Università di Genova | Dipartimento di Fisica

PhD Program in Physics

third Year 2025

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Cycle: XXXVIII
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Tentative Research Project title:

Quantitative STED Nanoscopy and Multidimensional Characterization of Chromatin Architecture

Annual Report of Year 3

1. Research Activity

During the third year of my PhD, my research activity focused on establishing a quantitative, high-resolution framework for characterizing chromatin architecture in human cell nuclei using STED nanoscopy combined with advanced image-analysis tools. After the availability of NucSpot Live 650 fluorophore and the full implementation of the Leica Stellaris 8 STED system, I was able to transition from diffraction limited confocal imaging (performed in year 2) to true nanoscale imaging, enabling the extraction of new structural descriptors with significantly improved spatial fidelity.

A major outcome of this year is the completion of a manuscript entitled “Quantitative Characterization of Chromatin Architecture Using Fractal Dimension, Radial Profile, Total Perimeter of Chromatin Domains (TPD) and IsoConcentraChromJ Parameter from Fluorescence STED Microscopy Images.” The work introduces a multiscale analytical pipeline for chromatin quantification in two human cell types: HeLa (cancerous) and fibroblast (normal) cells.

The research activity consisted of four interconnected tasks:

a) Acquisition of nanoscale chromatin images (STED mode)

I performed systematic STED imaging on fixed nuclei from both cell types, using NucSpot650 as a DNA marker. I optimized the imaging conditions (excitation, depletion laser power, pixel sampling, FOV selection) and verified the quantitative reliability of the images through FRC-based resolution estimation. We have reached an acceptable resolution.

b) Development of a four-parameter chromatin quantification framework

Based on the STED images, I extracted four structural descriptors:

1. **Fractal Dimension (FD):** describing hierarchical chromatin complexity.

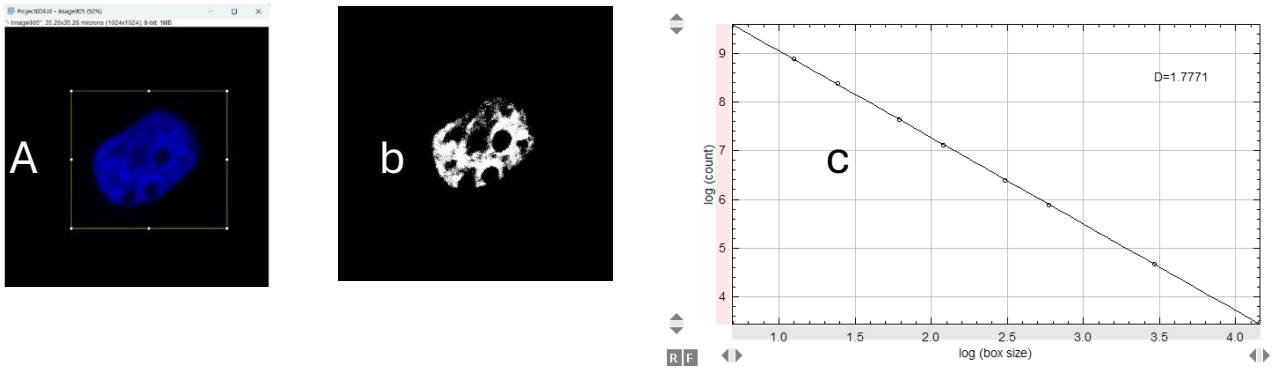

Fig.1: Extraction of FD value. **a)** Original image, with selection of ROI to be cropped. **b)** Resulting binarized image. **c)** Log-log plot of intensity versus box size, the slope is the FD.

2. **Total Perimeter of Chromatin Domains (TPD):** measuring domain abundance and edge topology.

