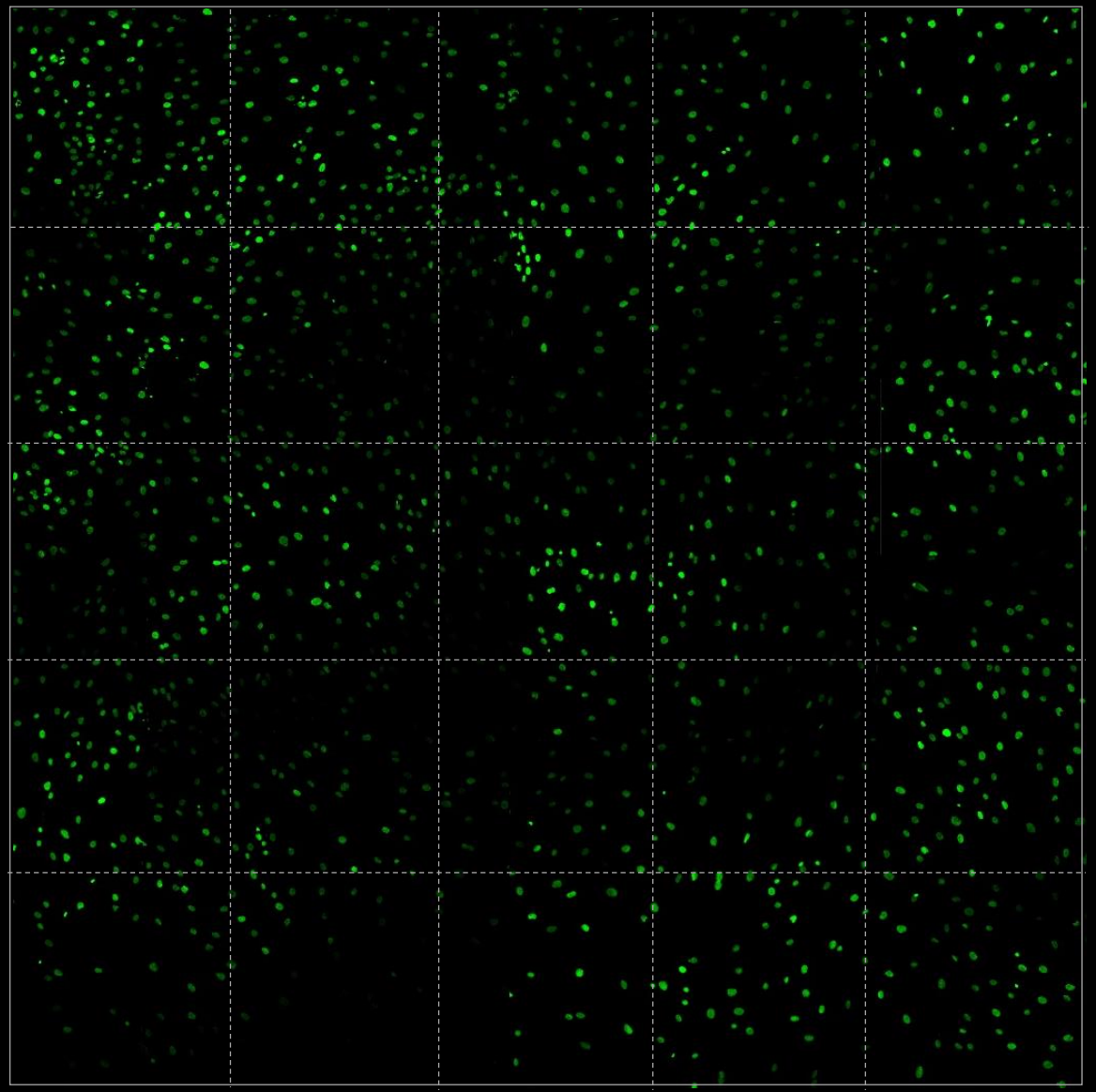
NB:
Protocol document distribution: After March 2023 meeting
Sample preparation complete
Contact person from each institute will be contacted prior to the shipment

