World Premier International Research Center Initiative (WPI)
FY 2022 WPI Project Progress Report (The center selected in and before FY2020)

Host Institution	Kanazawa University	Host Institution Head	Takashi Wada
Research Center	Nano Life Science Institute (NanoLSI)		
Center Director	Takeshi Fukuma	Administrative Director	Masafumi Iwami (as of April 2023)

Common instructions:
* Unless otherwise specified, prepare this report based on the current (31 March 2023) situation of your WPI center.
* So as to execute this fiscal year's follow-up review on the "last" center project plan, prepare this report based on it.
* Use yen (¥) when writing monetary amounts in the report. If an exchange rate is used to calculate the yen amount, give the rate.
- Prepare this report within 10-20 pages (excluding the appendices, and including Summary of State of WPI Center Project Progress (within 2 pages)).

Summary of State of WPI Center Project Progress (write within **2 pages**)

1. Research Progress

At NanoLSI, we have been working on three major projects: (1) the development of novel nanoprobe technologies especially for live-cell imaging, (2) nano-level understanding of basic cellular functions and cancer, and (3) the establishment of a new research field "nanoprobe life science" (Fig. 1).

(1) Development of novel nanoprobe technologies

Imaging at surfaces and inside of living cells: After establishing proof of principle, we made great efforts to extend the capabilities of the newly developed live-cell imaging techniques and to develop practical techniques for life science research. **Fukuma** extended the imaging capability of 3D nanoendoscopy AFM from the imaging of static structures to dynamic processes such as growth dynamics of focal adhesions and actin fibers. Using SICM, **Watanabe** succeeded in visualizing nanoparticle-cell interactions (*Small* **2022**) and established a method to observe nanodynamics of physical properties at the basal surfaces of spherical organoids through a collaboration with **Oshima** (*Small* **2023**).

Nanoendoscopic analysis and manipulation: We devoted continuous effort to develop fundamental technologies relating to nanoendoscopic analysis and manipulation. **Takahashi** established a technique for injecting reagents into cells using a glass nanopipette and developed a technology for automatic cell identification using machine learning and for automatic collection at the single-cell level. **Arai** demonstrated local heating and thermometry at the single cell level (*ACS Nano* **2022 &** *Materials Today Bio* **2022**) and established a methodology to manipulate the dynamics of lipid membranes. **Akine, Ogoshi, Maeda, & MacLachlan** developed new cyclic or helical compounds that could be utilized as molecular sensors and machines.

Modeling & simulation for nano life science: To provide a theoretical understanding of the AFM data, several mathematical models have been developed for multiscale structures such as proteins, cell membranes, chromosomes, and cell populations. **Foster & Hall** developed a new coarse-grained model of cell membrane dynamics to understand AFM data *(BPPB* **2022***)*. **Sumikama** developed a new theory based on polymer physics for 3D-AFM and applied it to chromosome models during inter and mitotic phases (*J Phys Chem Lett* **2022**). **Flechsig** developed a unique method to reconstruct full 3D atomistic structures from AFM topographic images (*PLoS Comput. Biol.* **2022,** *ACS Appl Mater Interfaces 2022, PLoS ONE* **2022,** *ACS Nano* **2023**). **Okuda** proposed new mathematical models that describe the dynamics of stress fibers, cell membrane, cell adhesion, and collective cell migration (*Biophys J* **2022**, *Euro Phys J E* **2022**, *iScience* **2023,** *Phys Rev E* **2023**).

(2) Nano-level understanding of cellular functions and cancer

Life science research using HS-AFM, 3D-AFM, or bio-SICM continued to produce many impactful publications. Meanwhile, we also actively explored applications of the newly developed live-cell imaging techniques in molecular cell biology and cancer research.

Basic cell functions: Using HS-AFM and protein engineering technology with MET-binding peptides, **Matsumoto & Shibata** created a designer receptor agonist capable of dimerizing and activating MET receptors (*Nat Biomed Eng*, **2023**). **Wong, Hanayama & Ando** discovered that the structure of small extracellular vesicles (sEVs) is substantially altered at high temperature, high pH, or hypertonic conditions and that the spherical shape of the sEVs is maintained in acidic or hypotonic environments (*J Extracell Vesicles* **2022**). Using HS-AFM, **Hanayama & Kodera** found how extracellular vesicles contribute to the aggregation and deposition of transthyretin in amyloidosis (*Front Mol Biosci* **2022**). **Toda & Watanabe** started a new project to study how chimeric

cadherins change cell membrane dynamics to induce cell sorting by using scanning ion conductance microscope (SICM). **Miyanari** has succeeded in simultaneously visualizing chromatin accessibility and epigenetic modifications; both are key chromatin signatures in the regulation of transcription (*Methods Mol Bio* **2023**).

Cancer research: **Oshima & Watanabe** found that metastatic malignant cells have a specific morphology of micro-ridge-like structures with active movement on the membrane surface by using SICM (*Biomaterials* **2022**). They also found that the basal surface of metastatic intestinal tumor organoids showed similar ridge-like structures and softer cell membranes (*Small* **2023**). **Nakajima** found that the DNA aptamer significantly enhanced the vitamin D_3-mediated inhibition of cancer cell proliferation (*ACS Appl Mater Inter* **2022**). **Hirao** established that endo-lysosomal activity as a metabolic biomarker of malignancy and an important therapeutic target for brain tumors (*Cancer Sci.* **2022**). **Yano** developed a therapeutic approach to ALK-rearranged lung cancer targeting with a transcription factor STAT3 (*NPJ Precis Oncol.* **2022**). They also found that deficiency of the splicing factor RBM10 limits EGFR inhibitor response in EGFR-mutant lung cancer (*J Clin Invest.* **2022**). These results have promoted further collaborations with experts in nanotechnology, leading to a deep understanding of the nature of cancer-specific abnormalities.

(3) Establishment of the novel research field "Nanoprobe Life Science"

Extending Capabilities of Various Bio-SPM Techniques: To maintain our current world-leading position in Nanoprobe Life Science, we have been improving the performance and functionality of our cutting-edge bio-SPM technologies. **Ando, Kodera & Shibata** developed several high-speed AFM (HS-AFM) techniques to further improve the scanning speed, low-invasiveness and assay system: a mass-controller for the cantilever to achieve higher resonant frequency, a new optical system to obtain a more accurate deflection signal of a small cantilever, and a new AFM observation substrate using pillar[5]arene molecules. For high-resolution SICM imaging of live cell surfaces with a high S/N ratio, **Takahashi** developed a method for controlling the inner/outer diameter ratio of glass capillaries, and **Watanabe** developed an ultra-low-noise wide-bandwidth transimpedance amplifier. Meanwhile, **Fukuma** continued to expand the application area of 3D-AFM. In addition to the hydration structures and living cells, he succeeded in visualizing the internal structure of chromosomes using an originally developed carbon nanotube probe.

Bio-SPM Collaborative Research on Various Life Phenomena: To lead the development of the Nanoprobe Life Science field, we worked on various transdisciplinary collaborations among the four major disciplines: nanometrology, life science, supramolecular chemistry and computational science. The published examples include Bio-SPM studies of pH around phycosphere (*ISME Journal* **2022**), cellulose and chitin structures and hydration (*Sci. Adv.* **2022 &** *Small Methods* **2022**), water-driven structuring of adenine assemblies (*JACS* **2022**), cellular dynamics induced by the interaction with phospholipid nanoparticles (*Small* **2022**), DNA-edit dynamics by CRISPR-Cas3 and Staphylococcus aureus Cas9 **(***Nat. Commun.* **2022 &** *ACS Nano* **2023)**, and the structure and the function of nucleolar protein, PQBP5 (*Nat. Commun.* **2023**).