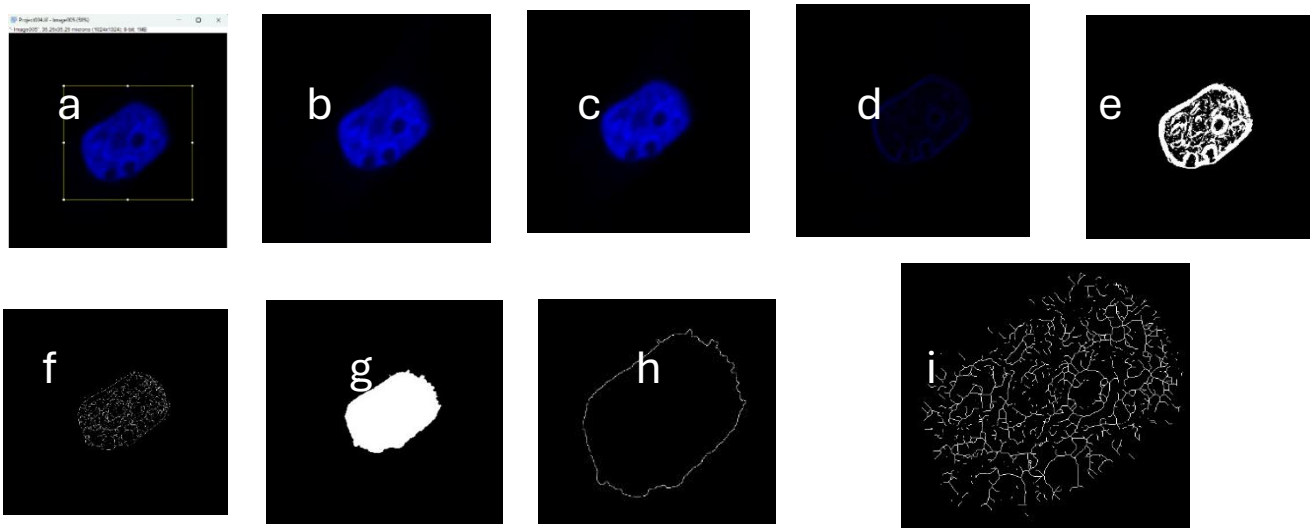

Fig. 2 : Extraction of TPD value . **a)** Raw image . **b)** Result of enhancing contrast . **c)** Result of gaussian blur filter. **d)** Result of sobel edge detection filter. **e)** After thresholding . **f)** Result of skeletonize. **g)** same image as in e after filling holes operation. **h)** Result of erosion of g and outline detection **i)** Result of subtraction of h from f, the TPD is calculated after the total length of these white lines , divided by the nucleus area in g (for normalization purposes)

3. **Radial Peak Intensity Position (Rmax):** identifying where chromatin density is maximized relative to the nucleus center.