Confirmed participation:
INRIM, NIST, NRC, LGC, KRISS, INMETRO, NIM-C and Tubitak

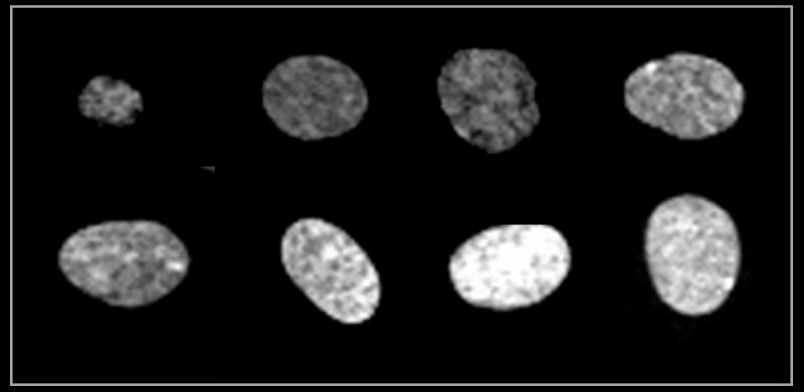
List of completed characterization

Chamber slide dimensional characterisation: Area measurement of the quadrates (25 grids)	**Laser profilometry (INRIM), Optical microscopy (NPL)**	√
Cell distribution on the grid Optimal mounting media selected Florescence stability tested (>1 year) Storage condition tested (>1 year) Automated and manual counting	**Optical microscopy (NPL) and a slide each was provided to INRIM and NIST**	√
Intensity bead testing (colocalization, intensity) Imaging parameter requirements for microscope Automated and manual counting	**Optical microscopy (NPL & NIST) and EM (NPL)**	√
Shipping conditions tested	**Slides were successfully packaged and shipped to INRIM and NIST**	√