2. Generating Fused Disciplines

Both top-down and bottom-up approaches have been continuously taken to promote fused disciplines. The top-down set out and executed three priority research themes, and the bottom-up supported interdisciplinary research by teams consisting of young researchers.

3. Realizing an International Research Environment

The total number of papers by 16 PIs in 2017-2022 was 697, of which 317 (45.5%) were internationally co-authored papers. The total number of papers co-authored by one of the four overseas PIs with resident researchers in NanoLSI has reached 24 since 2017. Various measures have been executed such as outreach programs for external researchers, mobility and career path for young researchers, and support for young foreign researchers to acquire research funds.

4. Making Organizational Reforms

The successful reforms of NanoLSI have been continued such as research professorships for concentrating on research, a rigorous evaluation-based salary system, integrated management of NanoLSI and the Graduate School "Division of Nano Life Science," the tenure-track junior PI program, and English-based administration.

5. Efforts to Secure the Center's Future Development over the Mid- to Long-term

Roadmaps on 6 nanotechnology and 7 life sciences have been updated. The total amount of external funds acquired in FY2022 by 82 NanoLSI researchers was 1,356 million yen (1,288 million yen in FY2021). Policy and practice of fostering next-generation researchers have been featured.

6. Others

Outreach activities have been described, such as press releases of research outcomes, media coverage, visitors to NanoLSI, and approaches to high school students.

* Describe clearly and concisely the progress being made by the WPI center project from the viewpoints below.
- In addressing the below-listed 1-6 viewpoints, place emphasis on the following:
  (1) Whether research is being carried out at a top world-level (including whether research advances are being made by fusing disciplines).
  (2) Whether a proactive effort continues to be made to establish itself as a "truly" world premier international research center.
  (3) Whether a steadfast effort is being made to secure the center's future development over the mid- to long-term.

1. Advancing Research of the Highest Global Level

* Among the research results achieved by the center, concretely describe those that are at the world's highest level. In Appendix 1, list the center's research papers published in 2022.
* Regarding the criteria used when evaluating the world level of center, note any updated results using your previous evaluation criteria and methods or any improvements you have made to those criteria and methods.

Outline

Continuing from the last year, we worked on the three projects shown in Fig. 1.

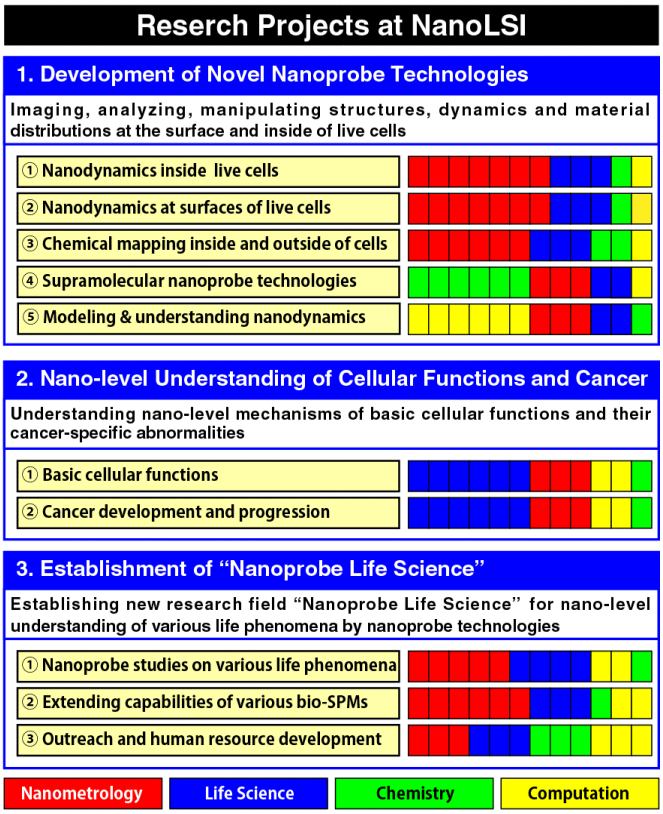

Fig. 1: Research projects at NanoLSI and contributions from the four major disciplines to each project.

(1) We have been developing Bio-SPM technologies for visualizing the structures, dynamics and material distribution inside and at the surfaces of living cells. We had largely completed proof of principle during the previous fiscal year. In FY2022, our focus has shifted to extending the performance and functionalities of the newly developed techniques and the development of practical application techniques for life science research. For example, we have successfully extended the imaging capability of nanoendoscopy AFM from in-cell imaging of static structures to dynamic processes such as the growth of focal adhesions and actin fibers. Furthermore, we have developed a sample preparation technique enabling SICM imaging of cellular surfaces constituting a spherical organoid (***Small*** **2022**).

(2) We have been investigating nanoscale mechanisms of cellular functions and cancer using bio-SPM techniques. Life science research with our world-leading bio-SPM technologies (e.g., HS-AFM, 3D-AFM and bio-SICM) continued to produce impactful publications. Examples include HS-AFM studies on the dynamics of MET receptor agonists (***Nat Biomed Eng*** **2023**) and extracellular vesicles (***J Extracell Vesicles*** **2022**), and 3D-AFM studies on molecular-scale surface structures and hydration of chitin and cellulose nanofibers (***Small Methods*** **2022 &** ***Sci. Adv.*** **2022**). Meanwhile, we have actively explored applications of the newly developed live-cell imaging techniques to different life science disciplines. Examples include SICM studies on the changes in cell surface dynamics and mechanics with cancer progression (***Small 2022*** **&** ***Small 2023***), and AFM studies on protein clustering on a cell surface induced by chemical fixation (***Comm Biol*** **2022**).

(3) We aim to establish the "Nanoprobe Life Science" field by creating a world-leading center for bio-SPM collaborations. Despite the COVID-19 pandemic, we made our best efforts to organize various symposiums, seminars and a summer school in person. In addition, all the overseas PIs managed to visit NanoLSI and engaged in collaborative research. Furthermore, we actively performed bio-SPM collaborations with external researchers and published many impactful papers. Now, the number of participants for the summer school and accepted proposals for the Bio-SPM collaborative research program have recovered to the levels before the COVID-19 pandemic. To continue to lead the bio-SPM research community, we have been working on extending the capabilities of our world-leading bio-SPM techniques. For example, we continued to improve the speed of HS-AFM to achieve 100 fps. In addition, we started to explore possibilities of 3D-AFM imaging of various 3D biological systems such as chromosomes.

Achievements in FY2022 are summarized as follows.
- Papers: 129 (41.1 % internationally co-authored; 46 with an IF > 10; 66 with an IF > 7),
- Invited talks in int'l meetings: 70,

- Funding: ¥1,356,624,224 overall (36grants > ¥10,000,000).
These achievements are of the highest global level for an institute with 82 researchers (as of March 2023).

(1) Development of techniques for measuring nanodynamics on the cell surface and in the interior

(Development of nano-imaging techniques)

･Measurement of nanodynamics on cell surfaces:

In 2021, Watanabe *et al.* developed a method to measure dynamic changes in nanomechanical properties of living cells based on high-speed scanning ion conductance microscopy (HS-SICM) and investigated the relationships between gene mutations, colon cancer phenotypes and nanomechanical properties in genotype-defined mouse intestinal tumor-derived cells. In FY2022, his group not only applied this technique to investigate nanoparticle-cell interactions (***Small* 2022**) but also extended the capability of HS-SICM nanomechanical measurement from 2D-cultured cells to 3D-tissure-like cellular structures to gain further insight into the nanomechanical properties of cells. The 3D cultured organoids were prepared so that the basal surface (BS) faces toward the outside surface of organoid cells and is accessible to the SICM probe. In addition, 3D living organoids of genotype-defined metastatic intestinal cells were partially embedded into collagen gel to immobilize and stabilize the organoid structures for long-term time-lapse HS-SICM measurement (Fig. 2a). In this setup, they succeeded in measuring the long-term dynamics of subcellular structures, such as ridge-like, stress-fiber, and local elastic modulus distributions on BS (Fig. 2b). Furthermore, they demonstrated the possibility that not only the averaged elastic modulus of cells (Fig. 2c) but also local correlations between topography and elastic modulus mapping provide a physical marker to categorize cancer progression (Fig. 2d) (***Small* 2023**).