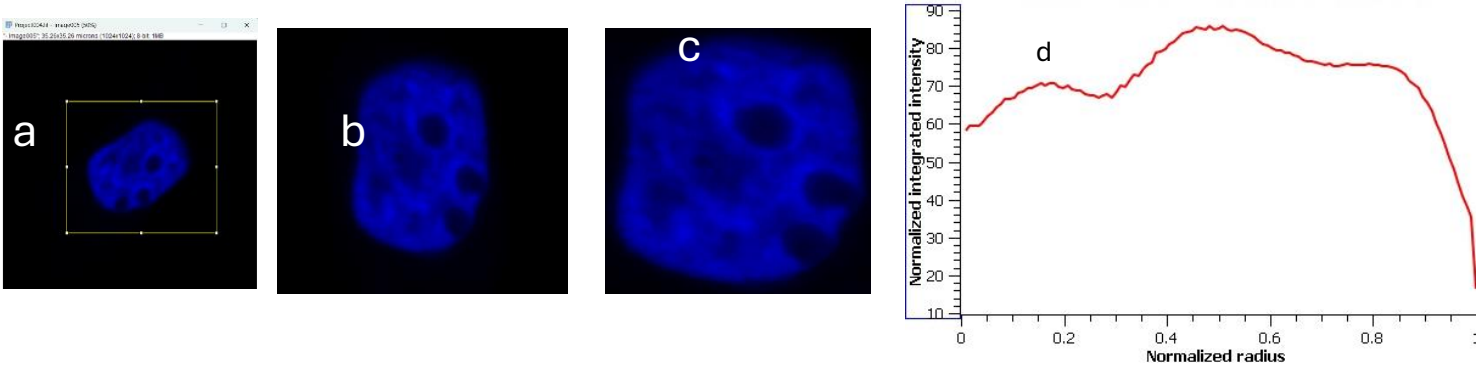

Fig.3: Extraction of R max value. **a)** Raw image has been selected. **b)**The same nucleus as in a after rotation to align the main axis of the ellipsoidal nucleus to the horizontal and vertical axis of the image, and cropping further close to the nucleus edges (1 pixel background only). **c)** Image after stretching the short side in b to the same size as the long side to make it square (nucleus circularization). **d)** Mean radial profile averaged across all directions at 360° (step 1°) around the center of the circle in c, with renormalization of circle radius to 1.

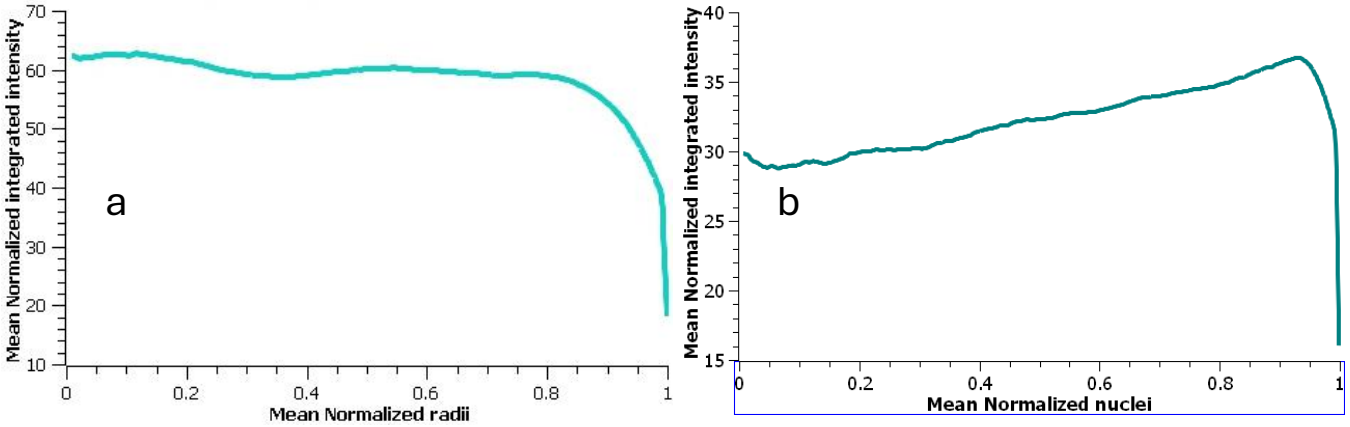

Figure.4: a) Normalized integrated average intensity plot as a function of normalized average radius for 20 HeLa cell nuclei. This plot shows that the signal intensity remains nearly uniform in the inner regions of the nucleus and decreases significantly near the nuclear boundary, which may indicate a specific spatial organization of the chromatin.Based on the plot, the maximum value of the normalized integrated average intensity is approximately within the range of 65 to 70. This value remains nearly constant in the inner regions of the nucleus and decreases near the nuclear boundary. In other word, the maximum of the normalized integrated mean intensity in the radial range is approximately between 0 and 0.8 (especially between 0-0.2). In this range, the intensity remains almost constant and close to its maximum value (~65 to 70). However, in the range from 0.8 to 1, a significant decrease in intensity is observed.The maximum intensity in this plot is observed around the normalized radius range of 0.3 to 0.5. In this region, some curves reach an intensity close to 100. This indicates that the highest chromatin density or the maximum detected signal occurs in this area.**b)** The radial profile plot displays the mean normalized integrated intensity across 20 nuclei of Fibroblast cell . The curve indicates a relatively stable intensity at the center, followed by a gradual increase toward the periphery, peaking near the edge (between 0.8-1) , then sharply dropping at the outermost region. This suggests a concentration of intensity near the nuclear boundary.

