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# Correlation-Based Nanometric Localization Using Lattice SIM<sup>2</sup> and dSTORM in PML Nuclear Bodies validated by Calibration Spheres

Corentin Rousset<sup>1</sup>, Rémi Neplaz<sup>1</sup>, Pelin Catal<sup>2</sup>, Elodie Chatre<sup>3</sup>, Christophe Place<sup>4</sup>, Patrick Lomonte<sup>1</sup>, Franceline Juillard<sup>1,5</sup>, Arnaud Favier<sup>2</sup>, Karine Monier<sup>1#</sup>.

<sup>1</sup>Laboratoire Physiopathologie et Génétique du Neurone et du Muscle-Institut NeuroMyoGène, Team « Chromatin dynamics, nuclear domains, virus », PGNM-INMG, UCBL - CNRS UMR5261 - INSERM U1315, LabEx Dev2CAN, Faculté de Médecine et de Pharmacie - F69008 Lyon, France.

<sup>2</sup> Université Claude Bernard Lyon 1, INSA Lyon, Université Jean Monnet, CNRS, UMR 5223, Ingénierie des Matériaux Polymères F-69622 Villeurbanne Cedex, France.

<sup>3</sup>PLATIM-LYMIC Microscopy platform, CNRS UAR3444, Inserm US8, ENS de Lyon, SFR Biosciences, Lyon F69007, France.

<sup>4</sup> Laboratoire de Physique, ENS de Lyon, CNRS UMR 5672, F69007 Lyon, France.

<sup>5</sup>SupBiotech Research Department - CellTechs Laboratory, SupBiotech, F69003 Lyon, France.

# Corresponding author

## **Abstract:**

We evaluated the Lattice di-SIM 3D structural illumination method with a focus on Promyelocytic Leukemia Nuclear Bodies (PML NBs). Lattice SIM<sup>2</sup>'s performance was compared to its predecessor using biological samples and calibration beads. Optimized SIM<sup>2</sup> parameters revealed PML NBs with a distinct ring-like morphology. Furthermore, we compared Lattice-SIM<sup>2</sup> with dSTORM, finding a strong correlation between blinking events and the SIM<sup>2</sup> mask. For 2D nanoscopic correlation, we used the long-lived imaging buffer Eternity, while the modified variant, Eternity-SIM, with a higher refractive index, provided improved 3D correlation for SIM<sup>2</sup> imaging.

## **Highlights:**

- ⇒ Optimized Lattice SIM<sup>2</sup> parameters to reveal the ring-like circular morphology of Promyelocytic Leukemia Nuclear Bodies (PML NBs)
- ⇒ Correlation of Lattice SIM<sup>2</sup> to Lattice SIM and to dSTORM imaging using biological samples and fluorescent spheres.
- ⇒ Investigated the effectiveness of Lattice SIM<sup>2</sup> to minimize Z-elongation compared to Lattice SIM
- ⇒ Correlative Lattice 3D SIM<sup>2</sup>-STORM nanoscopy with Eternity-SIM buffer to minimize refractive index differences.

**Authorship:** All authors should have made substantial contributions to all of the following: (1) the conception and design of the study (KM, PL, FJ, AF, CP), Sample preparation (CR, RN, PC), acquisition of data (CR, RN, KM, EC), or analysis (CR, RN, KM), interpretation of data (CR, RN, KM, PL, FJ, CP); (2) drafting the article (CP, KM, FJ) or revising it critically for important intellectual content (CP, KM, PL, AF); (3) final approval of the version to be submitted (all).

**Contribution:**

CR: Data curation; Formal analysis; Investigation; Methodology; Visualization, Resources

RN: Data curation; Formal analysis; Investigation; Methodology; Visualization, Resources

PC: Data curation; Methodology; Resources

EC: Data curation, Resources, Methodology

FJ: Conceptualization; Methodology; Project administration; Supervision; Validation;

Roles/Writing - original draft;

CP: Conceptualization; Methodology; Validation; Roles/Writing - original draft

PL: Conceptualization; Project administration; Supervision; Roles/Writing - original draft;

AF: Conceptualization; Methodology; Supervision; Validation; Roles/Writing - original draft;

KM: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Roles/Writing - original draft

AI: We used generative artificial intelligence (AI) only to improve readability and language.

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Keywords: Eternity-SIM buffer, super-resolution microscopy, membraneless-organelles, Fluorescent beads, Correlative nanoscopy, refractive index

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Mails:

[Corentin.Rousset@bruker.com](mailto:Corentin.Rousset@bruker.com)

[remi.neplaz@univ-lyon1.fr](mailto:remi.neplaz@univ-lyon1.fr)

[pelin.catal@univ-lyon1.fr](mailto:pelin.catal@univ-lyon1.fr)

[franceline.juillard@univ-lyon1.fr](mailto:franceline.juillard@univ-lyon1.fr)

[patrick.lomonte@univ-lyon1.fr](mailto:patrick.lomonte@univ-lyon1.fr)

[karine.monier@univ-lyon1.fr](mailto:karine.monier@univ-lyon1.fr)

[arnaud.favier@univ-lyon1.fr](mailto:arnaud.favier@univ-lyon1.fr)

[elodie.chatre@ens-lyon.fr](mailto:elodie.chatre@ens-lyon.fr)

[christophe.place@ens-lyon.fr](mailto:christophe.place@ens-lyon.fr)