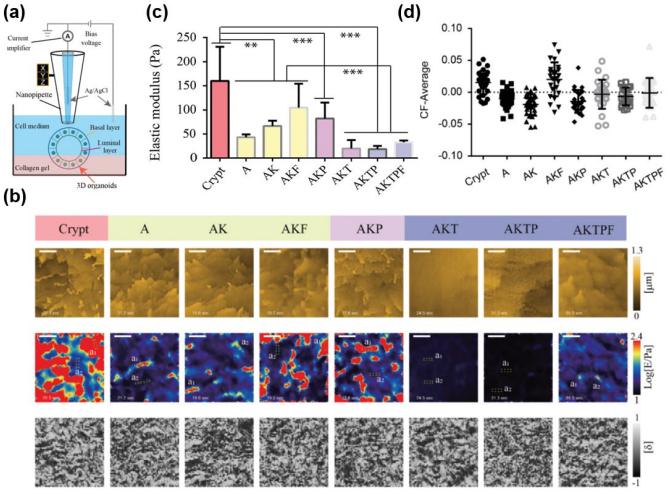

Fig. 2: (a) HS-SICM setup for 3D cell measurement. (b) Snapshots of time-lapse images of height and elastic modulus in genotype-defined (A, AK, AKF, AKP, AKT, AKTP, and AKTPF) 3D organoids of metastasis cancer cells and normal Crypt cells. (c) Elastic modulus of various 3D organoids. (d) Local correlation factor for various 3D organoids.

Ichikawa *et al.*, following on from last year, conducted cell surface observation using AFM. By culturing cells on the microporous silicon nitride membrane (MPM) and observing the cell surface through the 3 or 5 mm diameter holes of MPM, they successfully observed protrusions of less than 10 nm in diameter on the living colon cancer cell surface (collaboration with Prof. Oshima, Kanazawa University Cancer Research Institute). They investigated the nanoscale effect of chemical fixation reagents on the colon cancer cell surface using this technique. They tested commonly used fixation reagents, such as 2% glutaraldehyde, 4% paraformaldehyde, and 100% methanol. After treatment with all these fixation reagents, they found that most of the small protrusions on the living cell surface disappeared, and there were only large protrusions whose size was 10 - 50 nm on the cell. They also measured the distances between membrane molecules before, after, and during fixation, and concluded that these large protrusions were created by aggregating membrane proteins which move freely to some extent in the presence of fixation reagent. These results have been published (***Comm Biol* 2022**). Ichikawa *et al.* also investigated the binding affinity of Cassiicolin 1 (Cas1) and Cassiicolin 2 (Cas2), which are toxins causing Corynespora leaf fall disease, to the phospholipid. They directly observe the Cas1 and Cas2 binding to the phospholipid layer on mica and the lipid layer degradation using high-speed AFM. They found that Cas1 has a low affinity to the neutral phospholipid (1,2-dipalmitoyl-sn-glycero-3-phosphocoline, DPPC) but high affinity and degradation to negative phospholipid (1,2-dipalmitoyl-sn-glycero-3-phosphate, DPPA), glycerolipids (monogalactosyldiacylglycerol) and sterols (stigmasterol, sitosterol, MGDG). Cas2 has a high affinity to betaine lipid (1,2-dipalmitoyl-sn-glycero-3-O-4'-[N,N,N-trimethyl]-betaine, DGTS-d9) in addition to DPPA, MGDG, and sterols. They also confirm these properties with a confocal microscope using GFP-Cas1 and rhodamine-stained phospholipids. They further observed the Cas1-treated leaf with cryo-SEM and found that purified Cas1 caused lesions to the rubber leaf PB 255 clone, but Cas2 did not

cause large lesions. These data have been published (*Phytopathology* **2022**).

·Visualization of intracellular nanodynamics (nano-endoscopic observation):
(i) Nanoendoscopy AFM: In this WPI project, Fukuma *et al.* developed nanoendoscopy AFM, where a long needle probe is inserted into a live cell for the measurement of intracellular nanodynamics and nanomechanics. Last year, they reported in-cell imaging of the focal adhesion (FA) and measurement of nuclear stiffening caused by cancer progression. This year, they made significant progress in these projects.

As for the FA imaging, they succeeded in the in-cell imaging of the growth of the FA and actin fibers by nanoendoscopy AFM and confocal fluorescent microscopy (FM). These images reveal that the FA was initially thick but became thinner as they grew. In addition, the actin fiber was initially in contact with the upper cell membrane and detached during the fiber growth. Furthermore, the paxillin distribution was broadened to fill the gap between the newly formed actin fiber and the cell membrane. These findings were obtained owing to the 3D imaging capability of 3D-AFM with a high vertical resolution. As shown here, they have extended the capability of nanoendoscopy AFM from imaging a static structure to dynamic processes in a live cell.

As for the nuclear stiffening, they continued to work on the in-cell nuclear elasticity measurements and confirmed the nuclear stiffening with cancer progression for different cell lines and different softness of the underlying substrate. In parallel, they performed various biochemical analyses to determine the cause of the stiffening. The flow cytometry and Western blotting revealed that lamin A, B1/2, and C expression levels do not increase. The fluorescent microscopy analyses suggested that the total DNA amount or histone modifications did not change. Finally, the MNase analysis revealed a clear increase in the DNA compaction level with cancer progression. Based on this finding, they are now preparing cells with different DNA compaction levels to clarify their correlation with nuclear elasticity. Nuclear stiffness is strongly related to various diseases known as nuclear envelopathies and cell aging, and the method developed here can greatly help understand these phenomena.
(ii) Deroofed cells: Molecular-resolution analysis of intracellular structures and organelles can benefit from prior removal of the plasma membrane to enable direct nanoprobe/sample contact. Franz (Jr. PI) is developing experimental tools for opening the cell interior for SPM exploration, while maintaining the functionality of the exposed intracellular protein complexes. For instance, microsonication-based cell de-roofing methods were used for nanostructural and biomechanical characterization of contracting actomyosin stress fibers. Complementary experiments using both AFM and SICM imaging and elasticity mapping revealed complex patterns of stiffness changes along contracting actin stress fibers. Furthermore, the contribution of different myosin II isoforms on stress fiber ultrastructure and contraction mechanics were evaluated using a panel of myosin knockout cells. While mechanical de-roofing methods are suitable for exposing relatively stable intracellular compartments like actin stress fibers, more delicate intracellular protein assemblies and organelles require non-mechanical methods for minimally invasive cell de-roofing. Experiments in the group show that short treatment with bee venom phospholipase A2 introduces transient micrometer-sized pores into the plasma membrane of living cells, providing temporary windows into the cell interior for nanoprobe exploration. Using such enzymatic cell membrane digestion in combination with high-aspect-ratio AFM tips, it has now become possible to image intracellular components, including dynamic submembranous cytoskeletal networks or the nuclear lamina, with nanometer-resolution directly within living cells.