Representative micrographs: MSC with EdU on native protein substrate - full scan



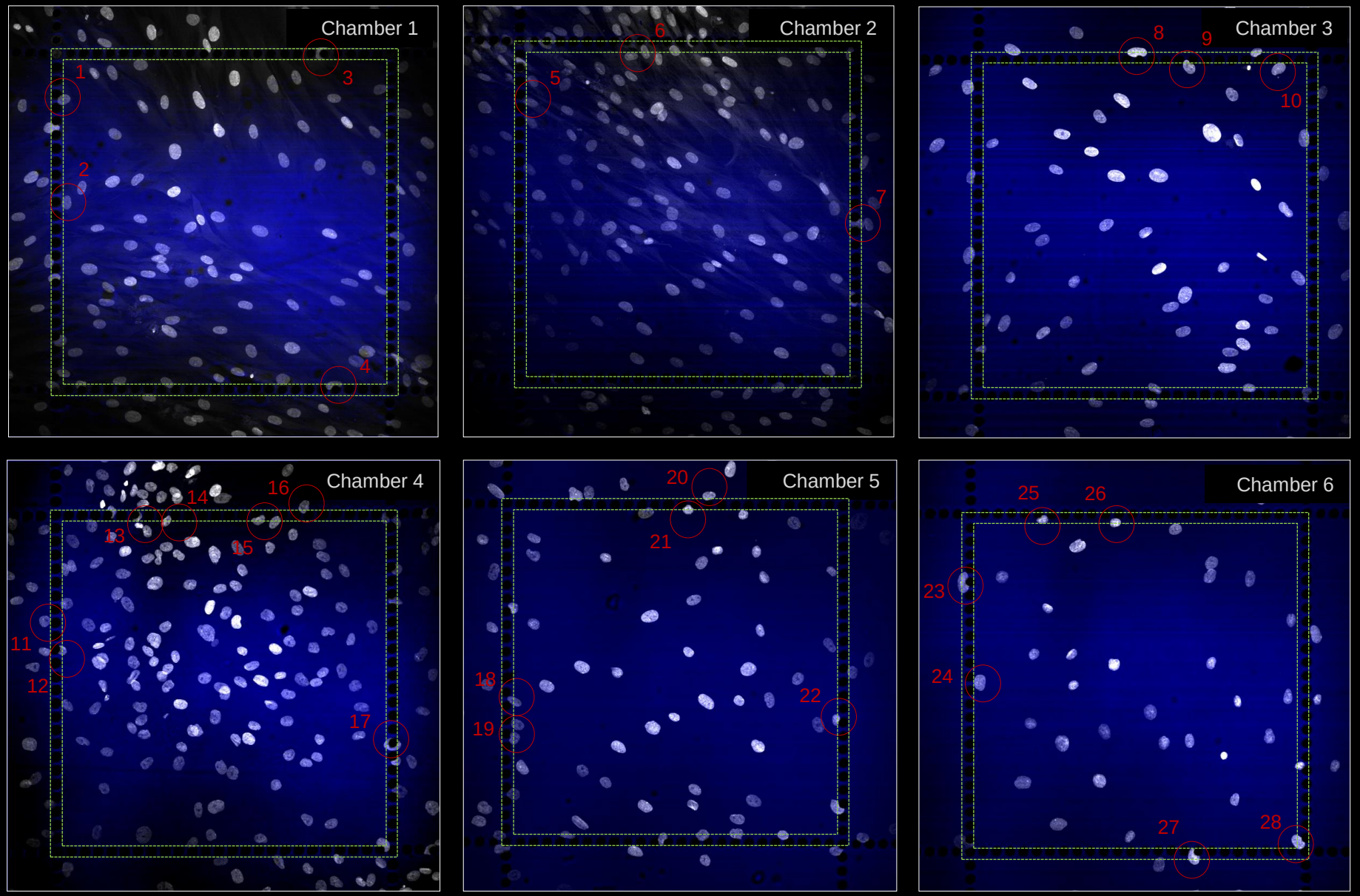
High-resolution images of EdU stained nuclei showing different proliferative status of cells



Change in intensity

Olympus IX81 inverted
UPLSAPO 10x (air) NA 0.40
800 pixels x 800 pixels
in 5x5 mosaic covering area 2.5 mm x 2.5 mm

Quality control: MSC with EdU on synthetic peptide coating – sub-region



Calibration needed:
- Fluorescent intensity on ex. 488 nm
- Colocalization of signal on ex. 405 nm vs. 488 nm

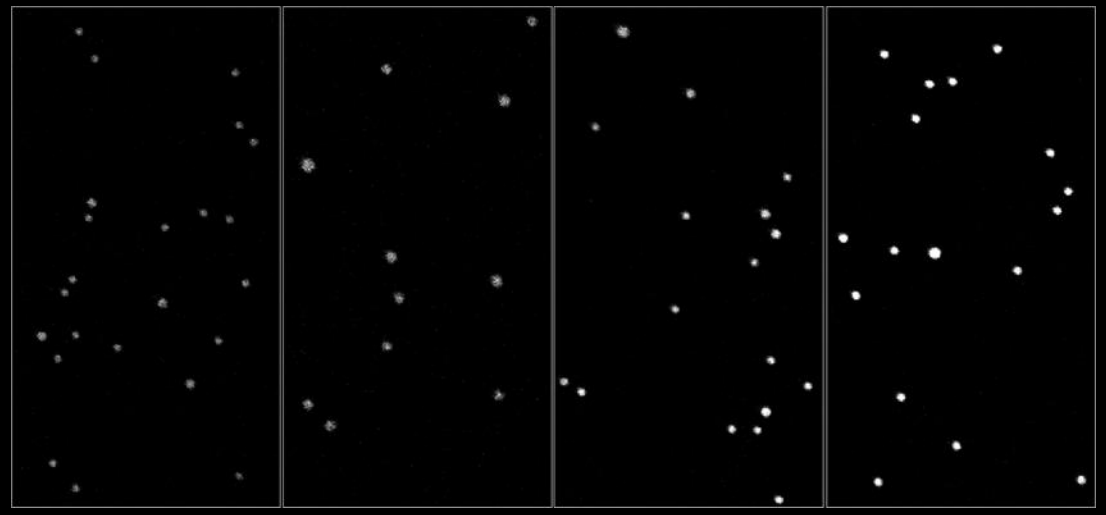
Olympus IX81 inverted
UPLSAPO 20x (air) NA 0.75
1600 pixels x 1600 pixels
acquisition 8 µs/pixel, frame averaging 2