## Introduction

In this investigation, our primary goal is to evaluate the effectiveness and capabilities of a novel implementation of the 3D structural illumination method [1], known as Lattice di-SIM (SIM<sup>2</sup>), [2], that has been deployed on the Zeiss Elyra 7 imaging system. Our initial focus at PGNM-INMG lab is the application of Lattice SIM<sup>2</sup> on Promyelocytic Leukemia Nuclear Bodies (PML NBs), to test the suitability of this method on a specific type of biological nuclear membrane-less organelles [3]. Imaging subnuclear bodies is challenging due to their small size, which approaches the diffraction limit, and the densely packed nuclear environment, both of which complicate their visualization. Even after nearly 30 years of research, the exact structure of PML bodies, the processes involved in their formation and the functions of PML proteins are still not well understood [4]. In this respect, PML NB do not differ from other nuclear bodies, ranging from 1/10 to 1/100 of the nuclear size and being densely packed, making their internal structure difficult to decipher by conventional optical microscopy. With the discovery of super-resolution microscopy at the beginning of the century, the field of nuclear bodies can now benefit from these approaches bypassing the 250 nm diffraction limit. Choosing the best-suited for a particular research question remains a challenge [5].

We wanted to take advantage of the new Lattice SIM technology that encompass conventional SIM methodology (Fig. S1) by several aspects. Thanks to its different illumination pattern (Fig. S1B), Lattice SIM is faster than conventional SIM and therefore leads to decreased phototoxicity and photobleaching. As far as optical resolution goes, Lattice SIM performs as well as conventional SIM (Fig. S1A). Increase in resolution can be reached by applying a specific deconvolution process called SIM<sup>2</sup>, allowing a 60 nm axial resolution achievement measured with an Argo-SIM slide (Fig. S1C), outperforming the 120 nm axial resolution in Lattice SIM.

In this study, we compared the performance of Lattice SIM<sup>2</sup> to its precursor Lattice SIM [6], and with the established dSTORM super-resolution technique (direct Stochastic Optical Reconstruction Microscopy) [7]. Analyses were performed both on biological samples and using our crafted fluorescent spherical calibration beads developed at IMP Lab (FluoRef). Green-emitting FluoRef beads, with a size close to that of PML NBs, were selected and provided valuable insights for the comparative assessment of Lattice SIM<sup>2</sup> and its precursor Lattice SIM. Subsequently, spherical calibration objects were also used to undertake a correlative nanoscopy study between Lattice SIM<sup>2</sup> and dSTORM to on an Elyra 7 imaging system. For this purpose, we selected original FluoRef beads coated with a far-red emitting 647 blinking fluorophore generally used as reference for dSTORM [8, 9], which Lattice SIM<sup>2</sup> can also image. This correlative light-imaging technique on identical calibration objects became possible through the development of an innovative recipe of our original long-lived blinking buffer, termed Eternity [10]. Eternity-SIM exhibits a higher refractive index ( $n=1.46$ ) compared to the classical Eternity recipe ( $n=1.33$ ), to minimize differences in refractive indexes along the light path, thus limiting aberrations while still promoting fluorophore blinking necessary for dSTORM imaging [7].

### I. Lattice SIM<sup>2</sup> optimization on biological structures

First, we tested the Lattice SIM<sup>2</sup> on a relevant biological structure: the PML NBs (promyelocytic leukemia nuclear bodies). These nuclear organelles are membrane-less organelles formed by the PML protein. They are involved in multiple regulatory cellular functions, including DNA damage response, antiviral response, genome stability, and programmed cell death [3]. They generally range from 1 to 30 per nucleus and can be found in most human and mouse cells. Their diameters measure between 0.1 and 1.0  $\mu\text{m}$ , often close to the diffraction limit. PML NBs structure has been widely described using various microscopy systems. The reference structure was obtained by Transmission Electron Microscopy (TEM) where PML NBs appear as punctate ring-shaped structures (Fig. 1A left). These observations led to the establishment of a model where the PML NBs shape is a hollow sphere formed with an outer shell of PML. Cellular proteins can enter and leave the sphere through direct interactions with PML (Fig. 1A right). The size of the PML NBs, their ring-like shape, their abundance in cells, and the fact that the structure is well described, make them a compelling model to investigate a new super-resolution microscopy system like Lattice SIM<sup>2</sup>.

From a biological perspective, Lattice SIM<sup>2</sup> offers high-resolution 3D imaging with lower technical complexity compared to other super-resolution techniques like dSTORM. It enables detailed visualization of the morphology and structural organization of PML NBs, which is essential for understanding their structure, formation, and function. SIM<sup>2</sup> supports multicolor imaging with minimal spectral overlap, making it particularly effective for studying the colocalization of PML NBs with other proteins or nuclear components. Furthermore, it can capture large fields of view in a single frame, enabling the simultaneous analysis of multiple cells.

Optimal antibody coverage is key to Lattice SIM<sup>2</sup> detection. We therefore tested the concentration of primary antibody necessary to acquire an image of the PML NBs consistent with the known structure. We incubated fixed human primary fibroblast BJ cells, with different concentrations of a mouse anti-PML antibody. The primary mouse antibody was detected with a secondary anti-mouse Alexa 488. At low concentrations of primary antibody (dilution 1/500 and 1/250), we observe a few intense dots forming squared structures instead of ring-like structures (Fig. 1Bi and Bii). These structures were probably obtained because a low concentration of antibody only allows a partial coverage of the structure or a poor signal-to-noise ratio (SNR) leading to misshaped reconstructions in Lattice-SIM<sup>2</sup>. However, when the primary antibody is more concentrated (dilution 1/100), the ring-shaped structure is visible (Fig. 1Biii). Our results are in agreement with the shell structure of PML NB described earlier with 4PI microscopy [11, 12].