(Development of nano-endoscopic analysis and manipulation techniques)

·Injection and sampling of substances using a nanopipette:

Takahashi *et al.* established a technique for injecting reagents into cells using a glass nanopipette to administer reagents such as inositol phospholipid to organelles, and observed changes in the dynamics of the organelles (Fig. 3). In this process, they established a technique to control the dosage of the reagent by applying a potential to the electrode in the glass nanopipette and inducing electroosmotic flow. Furthermore, they developed a technology for automatic cell identification using machine learning and have already established a technology for automatic collection at the single-cell level. In order to apply this

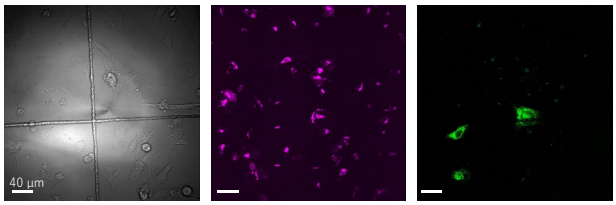

Fig. 3: Intracellular injection of inositol phospholipids by nanopipette. Confocal microscope image of Mouse Embryonic Fibroblast (MEF) cells, (left) bright field image, (center) Golgi apparatus labeled with fluorescent tag, (right) BODYPY-labeled inositol phospholipids.

technology to organelle pick-up, they are currently establishing a technology that enables observation of organelles without staining, such as holographic microscopy, and a collection technology using a glass nanopipette. A collaborative team (Arai and Takahashi) constructed the microscopic system coupling robust fluorescence lifetime imaging (FLIM) with a nanopipette for the combination with metabolomics and transcriptomics. So far, they have succeeded in accessing a single cell, followed by mRNA amplification.

·Analysis of nano-distribution of physical properties using a molecular sensor:

They are developing unique molecular sensors that can selectively recognize target compounds using their expertise in supramolecular chemistry. Ogoshi *et al.* have already succeeded in the selective recognition of 1-MNA (1-methylnicotinamide), which is one of the oncometabolites, by using a carboxylate-modified pillar[6]arene derivative. They have successfully improved the binding constant ca. 700-fold by changing the interaction sites.

·Nano-manipulation using molecular machines:

To expand the variety of ways for nano-manipulation, Arai *et al.* generated a library of functional chemical dyes toward a near-infrared (NIR)-driven supramolecular machine system. More specifically, using photothermal and thermosensitive dyes, they demonstrated local heating and thermometry at the single cell level (Fig. 4) (***ACS Nano* 2022 & *Materials Today Bio* 2022**). Furthermore, they established a methodology to manipulate the dynamics of lipid membrane using local heating at the molecular level, leading to a milestone in the development of future molecular machines.

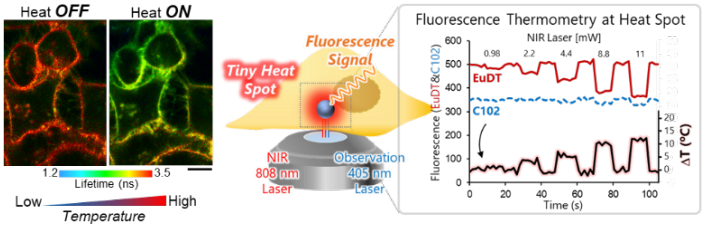

Fig. 4: Local thermometry (FLIM) and heating at subcellular scale.

Akine *et al.* have developed a new helical molecule that shows a unique time-dependent chirality change. The molecule can bind a chiral amine molecule at the constituent cobalt(III) ions to give a right-handed helix, which was inverted to the left-handed form before racemization upon the six-step amine exchange reaction with piperidine (***PNAS* 2022**). This would be a good candidate as a molecular scaffold to enable time-dependent functions. They also developed two types of metal-containing rotaxane molecules that could be utilized as a molecular machine due to their dynamic nature. One is obtained from a metal-containing dumbbell molecule and a crown ether derivative. The dynamic nature of the rotaxane can be easily modulated by additives (***Angew. Chem. Int. Ed.* 2023**). The other was obtained from a metallomacrocycle and a dumbbell molecule (***Dalton Trans.* 2022**). They also developed new amphiphilic metal complexes that can form a monolayer at the air-water interface. The chiroptical properties can be modulated by changing the surface pressure at the interface (***Dalton Trans.* 2022**). Ogoshi *et al.* have achieved pillar[n]arene-based chiral supramolecular assemblies (***Chem. Sci.* 2022 ×2, *Angew. Chem. Int. Ed.* 2022, *Nat. Comm.* 2022**), tubes and cavitands by covalent bonds (***Cell Rep. Phys. Sci.* 2022 & *JACS* 2022**), and ring-opening polymerization by pillar[5]arene crystals (***Angew. Chem. Int. Ed.* 2022 & *Chem. Sci.* 2022**). Maeda *et al.* have developed functional helical polymers (***J. Mater. Chem. C* 2022 & *Angew. Chem. Int. Ed.* 2023**). They also developed facile and versatile methods to synthesize end-functionalized helical polymers (***Angew. Chem. Int. Ed.* 2022**), which could be used to immobilize stimuli-responsive helical polymers on the surface of probes to be used as functional nanoprobes. MacLachlan *et al.* have developed a family of platinum-containing macrocycles functionalized with crown ether-like receptors. When these molecules bind to guests, such as alkali cations, they change color and luminescence, demonstrating their potential as molecular sensors (***Angew. Chem. Int. Ed.* 2023 & *Inorg. Chem.* 2022**). The size of the cavity in the molecules can be tuned using redox chemistry, allowing one to switch the selectivity of the molecule in situ. MacLachlan and Akine *et al.* developed Schiff-base macrocycles with well-defined cavities and investigated the selective guest binding of organic cations in the interior (***Org. Biomol. Chem.* 2022**).

(Understanding measurement principles of newly-developed nano-probe techniques and life phenomena by means of mathematical/computational sciences)

·Multiscale modeling at the nano-life science interface (Hall, Foster)

Foster *et al.* uses a range of simulation methodologies (including machine learning, molecular dynamics and Brownian dynamics approaches) to investigate various problems at the nano-life science interface. Recent work has focused on using convolutional neural networks to analyze and

interpret the molecular nature of adsorbate interrogated by high-resolution AFM measurements and the use of all-atom molecular dynamics to predict hydration patterns on biopolymers such as chitin and cellulose. Within the Foster laboratory, Assist. Prof. Damien Hall employs multiscale modeling to investigate both fundamental and disease-related life processes at the cellular to molecular level of detail. He is currently engaged with the modeling of AFM-based cell membrane topology, elasticity and penetration measurements (***BPPB* 2022**) and is also attempting a mathematical assignment of cancer versus healthy cells based on SPM measurements. The group is also conducting simulations of intracellular diffusion in complex cellular fluids as well as using multiscale cell automata models to investigate the cell life cycle in terms of growth, division and epigenetic transmission stages (Fig. 5).

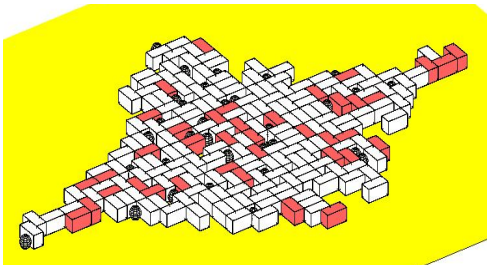

Fig. 5: Modeling cellular growth and transfer of epigenetic elements within growing colonies of the budding yeast *Saccharomyces cerevisiae* (white/red = +/- epigenetic factor).