Representative test samples in 6 IBIDI chambers and cell counting
Substrate: NPL CRM filaments on polymer (specific oil requires to be used) coverslip bottom with grids

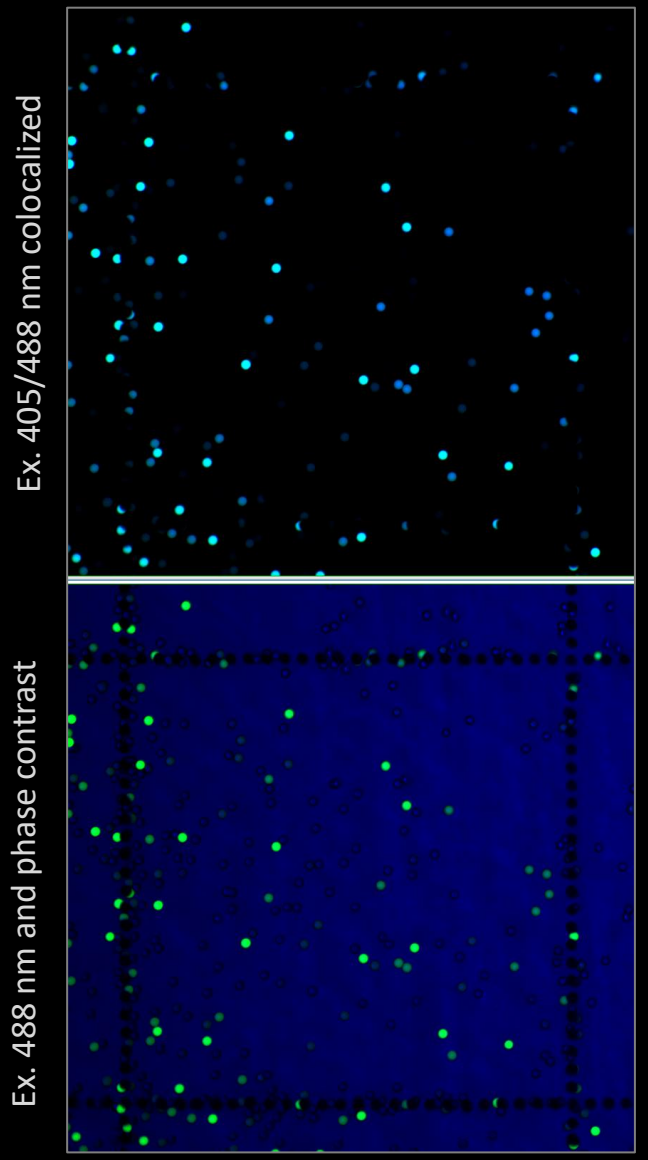
FoV: 635.2 µm x 635.2 µm

Calibration and calibration checks on fluorescent intensity and for colocalization