We then further optimized the Lattice SIM<sup>2</sup> processing. An important element of the Lattice-SIM<sup>2</sup> microscopy is the SIM<sup>2</sup> algorithm processing that allows a 4-time XY-resolution improvement compared to epifluorescence. In epifluorescence, PML NBs are visible as filled spots and neither the PML shell nor the central gap can be resolved (Fig. 1Ci). In SIM, the structure is still poorly resolved and the ring shape is not fully defined (Fig. 1Cii). We tested different Lattice-SIM<sup>2</sup> processing parameters, among Zen 3.0 SR Black pre-recorded parameters: Weak fixed (Fig. 1Ciii), Strong fixed (Fig. 1Civ), and Standard fixed parameters (Fig. 1Cv). Each configuration is defined by the number of iterations that will be applied during the processing, the strength of the filter applied after each iteration called the regularization weight and the signal to noise ratio to adapt to the quality of the acquired image. The “weak fixed” parameters have an intermediate iteration number of 15 and a high regularization weight of 0.0650. The PML NB image obtained with these parameters (Fig.

1Ciii) is very close to the one obtained in SIM (Fig. 1Cii). The “strong fixed” parameters have a high iteration number of 25 and a low regularization weight of 0.007. The processed image barely allows for reconstruction of the ring-shaped structure of PML NBs due to elimination of the lower intensity area of the structure (Fig. 1Civ). The “standard fixed” parameters have intermediate iteration numbers (20) and regularization weight (0.0250). These parameters are sufficient to reconstruct the expected ring-shape structure but the background inside the central gap remains visible (Fig. 1Cv). We then defined optimal parameters consisting of a low iteration number of 5 and no regularization weight associated with medium signal to noise (Fig. 1Cvi).

By using these optimal parameters, we were able to obtain images of the PML NBs structure properly resolved with a ring-shaped structure and a clearer gap at the center (Fig. 1Cvi), compared to the Lattice SIM reconstruction (Fig. 1Cii). Hence, the largest thickness of this PML NB can be gauged via the Lattice SIM<sup>2</sup> reconstruction, measuring 0.15  $\mu\text{m}$  using full width at half maximum, as depicted in Fig. 1Cvi. As exemplified with PML NB, the processing parameters significantly influence the quality of the final image. It is vital to define these parameters with precision, comparing different sets and evaluating the chosen configuration against a reference standard, such as TEM (Fig. 1A) in our specific case.

Comparison of Lattice SIM and Lattice SIM<sup>2</sup> with optimized parameters was obtained on several other PML NB structures (Fig. S2) to illustrate the gain in the definition of the structure with Lattice SIM<sup>2</sup> using optimized parameters (Fig. S2A). Intensity profiles in Lattice SIM<sup>2</sup> (blue lanes) reveal shallower curves compared to that of lattice SIM (red lanes), indicative of better defined structures. When segmentation was performed on 24 of these structures, surface measurements were significantly lower with Lattice SIM<sup>2</sup> than with Lattice SIM (Fig. S2B). Therefore our results indicate that Lattice SIM<sup>2</sup> with optimized parameters are well suited to define PML NB structures more finely than Lattice SIM.

## **II- Correlation-based nanometric localization in PML nuclear bodies using Lattice SIM<sup>2</sup> and dSTORM**

Correlation-based nanometric localization was previously performed on cytoskeleton and basal bodies using an aqueous STORM buffer based on enzymatic oxygen depletion [13]. However, the short life of 2 hours of this so-called GLOX-buffer can be a drawback when correlation needs to be performed sequentially. For nanoscopy correlation on PML NB, we thus chose to use our physically depleted oxygen buffer, called Eternity, and its variants, with a long-life of 2 months [10]. To optimize SIM<sup>2</sup>-STORM imaging, we chose to perform SIM<sup>2</sup> imaging in the green channel to obtain the best resolution and dSTORM in the far-red channel with AF647, one of the best blinking fluorophores. We thus used 2 secondary antibodies coupled to green or far-red emitting fluorophores to recognize the same primary antibody against PML NB (Fig. 1 Di). Lattice SIM<sup>2</sup> allowed to image a green ring-shaped structure (Fig. 1 Dii).

The correlative image (Fig. 1Diii) showed that the majority of these blue events detected with dSTORM are located within the green ring-shaped PML NB detected with 3D-Lattice SIM<sup>2</sup>, allowing a molecular detection of PML proteins with the full PML NB structure detected by SIM<sup>2</sup> (Fig. 1 Div). With Eternity-SIM buffer, a total of 632 single localization events were detected by dSTORM on 20 000 images for this PML NB with a median localization precision of 13 nm. To eliminate the impact of surface variations in PML NB's (with an average size of 0.18  $\mu\text{m}^2$  and a coefficient of variation of 46% across 25 PML NB),

localizations were expressed as densities based on surface measurements from the SIM<sup>2</sup> images. This allowed to calculate a localization density of 4127 localization / $\mu\text{m}^2$  (Fig. 1E). To analyze the mounting buffer influence on localization density, different buffers were tested for PML NB imaging by 2D dSTORM, using the same correlative strategy as above (Fig. S3). Four buffers suitable to record blinking and z-stage maintenance (Fig. S3A) were tested to image PML NB. It should be noted that even if Prolong Glass mounting medium is a reference for SIM imaging (Fig. S3B), it is not suited for dSTORM imaging because it does not leave any space for refractive index mismatch necessary to avoid Z-stage drifting during long dSTORM acquisitions. The average and standard deviation obtained to image 4 to 6 PML NB for each buffer were reported for localization densities and XY precision (Fig. S3B).