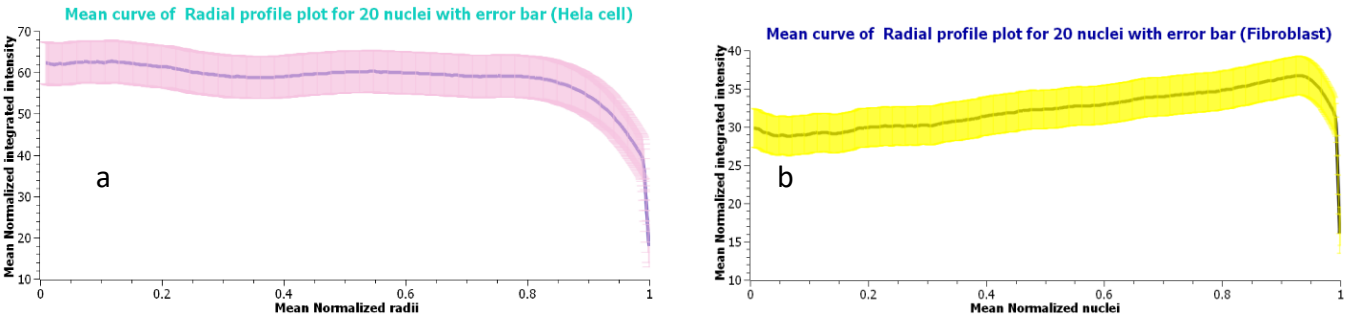

Figure.5: a) Normalized integrated average intensity plot as a function of normalized average radius for 20 HeLa nuclei with error bar. **b)** Normalized integrated average intensity plot as a function of normalized average radius for 20 Fibroblast nuclei with error bar.

4. **IsoConcentra Gradient Index (IGI):** a novel parameter quantifying radial chromatin distribution derived from the IsoConcentraChromJ plugin.

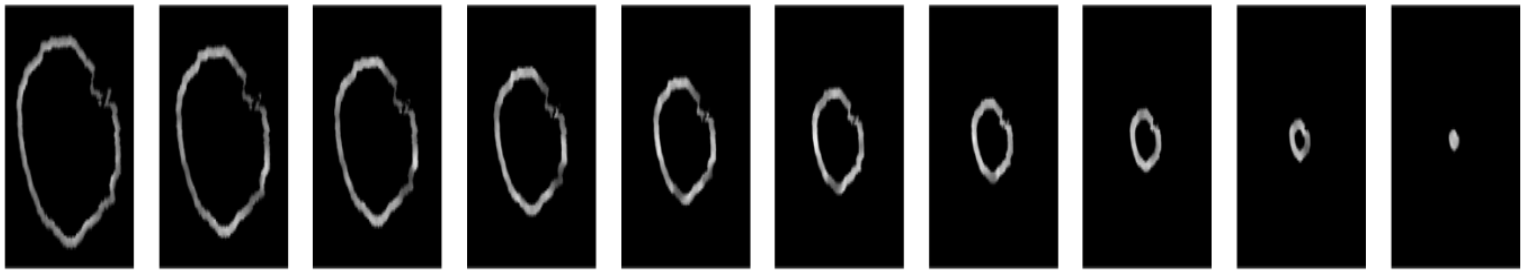

Figure.6: Representative visualization of concentric nuclear segmentation using the *IsoConcentraChromJ* plugin in ImageJ. Each nucleus was divided into ten concentric regions extending from the nuclear center to the periphery. These regions served as the basis for calculating the Normalized Integrated Intensity (A_n/A) to generate the radial chromatin distribution profiles, following the approach described by Zeaiter et al. (2024). This method enables quantitative assessment of chromatin density variations from the nuclear core toward the envelope.