·Developing a chromosome model and analyses on HS-AFM movies (Sumikama)

They have published a paper describing a theory for the computation of 3D-AFM images of biopolymers such as chromosomes (***JPCL 2022***). To apply this theory to chromosome models and compare their simulated 3D-AFM images with experimental ones, it is necessary to develop more realistic chromosome models that account for heterochromatin and euchromatin. As such, they also developed a classification method called SCN of heterochromatin and euchromatin based on Hi-C experiments (Fig. 6), which is now on ***bioRxiv***. This classification further provides a mechanistic insight into the differences in contact probability between heterochromatin and euchromatin: euchromatin has more contact than heterochromatin (Fig. 6).

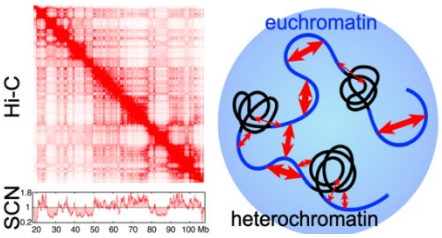

Fig. 6: A classification method (SCN) based on the Hi-C map (left). Schematic representation of heterochromatin and euchromatin (right).

To advance HS-AFM research to the next stage, it is important to theoretically analyze HS-AFM movies to reveal the biological function from the dynamics of molecules. Therefore, they analyzed HS-AFM movies of 1) CaMKII, 2) AMPA receptor, 3) TRPV1 channel, and 4) Na^+ channel based on statistical mechanics. Molecular dynamics simulations also help to validate HS-AFM measurements at the atomic level, and this has been performed on 5) AMPA receptor, 6) nucleosome, 7) Na^+ channel, and 8) lipase systems.

·Reconstruction of 3D atomistic biomolecular dynamics from HS-AFM imaging (Flechsig)

Their previously developed methods of data-driven simulation AFM and automatized fitting (***PLoS Comput Biol* 2022**) have been applied to provide atomistic level interpretation of HS-AFM experiments (Fig. 7) for 1) the aptamer-Cyp24 protein complex (cancer research) (***ACS Appl Mater Interfaces* 2022**), 2) Annexin V lattices (***PLoS ONE* 2022**), 3) TMEM16 membrane transporters, 4) EML4-ALK protein complex (cancer research), 5) Cas9-RNA-DNA endonuclease (***ACS Nano* 2023**), 6) MET-receptors (cancer research), 7) E6AP ligase (***bioRxiv* 2022**). Moreover, multiscale molecular dynamics simulations provide atomistic explanation of basement membrane laminin-integrin dynamics observed under HS-AFM. AI based reconstruction of the 200 nm long atomic structure of EEA1 protein and molecular dynamics simulations complement HS-AFM experiments to understand self-organization and biomechanical properties of the endosomal membrane. Furthermore, an electrostatic model to understand sample-substrate interactions in AFM and predict stability of experimental observations was developed and implemented as software tools.

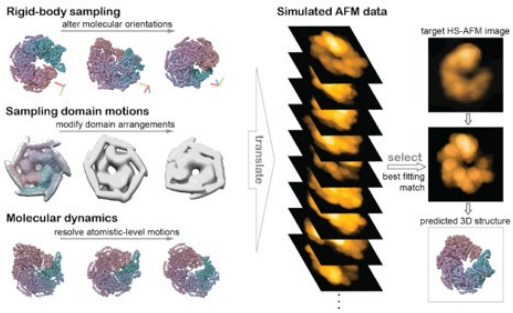

Fig. 7: Concept of inferring 3D atomistic biomolecular dynamics from resolution-limited AFM topographic imaging.

·Modeling multiscale cell dynamics from cytoskeletal to multicellular systems (Okuda)

Okuda (Jr. PI) proposed new mathematical models that describe the dynamics of stress fiber, cell membrane, cell adhesion, and collective cell migration. A developed 3D vertex model explained how cells collectively migrate as a cluster in 3D space as well as the variety of cell migratory modes observed in embryogenesis and cancer metastasis (***Biophys J*** **2022**). Moreover, a simple mathematical model revealed that alpha-actinin plays a key role in inducing viscous frictions between actin filaments within a stress fiber to propagate traction forces (***iScience*** **2023**). Furthermore, novel computational methods, referred to as a nonconservative fluid membrane model, succeeded in simulating long-term cell dynamics (***EPJE*** **2022**) as well as adhesive cell dynamics (***PRE*** **2023**). The integrated model revealed novel states of adhesive cell dynamics on a substrate under shear flow (Fig. 8).

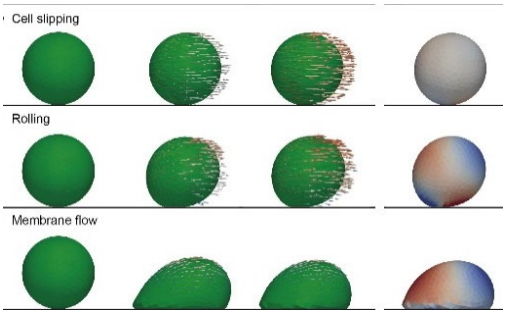

Fig. 8: Novel states of adhesive cell dynamics on substrate under shear flow.

(2) Nano-level understanding of basic cellular functions and cancer-specific abnormalities

·Cell membrane receptor engineering and dynamics (Matsumoto)

Matsumoto *et al.* have been investigating 1) dynamic structures for growth factor receptor activation and 2) creation and application of growth factor receptor agonists, focusing on MET/HGF receptor. In FY2022, they created a designer receptor agonist capable of dimerizing and activating MET receptor, using protein engineering technology with MET-binding peptides (Fig. 9) (***Nat Biomed Eng*** **2023**). The MET-activating agonists showed outstanding blood stability or the ability to cross the blood-brain-barrier in mice, indicating the creation of a high-performance biological drug for therapeutic purpose. The HS-AFM analysis revealed that the MET-agonist induced MET receptor dimerization/activation due to its bivalent properties (Fig. 9).

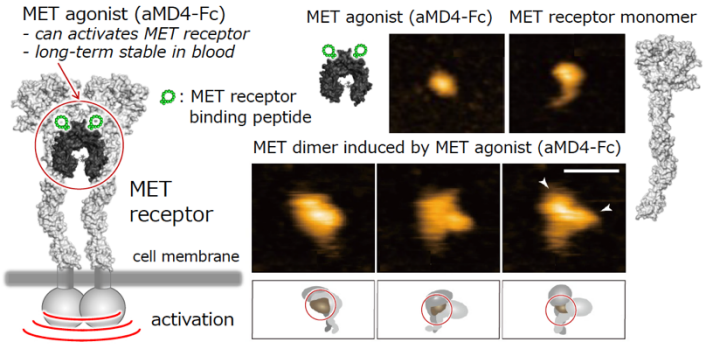

Fig. 9: MET receptor activation by newly created designer receptor agonist (aMD4-Fc).