The original S65VE buffer performed quite poorly in our hands for PML imaging as far as localization density was concerned (blue bar, Fig. S3B). Interestingly, anaerobic S65VE allowed a 6-time greater localization density for PML imaging, reaching a value of 2000 loc/ $\mu\text{m}^2$ . Even if anaerobic S65VE presents a higher refractive index than Eternity, its localization density remains lower (2000 instead of 3600 loc/ $\mu\text{m}^2$ ). We thus decided to make a version of Eternity-SIM containing sucrose, which turns out to present the highest localization density (almost 5000 loc/ $\mu\text{m}^2$ ). As far as median XY precision was concerned, Eternity-SIM presents a value of 11 nm, intermediate between S65VE and Eternity for PML imaging (orange curve, Fig. S3B). With a refractive index of 1.42, Eternity-SIM buffer is thus closer to the optimal value of 1.52 for 3D-SIM imaging. We are therefore anticipating more accurate measurements in 3D using the 1.46-refractive index of Eternity-SIM than with the 1.33 refractive index of Eternity.

In this context, the S65VE buffer containing 52% sucrose and 20% vectashield was first described for SIM imaging of thick biological samples [14]. With the original recipe of S65VE, blinking events were recorded for dSTORM imaging of PML NB, though averaging only 280 localization / $\mu\text{m}^2$  of PML NB. Removing oxygen allowed to improve by a factor of 7 times the localization density and to improve XY precisions from 12 to 10 nm compared to the same recipe without oxygen depletion (compare S65VE to anaerobic S65VE, Fig. S3B). Another improvement of a 2.5 factor was further obtained for localization density with Eternity-SIM, a combination of S65VE and Eternity, without significantly compromising XY precision (compare anaerobic S65VE to Eternity-SIM, Fig. S3B). Therefore, Eternity-SIM buffer is a good compromise to gather more than 4000 localization/ $\mu\text{m}^2$  and an XY precision of 13 nm, together with a refractive index of 1.46, compatible with definite focus usage (Fig. S3A). Evaluation of Eternity-SIM buffer for 3D imaging in comparison with Prolong Glass and Eternity buffer was therefore performed with real calibration objects, since PML NB are quite variable in size (see above).

### III- Lattice SIM<sup>2</sup> performances compared to Lattice SIM with calibrated spheres

The green-emitting crafted fluorescent spherical calibration beads developed at IMP Lab (FluoRef), with a size close to that of PML NBs, were selected to assess Lattice SIM<sup>2</sup> and its precursor Lattice SIM. The evaluation of Lattice SIM<sup>2</sup> involved a comprehensive analysis in both 2D and 3D using 1  $\mu\text{m}$  green-emitting spherical beads coated with lucifer yellow dye [15], FluoRef-LY, as depicted in Fig. 2A. In the realm of 2D perspectives, measurements of crown thickness and XY-maximum diameter were performed on intensity profil (Fig. 2E) on a single FluoRef-LY dataset for comparison purposes. This analysis aimed to gauge how Lattice

SIM<sup>2</sup> (Fig. 2C, D) enhances the clarity of 2D views in comparison to Lattice SIM (Fig. 2B, D). The merge view of Lattice SIM (green, Fig. 2D) and Lattice SIM<sup>2</sup> using optimized parameters (magenta, Fig. 2D) illustrate the marked difference between them.

Within the SIM data view, the XY-maximal diameter is 949 nm, when calculated from peak-to-peak. In the SIM<sup>2</sup> view, a similar value of 959 nm is observed (Fig. 2J). Both measurements closely align with the nominal 1  $\mu$ m diameter of the beads, considering that the expected thickness of the fluorescent layer is negligible in comparison to bead size. It is noteworthy that when opting for the full width at half maximum (FWHM), there is a discernible shift from 1130 nm in SIM to 1051 nm in SIM<sup>2</sup> for the maximal XY-diameter. This observation aligns with the notion that SIM<sup>2</sup> demonstrates greater precision (7%) in imaging plain 1  $\mu$ m structures. Regarding crown thickness, the full width at half maximum (FWHM) measurement yielded 188 nm on the SIM profile, whereas Lattice SIM<sup>2</sup> exhibited a significantly lower value of 103 nm (Fig. 2J). This represents a notable 45% reduction in thickness compared to Lattice SIM. Hence, the effectiveness of Lattice SIM<sup>2</sup> is fully realized for structures spanning several hundred nanometers in scale. Therefore, considering the accuracy of size verification for these fluorescent spheres, the measurements conducted using Lattice SIM<sup>2</sup> (Fig. 1Civ) to estimate the maximal diameter (approximately 600 nm) and the minimal crown thickness (around 100 nm) of PML NBs (Fig. 1E) are likely accurate.