Intensity

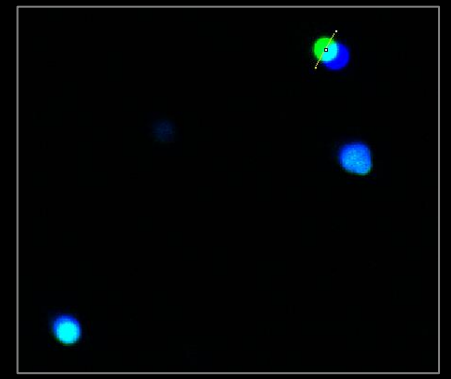


Type 2 beads: SpheroTech beads 3.9µm in diameter

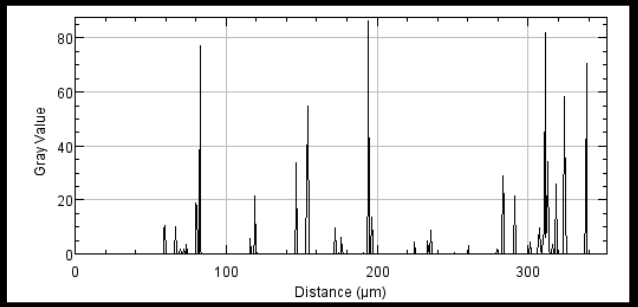


multispectral beads 10µm in diameter

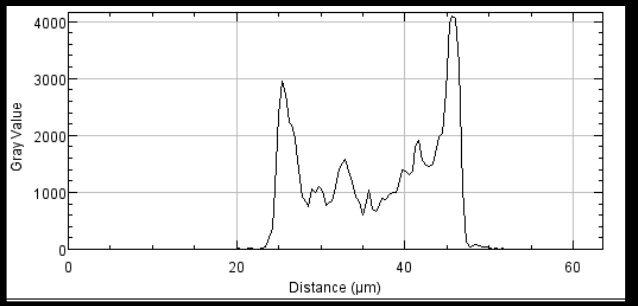
Colocalization



Shifting beads signal: fluorescent signal on ex. 405/488nm



Typical background signal (noise)



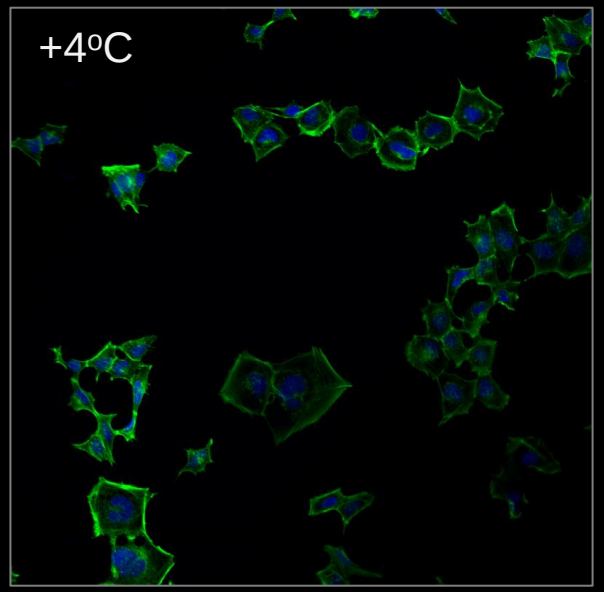
Typical signal from sample (ex. 488 nm)

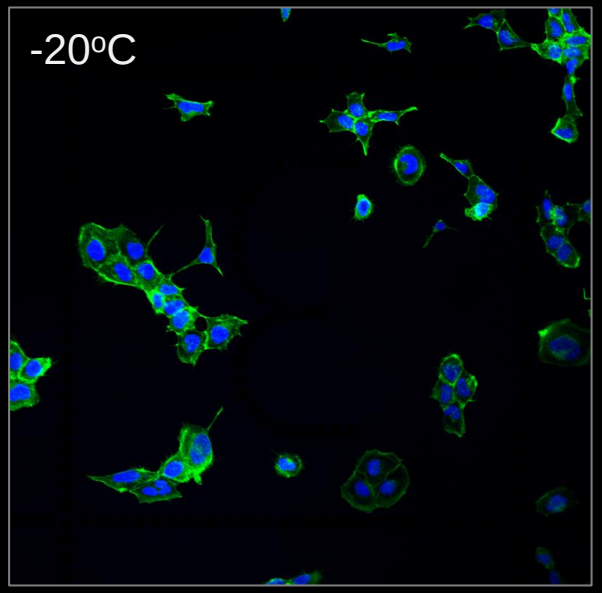
Stability of samples: mounted in Prolong Diamond and Glass