These parameters were calculated for 20 nuclei of each cell type, following a standardized image-processing pipeline developed by me (Gaussian filtering, Sobel edge detection, skeletonization, radial averaging, box-counting, concentric segmentation).

c) Multidimensional statistical analysis

I constructed 3D and 4D representations of the data (using SigmaPlot and python), demonstrating clear segregation between fibroblast and HeLa nuclei.

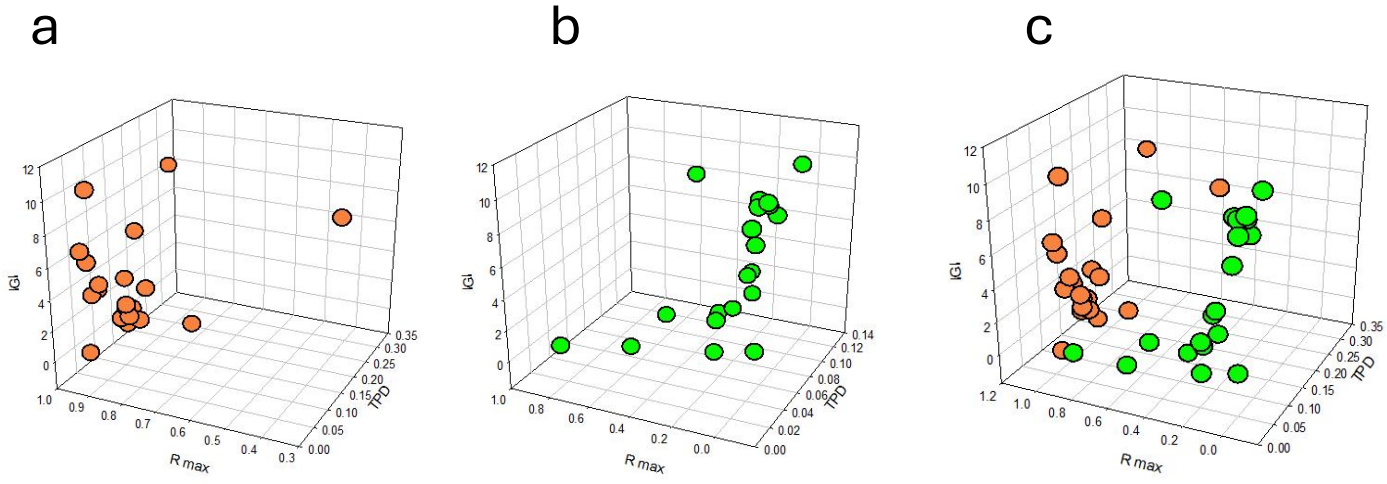

Figure.7: Three-dimensional embedding of TPD, Rmax and IGI, highlighting axis-wise parameter contributions. **a)** Fibroblast nuclei cluster in a region where: Rmax axis: Upper part → intensity peaks closer to nuclear periphery TPD axis: Upper part → more extended chromatin-edge structures IGI axis: Lower part → global outward radial gradient This tri-axial configuration uniquely maps Fibroblasts to a peripheral enrichment domain.**b)** HeLa nuclei form the opposite tri-axial pattern: Rmax: Lower region → peak intensity near nuclear center TPD: Lower-to-mid region → smoother domain distribution IGI: Upper region → inward-focused chromatin gradient Their combined shift defines a central chromatin enrichment domain. **c)** The combined plot shows clear tri-axial segregation Here also, IGI provides the strongest discriminatory axis.