After evaluating enhancements in the axial XY-views with the calibrated objects, there was a desire to study the contribution of Lattice SIM<sup>2</sup> in lateral XZ-views. This was particularly important because the axial resolution (XZ) is typically three to four times lower than the lateral resolution (XY). Accordingly, in the XZ-direction, orthogonal sections (Fig. 2F, H) and 3D surface reconstructions (Fig. 2G, I) visually depicted an elongation of the spherical beads along the Z-axis, measuring 2.12  $\mu$ m in the Lattice SIM reconstruction and 1.67  $\mu$ m in the SIM<sup>2</sup> reconstruction (Fig. 2G, I). This measured Z-diameter for Lattice SIM<sup>2</sup> indicates a 21% decrease compared to Lattice SIM (Fig. 2J). Although the measured value of 1.67  $\mu$ m surpasses the nominal bead diameter of 1  $\mu$ m, the Z-axis elongation is significantly reduced with Lattice SIM<sup>2</sup> comparatively to Lattice SIM or conventional widefield microscopy. Thus, considering the 1.67-fold elongation observed along the Z-axis with these fluorescent spheres, measurements of biological structures along the Z-axis should be adjusted by applying at a first glance a reduction factor of 1.67.

#### **IV- Lattice SIM<sup>2</sup>/dSTORM correlation**

To further evaluate the Lattice SIM<sup>2</sup> approach, we undertook a comparative analysis with the established dSTORM super-resolution technique. This technique is also available on our Elyra 7 system, enabling the imaging of the same structure using both modalities. A significant challenge in conducting a correlative nanoscopy analysis between Lattice SIM<sup>2</sup> and dSTORM was however the development of a compatible mounting medium for both techniques. Specifically, dSTORM requires a blinking buffer with a refractive index (n) close to 1.33, while Lattice SIM<sup>2</sup> ideally functions with mounting media having a refractive index close to 1.52. We tried a previously developed buffer [16] containing Vectashield, but the number of events collected was too low (data not shown). To address this challenge, we designed a new dedicated formulation called Eternity-SIM of our initial Eternity blinking buffer (as cited in [9]), by elevating its refractive index from 1.33 to 1.46. For the Lattice SIM<sup>2</sup>/dSTORM correlation, we selected 1  $\mu$ m Fluoref-647 calibration spheres coated with the reference 647



blinking dye for dSTORM (Fig. 3D, only 2D views are presented for clarity), that were developed in collaboration with Idylle Labs (now available as SpheroRuler).

Regarding Lattice SIM and SIM<sup>2</sup>, the initial optimized acquisition was used to image the Fluoref-647 beads (Fig. 3E) immersed in the Prolong Glass 1.52 refractive index medium, (Fig. 3A-D). Remarkably, the definition of the sphere in Lattice SIM (Fig. 3B) and SIM<sup>2</sup> using standard fixed parameters (Fig. 3C) exhibits significant enhancement compared to epifluorescence (Fig. 3A). In terms of measurements, the XY-diameter calculated from peak-to-peak at the equator (Fig. 3F) was 964±33 nm for Lattice SIM<sup>2</sup>, very close to the nominal diameter of 1000 nm for the beads. This peak-to-peak measurement obtained with Lattice SIM<sup>2</sup> for micrometric size does not vary with imaging modality (Fig. 3F) or with wavelength (Fig. 2J). When FWHM was used to determine the XY-diameter of the bead (Fig. 3F), the values were higher than the nominal diameter of 1000 nm for the bead (1055±25 nm for Lattice-SIM<sup>2</sup> and 10% more for Lattice SIM). Therefore, the fluorescence layer tends to slightly over-estimate the bead diameter in the micrometer range. Concerning sub-resolution measurements, such as crown thickness, full width at half maximum (FWHM) was 97 nm in Lattice SIM<sup>2</sup>, indicating a significant 134% reduction compared to Lattice SIM (Fig. 3E) and in the same range than in the green channel (Fig. 2J).

Regarding the SIM<sup>2</sup>-dSTORM nanoscopy correlation, the equatorial view of Lattice SIM<sup>2</sup> (Fig. 3H) conducted on a single bead immersed in the Eternity-SIM buffer (n=1.46) was similar than the one previously obtained with the Prolong Glass mounting medium (Fig. 3C, F). Both the XY-diameter and crown thickness show little difference not exceeding 4%. This consistency implies that the Eternity-SIM buffer, with its refractive index of 1.46, has negligible impact on Lattice SIM<sup>2</sup> reconstruction compared to the classical 1.52 refractive index of Prolong Glass.

The 2D dSTORM visualization of the same FluoRef-647 bead (Fig. 3J, purple) was reconstructed using 2000 events from 10,000 images, achieving a median localization precision of 19 ± 5 nm. The peak-to-peak XY-diameter at the equator measured 847 nm, a value slightly lower compared to that of Lattice SIM<sup>2</sup> (Fig. 3H, K). However, in the case of dSTORM, it is relevant to employ the full width at half maximum diameter of 974 nm to estimate bead size, as the reconstruction relies on single dots with a precision of about 19 nm. The comparable diameters of SIM<sup>2</sup> (934 nm, P2P) and dSTORM (974 nm, FWHM) were therefore remarkably close, both approximating the nominal size of 1000 nm quite well. In addition, the very good shape correlation was evident in the SIM<sup>2</sup>/dSTORM superimposition (Fig. 3I, cyan: SIM<sup>2</sup> and purple: dSTORM). Regarding crown thickness, dSTORM reveals a sharper measurement of 100 ± 13 nm compared to the 108 ± 16 nm of the Lattice SIM<sup>2</sup> in the far-red channel (Fig. 3K). In order to assure that standard fixed parameters for Lattice SIM<sup>2</sup> were the best suited for this correlative nanoscopy, correlative nanoscopy was also performed on the same FluoRef reconstructed with the different SIM2 configuration : weak fixed, Strong fixed, standard fixed and optimized (Fig. S4). The best colocalization is achieved with the standard fixed configuration (Fig. S3).