·Intracellular trafficking (Wong)

The control of intracellular traffic is critical for cell growth and differentiation (***Stem Cells*** **2022**). Nuclear pore complexes (NPCs) are multi-protein turnstiles that regulate nucleo-cytoplasmic traffic. Recently, Wong *et al.* developed novel aminocyclopropenone 1n (ACP-1n) and investigated its biological effects on epigenetic "readers" of histone acetylation, the bromodomain-containing protein 4 (BRD4) functions. ACP-1n blocked BRD4 functions by preventing its phase separation ability both in vitro and in vivo, attenuating the expression levels of BRD4-driven MYC. Notably, ACP-1n significantly reduced the nuclear size with concomitant suppression of the level of the NPC protein NUP210. Furthermore, NUP210 is in a BRD4-dependent manner and silencing of NUP210 was sufficient to decrease nucleus size and cellular growth. Their findings highlighted an aminocyclopropenone compound as a novel therapeutic drug blocking BRD4 assembly, thereby preventing BRD4-driven oncogenic functions in cancer cells. (***Cells*** **2022**). In an interdisciplinary project with the Hanayama and Ando groups, using HS-AFM, they evaluated nanotopological changes of small

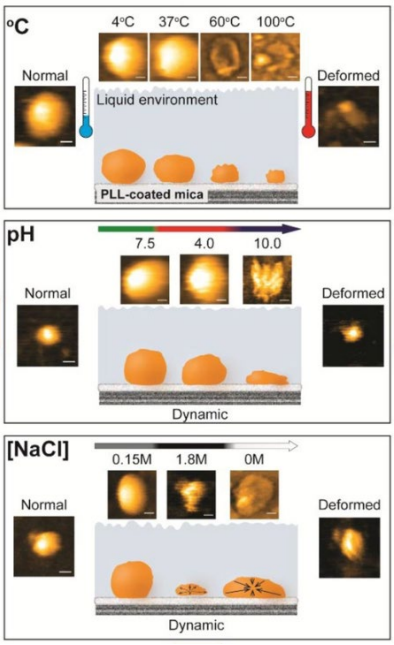

Fig. 10: HS-AFM imaging represents the continuity of the nanotopological dynamics of sEV in response to different physicochemical parameters.

extracellular vesicles (sEVs) with respect to different physicochemical stresses including thermal stress, pH, and osmotic stress. The sEV structure is severely altered at high-temperature, high-pH, or hypertonic conditions. Surprisingly, the spherical shape of the sEVs is maintained in acidic or hypotonic environments (***J Extracell Vesicles*** **2022**) (Fig. 10). In the near future, they may also be able to perform HS-AFM imaging to trace the non-canonical nuclear transport of sEV contents.

·Cell communications via extracellular vesicles (Hanayama)

Hereditary transthyretin amyloidosis, which is caused by variants in the transthyretin (TTR) gene, leads to TTR amyloid deposits in multiple organs. Using HS-AFM, Hanayama *et al.* showed that extracellular vesicles (EVs) are involved in the formation of TTR amyloid deposits on the membrane of EVs, as well as the deposition of TTR amyloid in cells, suggesting that TTR in serum-derived EVs is a potential target for future amyloidosis diagnosis and therapy (***Front Mol Biosci*** **2022**) (Fig. 11). In addition, they established engineered EVs that prevent SARS-CoV-2 infection by conjugating anti-spike nanobody and IFN-beta to EVs (***Pharm Res*** **2022**).

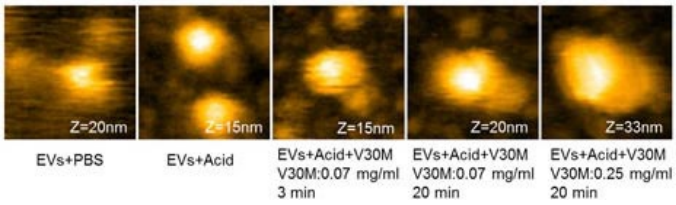

Fig. 11: The height of the EVs increased in a V30M-TTR-dependent manner due to the formation of TTR amyloid deposits on EVs.

·Morphogenesis - bottom up & mechanical approach (Toda)

Toda *et al.* have been working on the mechanisms of how interacting cells organize tissue morphologies. In FY2022, they engineered chimeric Cadherin proteins in which the cadherin intracellular domain is replaced with specific cytoskeletal regulators and showed that cytoskeletal activity at the Cadherin tail can determine whether cells are sorted into the center or to an outer layer of the cell aggregate. Now they have started a collaboration with the Watanabe group using scanning ion conductance microscope (SICM) to study how chimeric cadherins change cell membrane dynamics to induce cell sorting. They are also studying the mechanisms of how the diffusion of the signaling protein Wnt is regulated to generate stable tissue patterns. They developed a protein detection system that can distinguish a soluble form and membrane-tethered form of Wnt and are testing which proteins can tether Wnt on the cell surface to control its diffusion.

·Transcriptional regulation & epigenetics (Miyanari)

Miyanari *et al.* have been studying roles for chromatin dynamics in transcriptional regulation, which is crucial for cell lineage allocation in mammalian development. They have succeeded in simultaneously visualizing chromatin accessibility and epigenetic modifications; both are key chromatin signatures in the regulation of transcription (***Methods Mol Bio*** **2023**). They also developed a functional peptide binder to survivin, a regulator of chromosome segregation and universal tumor antigen. They found that the binder can selectively inhibit proliferation of surviving positive cells by inducing apoptosis, and significantly suppressed tumor growth in xenograft mice (***Bioconjug Chem***. **2022**).

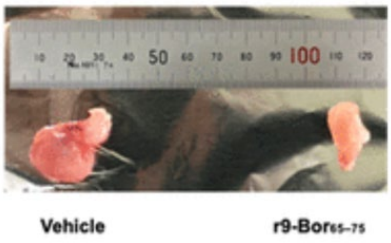

Fig. 12: Anticancer effect of the survivin binder on xenograft nude mouse model.

·Oncogenes and Cancer Cell Dynamics (Oshima)

Oshima *et al.* previously established intestinal tumor-derived organoid lines with defined genetic alterations and malignant phenotypes. Using the organoid lines, they examined apical cell surface structures and physical properties at nanoscale by high-speed (HS)-SICM. Notably, they found that metastatic malignant cells have a specific morphology of micro-ridge-like structures with active movement on the membrane surface (***Biomaterials*** **2022**). Moreover, the cell surface of metastatic cells

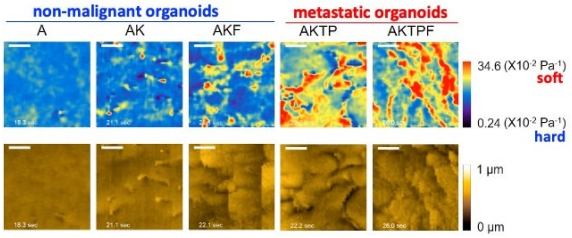

Fig. 13: Physical properties of non-malignant and metastatic organoid cell surface.

showed significantly softer characteristics compared to that of non-metastatic cells (Fig. 13). They next developed a unique organoid culture system to examine the basal surface of cells by HS-SICM. In this system, half of the organoids are embedded in collagen (Fig. 2a). Interestingly, the basal surface of metastatic intestinal tumor organoids showed similar ridge-like structures and softer cell membranes (***Small* 2023**). These data will contribute to understanding the mechanisms of malignant cancer cells for invasion, migration, and metastasis.

·Development of DNA Aptamers as Anti-cancer Molecules (Nakajima)

Nakajima *et al.* have successfully developed DNA aptamer molecules that inhibit CYP24A1, an enzyme degrading anti-proliferative vitamin D_3. By collaboration with the Kodera group, they clarified the molecular dynamics and inhibition mechanism of the DNA aptamer against CYP24A1 by HS-AFM. Interestingly, the DNA aptamer significantly enhanced the vitamin D_3-mediated inhibition of cancer cell proliferation (***ACS Appl Mater Inter* 2022**). They have also obtained preliminary data from an in vivo study using cancer-bearing mice, showing the therapeutic potential of the aptamers for cancer. In addition, they have explored DNA aptamer molecules targeting ADAR, an enzyme catalyzing RNA editing, the abnormal expression of which is relevant for cancer development.