Conditions: at +20ºC / +4ºC / -25ºC

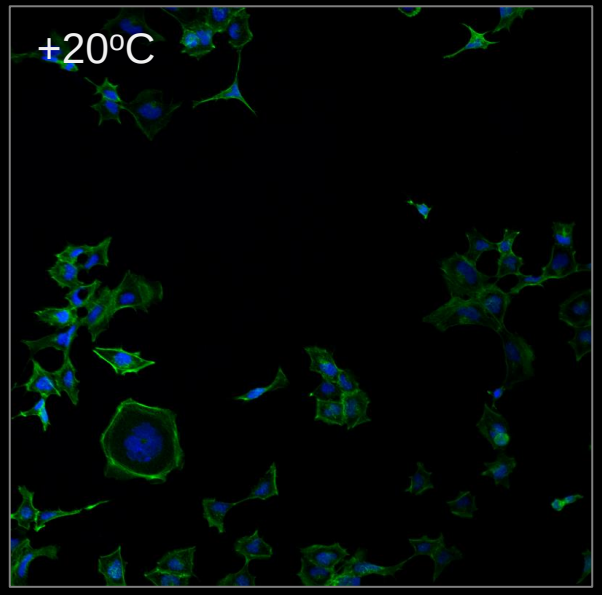
Prolong Diamond

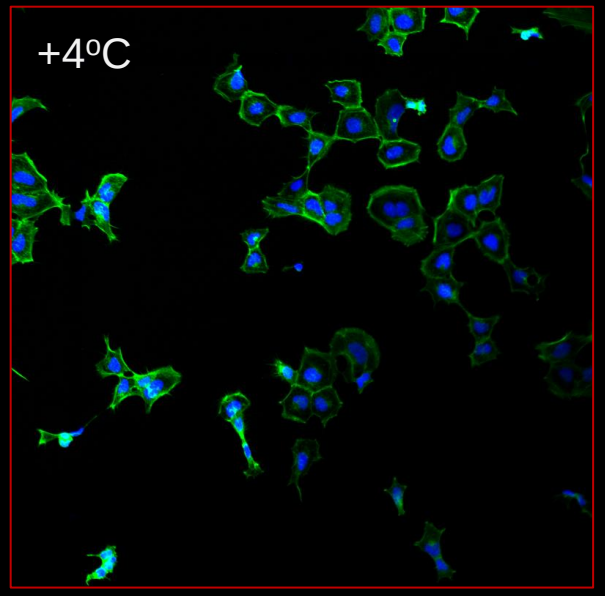
In 2 months fluorescent signal was significantly dropped reported by INRIM / NIST on samples shipped Q1 / 2021