Finally, to further assess the efficacy of Eternity-SIM buffer (n=1.46) for correlative SIM<sup>2</sup>-dSTORM imaging, the same measurements were performed on a FluoRef-647 bead mounted in the original Eternity buffer (n= 1.33) (Fig. 3I, second lane in blue). With Lattice-SIM<sup>2</sup> using standard fixed parameters, bead XY-diameter was a bit lower while crown thickness was a

bit higher with a value of  $108 \pm 16$  nm for Eternity against  $97 \pm 6$  nm for Eternity-SIM buffer. With dSTORM imaging, Eternity buffer displayed 1495 events, which is lower than the 2251 events obtained with Eternity-SIM and a localization precision that is quite comparable. The Chi-square fitting parameter is a bit lower for Eternity compared to Eternity-SIM. Thus, utilizing Eternity-SIM buffer with a refractive index of 1.46 improves fidelity of SIM<sup>2</sup> imaging, making it a game-changer for correlative SIM<sup>2</sup>/STORM imaging.

## Conclusions

In the broader context of SIM<sup>2</sup>/STORM comparison, it becomes evident that both the reduction in XY-diameter and the enhancement in crown sharpness achieved with Lattice SIM<sup>2</sup> are more closely aligned with the well-established dSTORM technique than Lattice SIM. Consequently, our study emphasizes that Lattice SIM<sup>2</sup> represents a promising approach for enhancing resolution of biological structures by a factor of 2 compared to Lattice SIM, while converging toward the reconstruction fidelity characteristic of dSTORM.

For optimal SIM<sup>2</sup>-STORM correlation, utilizing green-emitting fluorescence in Lattice SIM<sup>2</sup> imaging is ideal, though far-red emission performs quite well. Additionally, employing a high refractive index buffer such as Eternity-SIM ( $n=1.46$ ) provides an excellent balance, enabling accurate XY measurements similar to those obtained with a pure SIM mounting medium, while also achieving a higher density of blinking events than with the original Eternity buffer. 3D correlation with biological structures necessitates applying a correction factor for Z-axis elongation. This elongation is minimized when using the higher refractive index Eternity-SIM buffer ( $n=1.46$ ) compared to the lower refractive index Eternity buffer ( $n=1.33$ ). Applying this rule to estimate the 3D structure of PML NB mounted in Eternity-SIM would be a plus to determine their overall shape and dimensions in the nuclear space.

## Methods

### *Cell Culture*

Human BJ primary foreskin fibroblasts (ATCC, CRL-2522) were grown at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D6429) supplemented with 10% fetal bovine serum (Sigma, F7524), L-glutamine (1% v/v), 10 U/mL penicillin, and 100 mg/mL streptomycin (Sigma- Aldrich, P4458).

### *Immunofluorescence*

Cells were seeded on either a 12-well plate containing an 18 mm glass coverslip for SIM or on Willco GWST 35–22 for dSTORM. Cells were fixed for 10 min with 2% paraformaldehyde, washed three times with PBS, then permeabilized for 5 min in a PBS solution containing 0.5% Triton X-100. Slides were saturated with PBS1X 3% Normal goat serum (NGS) for 1h at RT. Primary antibodies were diluted in PBS containing 3% NGS (see antibodies section). After incubation at room temperature for 2 h, coverslips were washed three times with PBS, and then incubated with goat anti-mouse or anti-rabbit (H+L) cross-absorbed secondary antibodies Alexa-488, or Alexa-647 (Invitrogen). After a further 45 min incubation, coverslips were washed three times with PBS. Coverslips were mounted in Prolong glass (Invitrogen, ref. P36982.) for Lattice-SIM and SIM<sup>2</sup> following manufacturer's instructions.

Willco dishes were mounted with Eternity buffer and sealed air-tight with twinstil for dSTORM imaging and correlative SIM-STORM nanoscopy (see details in section below). Samples were stored in the dark and at 4°C before observation.

Primary antibodies:

Proteins	Suppliers	References	Species	Concentration	Dilutions
PML	Santa Cruz Biotechnology	sc-966 Clone PG-M3	Mouse monoclonal	200 µg/ml	2µg/ml (1/100)
PML	Sigma-Aldrich	PLA0172	Rabbit polyclonal	200 µg/ml	0,2µg/ml (1/1000)

Secondary antibodies:

Target	Suppliers	References	Species	Fluorochromes	Concentration	Dilutions	Fluorochrome/antibody
Mouse IgG	Invitrogen	A11001	Goat	AF488	2 mg/mL	2µg/ml (1/1000)	7
Mouse IgG	Invitrogen	A21236	Goat	AF647	2 mg/mL	2µg/ml (1/1000)	5
Rabbit IgG	Invitrogen	A32731	Goat	AF488	2 mg/mL	2µg/ml (1/1000)	2 to 8