·Development of Diagnostic and Therapeutic Technology (Hirao)

Hirao *et al.* have recently elucidated molecular mechanisms that regulate cell differentiation and stress responses under microenvironmental influence (***Leukemia* 2022**, ***Commun Biol*. 2022**). They have also uncovered the significance of endo-lysosomal activity as a metabolic biomarker of malignancy, and an important therapeutic target for brain tumors (***Cancer Sci.* 2022**). To advance innovative imaging technology, they evaluated the performance of a novel sensor for the detection of 1-MNA, a metabolite associated with malignant properties in various types of cancer. The sensor demonstrated notable advantages over the current sensor, P6A (CA), including the quantification of 1-MNA secreted into the cell culture supernate (Fig. 14). These findings are expected to further the development of nano-pipette imaging technology, leading to a deeper understanding of the mechanisms that regulate malignant traits in cancer cells.

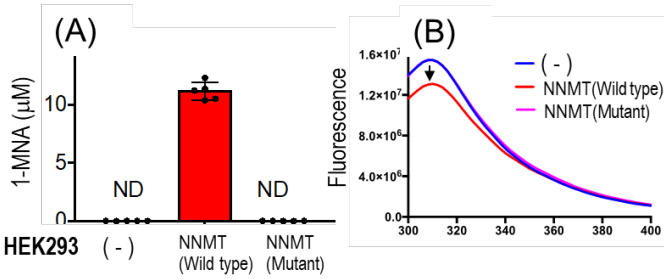

Fig. 14: Detection of 1-MNA secreted from 293 cells expressing NNMT by LC-MS/MS (A) and fluorescence quenching of the sensor (B).

·Development of Therapeutic Approach to Lung Cancer (Yano)

Yano *et al.* developed a therapeutic approach to ALK-rearranged lung cancer targeting with a transcription factor STAT3 (***NPJ Precis Oncol* 2022**) Ca. They found that the mechanisms underlying adaptive cancer cell survival in residual tumors of ALK-rearranged lung cancer were predominantly dependent on the activity of a transcription factor STAT3 and subsequent transcriptional regulation of apoptosis (Fig. 15). They also found that inactivating genetic alteration of the mRNA splicing factor RNA-binding motif 10 (RBM10) that co-occurs with mutant EGFR decreased efficacy of EGFR inhibitor in lung cancer by inhibiting apoptosis (*J Clin Invest* **2022**). These findings show new therapeutic approach consisting of ALK- or EGFR-inhibitors with apoptosis inducing drugs.

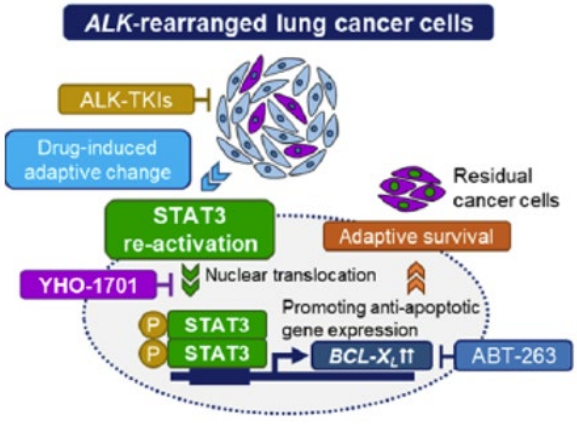

Fig. 15: STAT3 re-activation emerges adaptive survived lung cancer cells treated with ALK inhibitors.

(3) Establishment of a New Research Field: Nanoprobe Life Science (Further improvement of Bio-SPM technologies)

·HS-AFM technology

To expand the range of biological samples and dynamic phenomena that can be studied with HS-

AFM, further improvement of the low-disturbance and high-speed performance of HS-AFM is essential. However, the improvement capacity of instrumental components is already close to their limit. Therefore, HS-AFM group has been attempting to develop new scanning and control methods, while improving hardware components: scanners, amplitude detectors and small cantilevers. Fortunately, Ando *et al*. have made a breakthrough, i.e., retrace imaging during backward X-scanning is more disturbing to the sample because the feedback error signal during retrace imaging does not faithfully reflect the excessive tip–sample interaction. Based on this finding, they combined the Only Trace Imaging (OTI) mode and the dynamic PID control and succeeded in increasing the imaging rate from 10 fps to 50 fps. Furthermore, they worked on improving the optical system of HS-AFM to obtain a more accurate deflection signal of a small cantilever. By developing an evaluation system, the area of a focusing laser spot on the cantilever could be quantitatively measured. So far, Kodera *et al*. successfully reduced the area of the laser spot to ~29% of the conventional one by changing the laser diode and collimation lens. For producing smaller cantilevers with higher f_c and small spring constants, they have started a collaboration with a cantilever manufacturer.

Increasing the choice of AFM observation substrates is important for applying HS-AFM to a wide range of biological systems. In collaboration with the researchers of supramolecular chemistry, Shibata *et al*. prepared a new AFM substrate by immobilizing pillar[5]arene molecules on the mica surface. The pentagonal tubular structure of pillar[5]arene whose top and bottom surface have positive charges allowed DNA to be loosely immobilized on the substrate. Using this new substrate, they succeeded in capturing the nucleosome sliding of H2A.Z, a histone variant associated with various biological processes at 0.3 s (Fig. 16) (***Nano Letters*** **2022**).

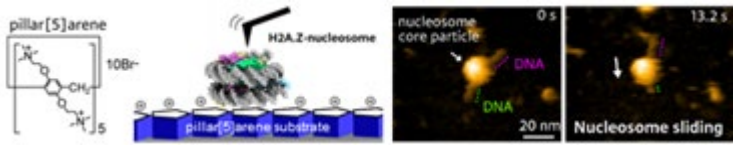

Fig. 16: Further improvement of HS-AFM. HS-AFM images of H2A.Z-nucleosome on the pillar[5]arene-modified mica surface.

·Scanning ion conductance microscopy (SICM)

Takahashi *et al.* have developed a method for controlling the inner/outer diameter ratio of glass capillaries for high-resolution scanning ion conductance microscopy, and established a method for fabricating nanopipettes with a radius of 20 nm or less. This new nanopipette fabrication method provides a simple but highly reproducible method of adjusting the inner/outer diameter ratio of a glass capillary by preheating the nanopipette prior to extension. The new capillary is capable of measuring ion currents with excellent S/N and has successfully visualized the dynamic cell surface topographic change related to endocytosis.

Watanabe *et al.* developed an ultra-low-noise wide-bandwidth transimpedance amplifier (TIA) to improve S/N in SICM measurements. A design of a low input capacitance interface and two-stage opamp configuration provides extremely low current noise performance in frequencies higher than 10 kHz. The background root-mean-square (RMS) noise of the TIA reaches ~3.5 pA at a bandwidth of 100 kHz, corresponding to ~30 percent of RMS current noise of commercially available low-noise TIAs for SICM use. In addition, extended input current range up to ~34 nA at a transimpedance gain of 1 GW assures the capability of developed TIA for most SICM applications. Such excellent performance of developed TIA will push the limits of the temporal resolution of SICM for visualizing dynamic structural changes on a cellular surface that are difficult with current instruments.

·3D-AFM imaging of Various 3D Self-Organizing Systems

Fukuma *et al.* have been developing 3D-AFM techniques to visualize the inside of various 3D self-organizing systems (3D-SOS). They developed 3D-AFM in 2010 and enabled the visualization of 3D hydration structures. Based on this technique, they developed 3D nanoendoscopy AFM and enabled the visualization of intra-cellular structures using a micrometer-scale needle probe in the past WPI project period. They are now exploring possibilities to visualize various 3D-SOSs with a thickness between hydration structures and living cells with various needle probes. For example, they developed a carbon nanotube (CNT) probe and applied it to visualize the inside of chromosomes. Their MD simulation results suggest that the obtained force contrasts largely represent the chromatin density distribution. They plan to use this technique for investigating the formation mechanism of the chromosome structure and its abnormalities caused by diseases or aging.