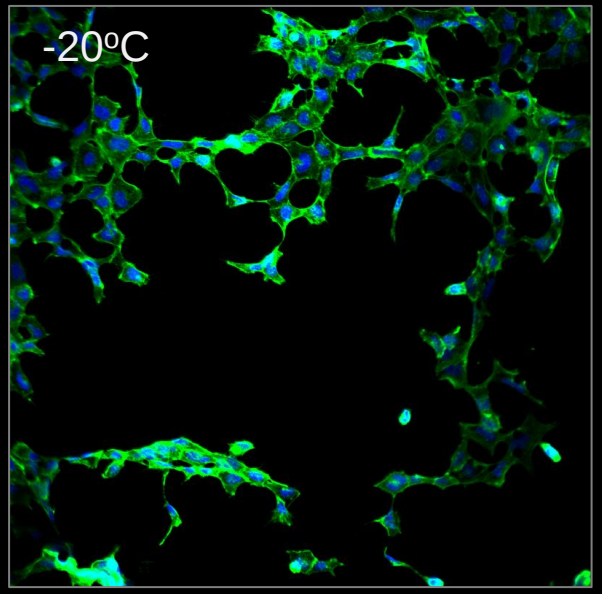




Prolong Glass





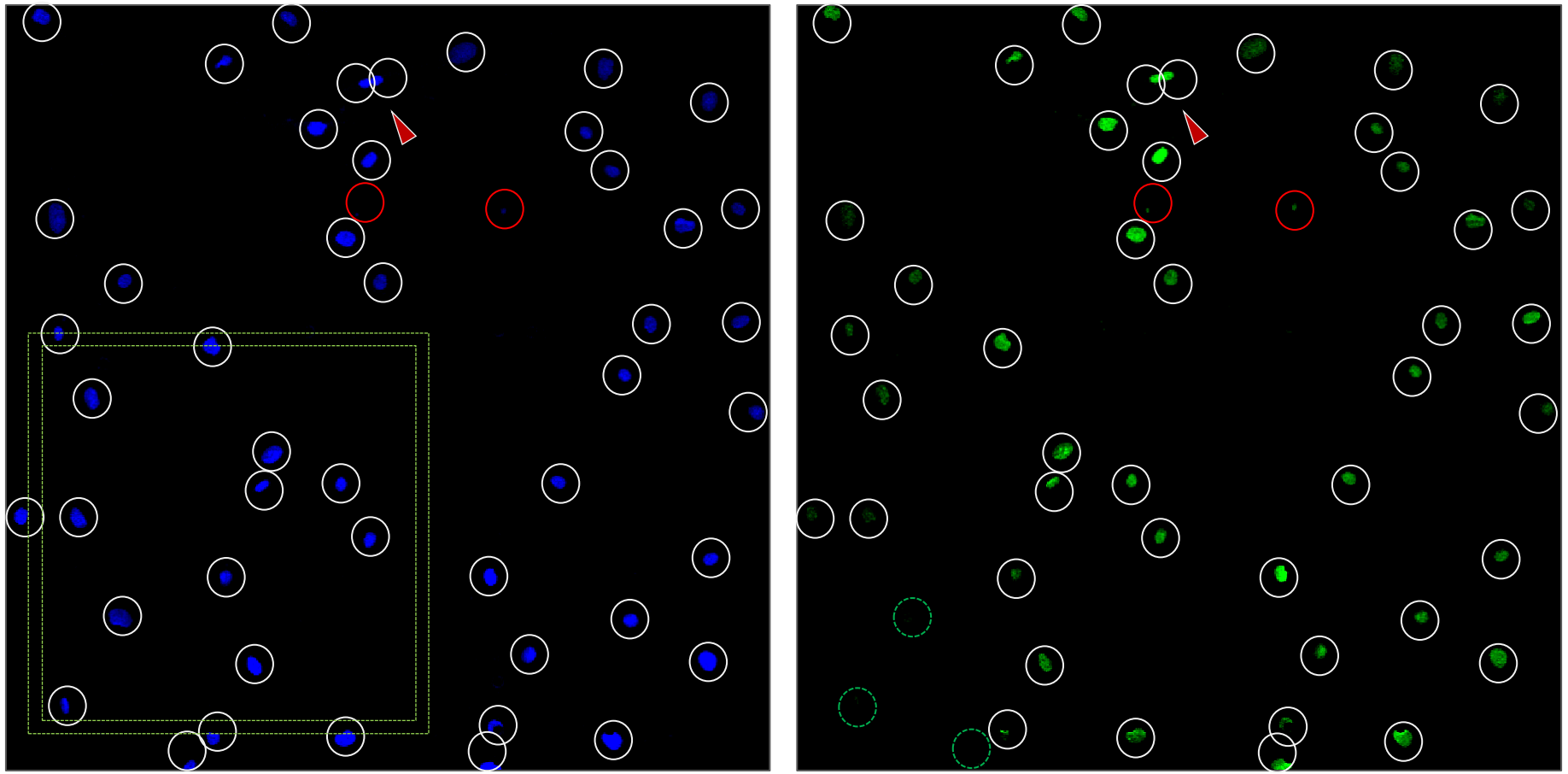


FoV: 635.2 µm x 635.2 µm

Representative example for cell counting and concept of analysis and colocalization