*Preparation of calibration sphere samples in Willco dishes*

Two types of fluorescent calibration beads (FluoRef) were used. Both are 1 µm polystyrene spheres coated with either lucifer yellow dye (FluoRef-LY) or a cyanine 5 dye (FluoRef-647, now available as SpheroRuler, [www.idylle-labs.com](http://www.idylle-labs.com)). FluoRef beads in suspension ( $7 \cdot 10^8$  particles/ml) were sonicated and vortexed before use (5 times: 5 seconds on and 5 seconds off). A volume of 5µl of suspension was dispensed at the center of a clean Willco Well (Willco Wells, ref HBST-3522). Immediately after, the selected imaging buffer (Eternity buffer or Eternity-SIM) was added on top. For Prolong glass conditions, the 5 µl of FluoRef suspension were first diluted in 300 µl of 1X PBS and then dispensed on the Willco dish for 10 minutes. After 3 washes with 1X PBS, Willco dishes were drained and then mounted with a drop of Prolong glass with a 20 mm glass coverslip following supplier's instructions.

*Buffer preparation and dSTORM sample mounting*

The original Eternity buffer, based on physical oxygen removal, was prepared as described previously [10] in 0.5 ml tubes inside an Argon-filled glove box. The S65VE buffer described previously [14] was likewise submitted to physical oxygen removal: anaerobic S65VE. A new

version of Eternity buffer, named Eternity-SIM, was prepared with 0.5X Eternity and supplemented with 50% sucrose and 20% Vectashield allowing it to reach a 1.46 refractive index. A volume of 450  $\mu$ l of buffer was added directly to the samples of the Willco dish. A clean 25 mm coverslip was added to spread the buffer by avoiding air-bubble formation. Excess buffer was removed with a kimwipe and air-tight sealing was performed with 0.7 ml of a pre-mix of blue and yellow twinsil (Rotec). To limit re-oxygenation of the buffer on the bench, these steps were performed in a time-course of less than 2 minutes. Sealed Willco dishes were left on the bench for 2 minutes for biological samples and 30 minutes for FluoRef samples to allow for bead sedimentation.

#### *Lattice SIM and SIM<sup>2</sup>*

Images (512x512 pixels) were acquired on a Zeiss ELYRA 7 which includes an Axio-Observer microscope equipped with a super-resolution module with a Plan-alpha Apochromatic 63x objective, NA=1.46, Corr M27 TIRF oil (df=0.10 mm). A PCO.edge 4.2 sCMOS camera (with 2048x2048 pixel field of view and 6.5  $\mu$ m pixel) was used to collect 3D SIM illumination settings on Zen Black 3.0 SR software correspond to 13 phases of a 27.5  $\mu$ m period grid for illumination at a wavelength of 488 nm and 32  $\mu$ m period for 642 nm.

Lattice 3D was obtained by acquiring a 41-section z-stacks using a 101 nm step for AF488 and 132 nm for AF647. 3D SIM processing was run with the following parameters: "sharpness": auto, no fit, no baseline cut. Lattice 3D SIM<sup>2</sup> processing with optimized parameters was best for PML NB (Fig. 1) and Fluoref in green (LY-FluoRef, Fig. 2) and was run with the following parameters: "Input SNR": Medium, "Iterations": 5, "Regularization weight": 0, Processing sampling and Output sampling: x3, No fit. For the other settings, chose the proper configuration in the SIM<sup>2</sup> setting menu: Weak fixed, Strong fixed or Standard fixed associated with Processing sampling and Output sampling: x3, No fit. Standard fixed configuration was best for Fluoref in far red (FluoRef-647) analyzed in 2D.

#### *dSTORM imaging*

Elyra 7 imaging system driven by Zen Black was used for acquisition with an EMCCD camera (Andor iXon Ultra 897, with 512x 512 pixel field of view and 16  $\mu$ m pixel). TIRF mode was used to collect 2D dSTORM serie on the equatorial plane of a FluoRef, by using the UHP-mode to concentrate laser power by a factor of 8 fold in the center of the field of view (128x128 pixels). Pumping step was performed by increasing the 642 nm laser to 100% until dark state (about 30 seconds) was reached on the whole fluorescent signal. Laser power was then decreased to 30% for acquisition of 20 000 images with an exposure time of 20 msec after activating the definite focus. Processing was performed in Zen Black using 2D gaussian fitting using the discard overlapping method, with a peak mask size set up at 7 and a peak intensity to noise at 6. Drift was corrected with automatic menu set up to a maximum of 30 segments. At the end, filtering was applied to discard the first 1000 images (pumping step). PSF filtering was also performed to restrict values between 220 and 280 nm for FluoRef images only.

#### *Correlative-nanoscopy imaging:*

Correlative images were acquired at the same focal plane for both SIM<sup>2</sup> (1 pixel = 16 nm) and dSTORM (1 pixel = 6 nm) to image the same structures with the same camera (sCMOS). The Lattice-3D SIM z-stack was first acquired at 488 nm wavelength (Fig. 1D) and 647 nm wavelength (Fig. 3F-H) and processed as described above, then a (128 x 128 pixels) ROI was selected around a structure of interest and 20,000 dSTORM images were acquired in HILO

mode at 647 nm wavelength and processed as described above. Correlative nanoscopy was observed on the same focal plane. Image overlay was performed using GIMP software with the fusion mode with a 60% opacity for the SIM<sup>2</sup> image.