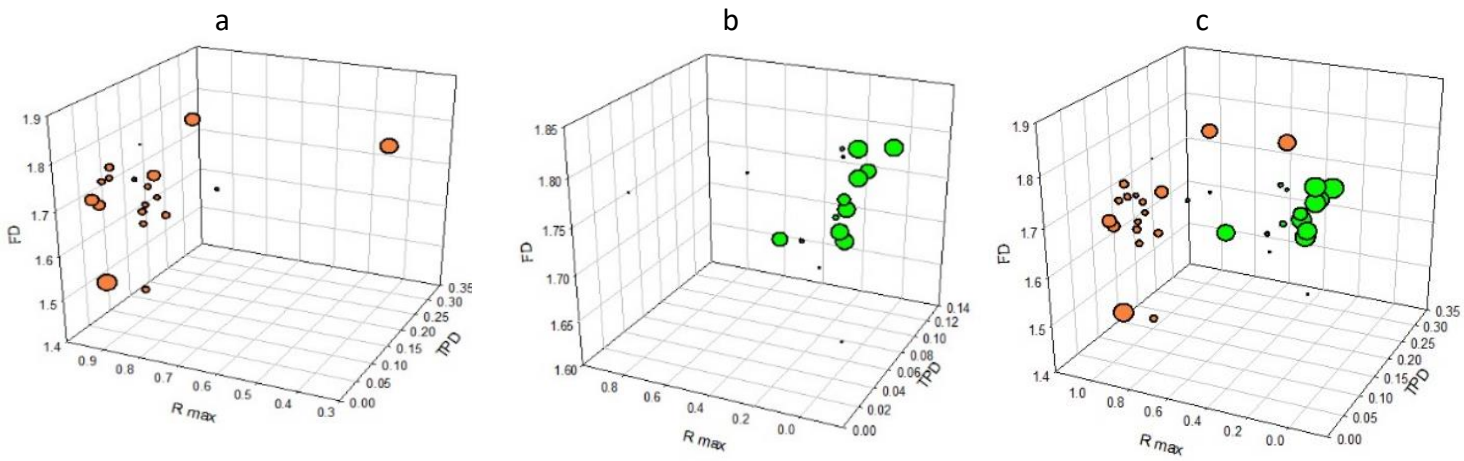

Figure.8: Four-dimensional distribution and multivariate clustering of the chromatin descriptors TPD, Rmax, FD and IGI in Fibroblast and HeLa nuclei. **a)** Fibroblasts:Form a compact 4D cluster. They show higher Rmax (peripheral intensity), higher TPD (larger perimeter), mid–high FD, and a very low, tightly grouped IGI reflecting stable peripheral heterochromatin.**b)** HeLa:Occupy an opposite region of the 4D space. They show lower Rmax (interior intensity), lower mid TPD, lower mid FD, and high, dispersed IGI consistent with heterogeneous cancer chromatin**. c)**Combined dataset: The two populations form fully separated, non overlapping clusters. IGI is the strongest contributor to the separation.

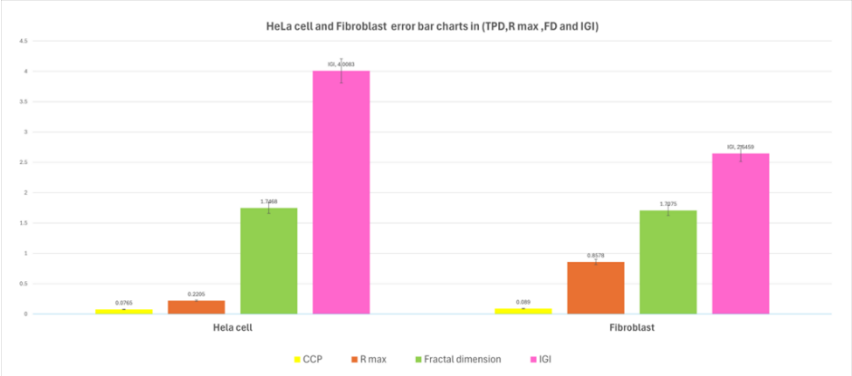

Fig.9:*) plot of means ± standard deviation for each of the three parameters for the two cell types; results of ANOVA showed significant difference in all cases (p<0.01).*