Hoechst (nuclei marker) ex. 405 nm

EDU (cell proliferation marker) ex. 488 nm



100 µm

Olympus IX81 inverted
UPLSAPO 10x (air) NA 0.40
800 pixels x 800 pixels
FOV: 980µm x 980µm

Area / measurands	Total number of cells (X)	Proliferative cell number (Y)	Ratio (X/Y)
1mm x 1mm	48	45	0.936
500µm x 500µm	homogeneity requirement: <5% in cell number (TNC) / unit area (under discussion)		

counted cells (X or Y)

non-proliferative cells

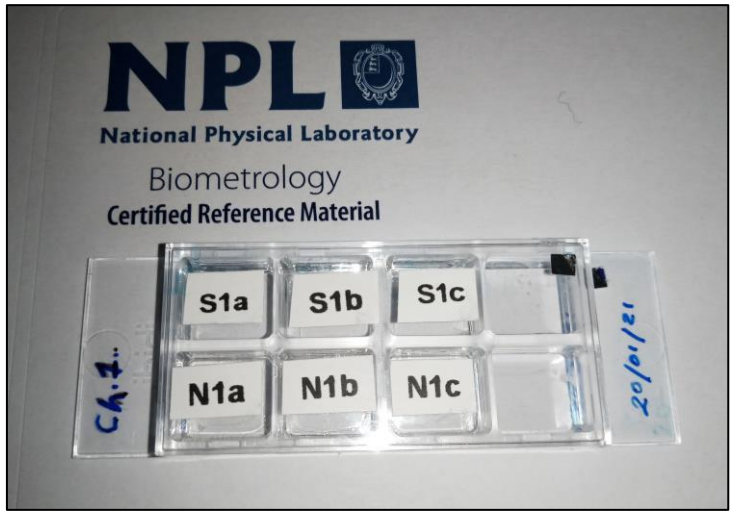
non-colocalized

Sample arrangement for shipment

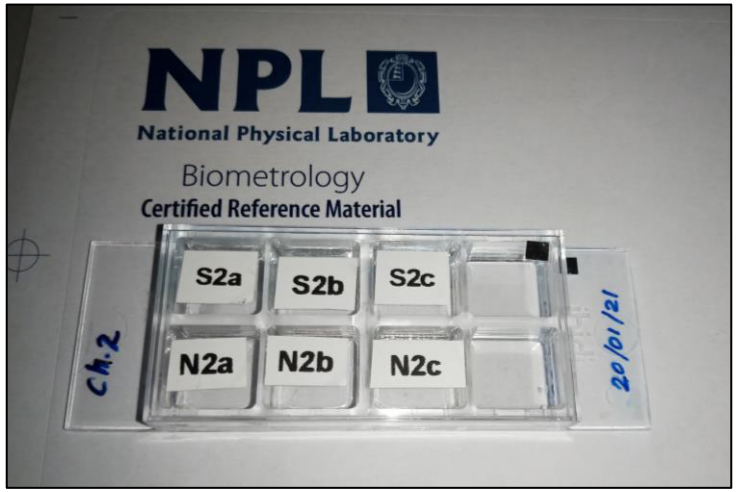
Based on first stage testing with INRIM and NIST being tested and optimized for extreme shipment conditions

Sample format:

NIST- chamber 1



INRIM – chamber 2



Sample packaging inside a plastic slide box with a bubble wrap