## REFERENCES

- [1] M. G. Gustafsson, Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy, *J. Microsc.* 198 (2000) 82-7. [https://doi: 10.1046/j.1365-2818.2000.00710.x](https://doi.org/10.1046/j.1365-2818.2000.00710.x).
- [2] A. Löschberger, Y. Novikau, R. Ralf Netz, M. C. Spindler, R. Benavente, T. Klein, M. Sauer, I. Kleppe, Super-Resolution Imaging by Dual Iterative Structured Illumination Microscopy, *BioRxiv* (2021) [https://doi: 10.1101/2021.05.12.443720](https://doi.org/10.1101/2021.05.12.443720).
- [3] A. Corpet., C. Kleijwegt, S. Roubille, F. Juillard, K. Jacquet, P. Texier and P. Lomonte, PML nuclear bodies and chromatin dynamics: catch me if you can! *Nucleic Acids Research* 48 (2020) 11890–11912. [https://doi: 10.1093/nar/gkaa828](https://doi.org/10.1093/nar/gkaa828).
- [4] S. A. Silonov, E. Y. Smirnov, I. M. Kuznetsova, K. K. Turoverov, A. V. Fonin, PML Body Biogenesis: A Delicate Balance of Interactions, *Int. J. Mol. Sci.* 24 (2023) 16702. [https://doi: 10.3390/ijms242316702](https://doi.org/10.3390/ijms242316702).
- [5] Schermelleh L, Ferrand A, Huser T, Eggeling C, Sauer M, Biehlmaier O, Drummen GPC. Super-resolution microscopy demystified. *Nat Cell Biol.* 2019 Jan;21(1):72-84. doi: 10.1038/s41556-018-0251-8. Epub 2019 Jan 2. PMID: 30602772.
- [6] E. Betzig, Excitation strategies for optical Lattice microscopy, *Opt. Express* 13 (2005) 3021-36. [https://doi: 10.1364/opex.13.003021](https://doi.org/10.1364/opex.13.003021).
- [7] M. Heilemann, S. van de Linde, M. Schüttpelz, R. Kasper, B. Seefeldt, A. Mukherjee, P. Tinnefeld, M. Sauer Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. *Angew. Chem. Int.* 47 (2008) 6172–6176. [https://doi: 10.1002/anie.200802376](https://doi.org/10.1002/anie.200802376).
- [8] G.T. Dempsey, J.C. Vaughan, K.H. Chen, K., M. Bates, X. Zhuang, Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging, *Nat. Meth.* 8 (2011) 1027-1036. [https://doi:10.1038/nmeth.1768](https://doi.org/10.1038/nmeth.1768)
- [9] L. Fabre, C. Rousset, K. Monier, F. Da Cruz-Boisson, P. Bouvet, M.T. Charreyre, T. Delair, E. Fleury, A. Favier, Fluorescent Polymer-AS1411-Aptamer Probe for dSTORM Super-Resolution Imaging of Endogenous Nucleolin. *Biomacromolecules* 23 (2022) 2302-2314. [https://doi:10.1021/acs.biomac.1c01706](https://doi.org/10.1021/acs.biomac.1c01706).
- [10] A. Provost, C. Rousset, L. Bourdon, S; Mezhoud, E. Reungoat, C. Fourneaux, T. Bresson, M. Pauly., N., Béard, L. Possi-Tchouanlong, B. Grigorov., P. Bouvet, J. J. Diaz, C. Chamot, E.-I. Pécheur, C. Ladavière, M.-T. Charreyre, A. Favier, C. Place, K. Monier, Innovative particle standards and long-lived imaging for 2D and 3D dSTORM. *Sci. Rep.* 9 (2019) 17967. [https://doi: 10.1038/s41598-019-53528-0](https://doi.org/10.1038/s41598-019-53528-0).
- [11] M. Lang, T. Jegou, I. Chung, K. Richter, S. Münch, A. Udvarhelyi, C. Cremer, P. Hemmerich, J. Engelhardt, S. W. Hell, K. Rippe, Three-dimensional organization of promyelocytic leukemia nuclear bodies *J. Cell Sci.* 123 (2010) 392–400. [https://doi:10.1242/jcs.053496](https://doi.org/10.1242/jcs.053496).
- [12] C. Cremer, A. Szczurek, F. Schock, A. Gourram, U. Birk, Super-resolution microscopy approaches to nuclear nanostructure imaging, *Methods* 123 (2017) 11-32. <https://doi.org/10.1016/j.ymeth.2017.03.019>.
- [13] V. Hamel, P. Guichard, M. Fournier, R. Guiet, I. Flückiger, A. Seitz, P. Gönczy Correlative multicolor 3D SIM and STORM microscopy, *Biomedical Optics Express* 5 (2014) 3326-3336. <https://doi.org/10.1364/BOE.5.003326>.
- [14] A; Szczurek, F. Contu, A. Hoang, J. Dobrucki, S. Mai, Aqueous mounting media increasing tissue

translucence improve image quality in Structured Illumination Microscopy of thick biological specimen, *Sci. Rep.* Sep 18 (2018) 13971. [https://doi: 10.1038/s41598-018-32191](https://doi.org/10.1038/s41598-018-32191).

[15] W. W. Stewart, Lucifer dyes-highly fluorescent dyes for biological tracing, *Nature*, 292 (1981) 17-21.

[16] N. Olivier, D. Keller, V. Sundar Rajan, P. Gönczy, S. Manley, Simple buffers for 3D-STORM Microscopy, *Biomed. Opt. Express*, 4 (2013) 885-899. [https://doi: 10.1364/BOE.4.000885](https://doi.org/10.1364/BOE.4.000885).