Key findings:

- Fibroblasts exhibit peripheral chromatin accumulation, higher TPD, high Rmax, and low-variance IGI (stable lamina-associated heterochromatin).
- HeLa nuclei show central enrichment, lower TPD, low Rmax, and highly dispersed IGI (chromatin plasticity typical of proliferative cancer cells).
- FD differences were small in magnitude but statistically significant with low intra-group variability, indicating robust measurement reliability.

d) Biological interpretation and integration

The combination of the four parameters revealed a multidimensional signature of chromatin phenotype. The results support the model in which:

- differentiated cells maintain a structured, lamina-associated heterochromatin shell,
- proliferative cancer cells reorganize chromatin toward a more open, centrally enriched configuration.

This year's work strongly advances the long-term PhD goal of linking nuclear structural metrics with biological cell state and provides a basis for future MINFLUX studies.

2.Exams of former course

No	Course	professor	CFU	status
i	Quantum Optics	Dario Ferraro	3	passed,18 july 2025
ii	Atomic force spectroscopy	Annalisa Relini	3	passed,16 august 2025
iii	Basics of applied statistics and probability: applications with R	Simone Barani	3	passed,21 july 2025
iv	Italian language and culture	silvano Tosi	3	passed,5 june 2025
V	Astrophysical Experimental Methods	Filippo Maria Zerbi	3	Passed december 2025

3.Conferences, workshops, schools

No	Conferences, workshops, schools	place	date
i	SEELIFE-Beyond the invisible	Genova	16-May-25
ii	12th International Weber Symposium	Genova	15-20 june 2025
iii	Nanoscopy Retreat	Venice	28-30 may 2025

4.Publications

No	Article title	status	DOI
i	Image analysis tools for improved characterization of nuclear chromatin patterns by confocal fluorescence microscopy	published	10.1007/s00249-025-01770-y
ii	MINFLUX Nanoscopy: A "Brilliant" Technique Promising Major Breakthrough.	published	10.1002/jemt.24765
iii	Quantitative Characterization of Nuclear Chromatin Architecture Using Fractal Dimension, Radial Distribution, Chromatin Domain Perimeter and IGI from STED Microscopy	Request to submit	-

5.References

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certificates

UNIVERSITÀ DEGLI STUDI DI GENOVA

DISTAV

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Basics of applied statistics and probability: applications with R

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Module of Basics of probability and statistics (3 CFU)

Professor: Dr. Simone Barani

CERTIFICATE OF ATTENDANCE

The present document certifies that Dr. Mohammadmehdi Roushenas attended the first module "**Module of Basics of probability and statistics**" of the course entitled "**Basics of applied statistics and probability: applications with R**" (duration of the module: 12 hours corresponding to 3 CFU) belonging to the PhD Course in Science and Technologies for the Earth and Environment and the PhD Course in Marine Sciences and Technologies, XL Ciclo.

Genova, 21 luglio 2025

Prof. Simone Barani

We hereby certify that

Mohammadmehdi Roushenas

Attended

"2025 Nanoscopy Retreat"

May 28 th 2025 – May 30th

At Palazzo Loredan, Venice

Genova, May 30th 2025

Prof. Alberto Diaspro

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SeeLife

We hereby certify that

Mohammadmehdi Roushenas

Attended

"SEELIFE-Beyond the invisible"

May 16th 2025 – 10.00 to 5.00 p.m.

At Palazzo Della Borsa, Genova

Genova, May 16th 2025

Prof. Alberto Diaspro

CERTIFICATE OF ATTENDANCE

This is to certify that

Mohammadmehdi Roushenas

attended the

12th International Weber Symposium

which was held in

Genoa, Italy, on June 15-20, 2025.

The Organizing Secretariat