(Nanoprobe studies on various life phenomena)

i) Quantification of phycosphere pH of marine nano-phytoplankton species at a single cell level (*ISME Journal*, 2022, IF: 11.217**) (Zhang, Korchev)**

Marine phytoplankton are at the base of the food chain and their productivity ultimately determines the maintenance of fisheries. It is well documented that ocean acidification is profoundly altering a range of phytoplankton processes including nutrient acquisition, biogenic calcification and silicification. However, the assessment and prediction of the impacts of ocean acidification on phytoplankton remains challenging, due to a poor knowledge of processes occurring in the phycosphere surrounding cells. In this study, they employ a novel nano-technology pH probe for a high spatial (50 nm) and temporal resolution (2 ms) *in situ* quantification of phycosphere pH of marine nano- and micro-phytoplankton species at a single cell level (Fig. 17). For the first time, they show that the phycosphere pH is consistently higher than bulk seawater, which is amplified under ocean acidification conditions. They also demonstrate that a phycosphere pH increase can significantly alter the chemistry of iron, a limiting trace metal nutrient in about 40% of surface oceans.

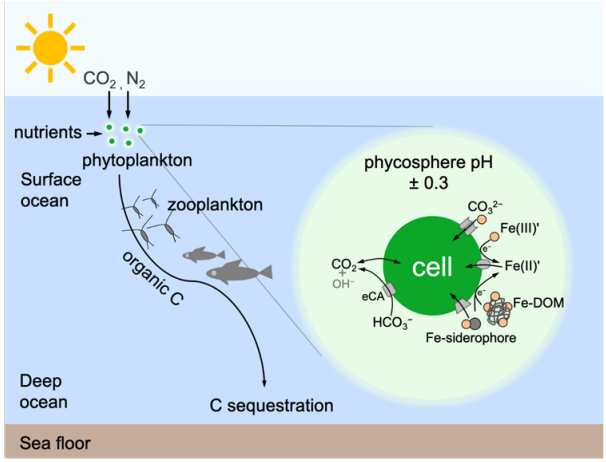

Fig. 17: Impact of an increase of pH in the phycosphere driven by algal uptake of CO_2 and extracellular enzymatic transformation of HCO_3- and a pH decrease driven by CO_2 release from the phytoplankton cell on Fe speciation and its availability to marine phytoplankton.

ii) Molecular insights on the crystalline cellulose/chitin-water interfaces via three-dimensional atomic force microscopy (*Science Advances*, 2022, IF: 14.98; ***Small Methods*, 2022,** IF: 15.367**) (Yurtsever, Miyata, Miyazawa, MacLachlan, Foster, Fukuma)**

Cellulose/chitin, a renewable structural biopolymer, is ubiquitous in nature and is the basic reinforcement component of the natural hierarchical structures of living plants, bacteria, tunicates, and many other organisms. However, a detailed picture of the crystalline cellulose/chitin surface at the molecular level is still unavailable. In this study, using AFM and molecular dynamics simulations, they revealed the molecular details of the cellulose/chitin chain arrangements on the surfaces of individual cellulose/chitin nanocrystals in water. They found substantial differences in the molecular details of water structure at different interfaces, reflecting the heterogeneous nature of the interactions between cellulose/chitin nanocrystals and water at the molecular level (Fig. 18). The inhomogeneous existence of these structured water layers on different crystalline planes may affect the cellulose/chitin nanocrystal surface adsorption and interaction behavior; thus, the degradation of cellulose/chitin by cellulase/chitinase-degrading enzymes could be affected differently.

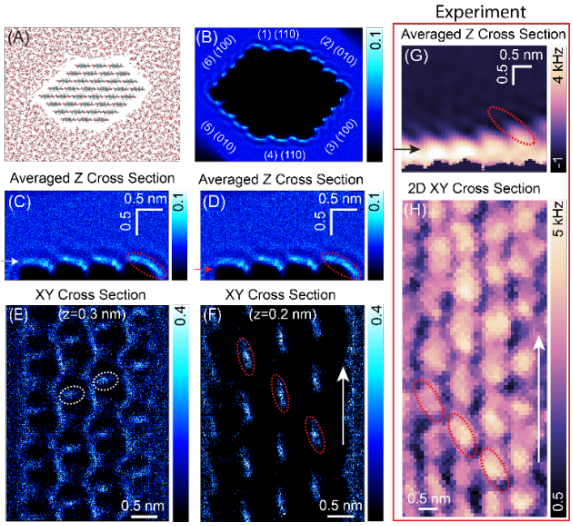

Fig. 18: Water-density distribution around different crystalline planes of a cellulose nanocrystal surface.

iii) Water dimer-driven DNA base superstructure with mismatched hydrogen bonding (*Journal of the American Chemical Society*, 2022, IF: 16,383**) (Foster)**

The existence of water dimers in equilibrium water vapor at room temperature and their anomalous properties revealed by recent studies suggest the benchmark role of water dimers in both experiment and theory. However, there has been a limited observation of individual water dimers due to the challenge of water separation and generation at the single-molecule level. Here, we use SPM to achieve real-space imaging of individual confined water dimers embedded inside a self-assembled layer of a DNA base, adenine, on a silver surface. The hydration of the adenine layers by these water dimers causes a local surface chiral inversion in such a way that the neighboring homochiral adenine molecules become heterochiral after hydration, resulting in a mismatched hydrogen-bond pattern

between neighboring adenine molecules. The observation of single confined water dimers offers an unprecedented approach to studying the fundamental forms of water clusters and their interaction with the local biological environment (Fig. 19).

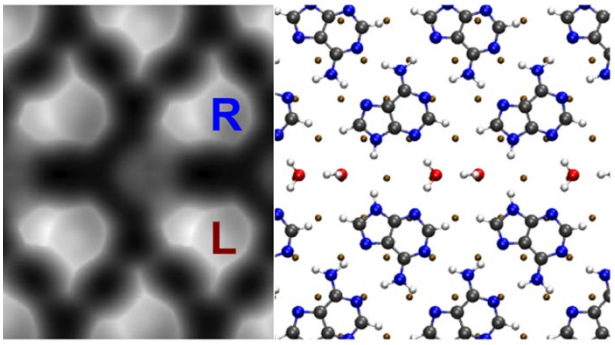

Fig. 19: Simulated AFM image of the hydrated adenine structure.

iv) In situ visualization of dynamic cellular effects of phospholipid nanoparticles via high-speed scanning ion conductance microscopy (*Small,* 2022, IF: 15.153**) (Sun, Watanabe)**

Phospholipid nanoparticles have been actively employed in numerous biomedical applications. A key factor in ensuring effective and safe applications of these nanomaterials is the regulation of their interactions with target cells, which is significantly dependent on an in-depth understanding of the nanoparticle-cell interactions. To date, most studies investigating these nano-bio interactions have been performed under static conditions. It is, however, noteworthy that such nanoparticle-cell interactions are highly dynamic. To gain a deeper insight into the cellular effects of phospholipid nanoparticles, they investigated the dynamic cellular effects of sub-100 nm phospholipid nanoparticles using high-speed scanning ion conductance microscopy. It was revealed that upon introduction into the cellular environment, within a short timescale of hundreds of seconds, phospholipid nanoparticles can selectively modulate the edge motility and surface roughness of healthy fibroblast and cancerous epithelial cells. Furthermore, the dynamic deformation profiles of these cells can be selectively altered in the presence of phospholipid nanoparticles. This work should shed further light on real-time nanoparticle-cell interactions for improved formulation of phospholipid nanoparticles for numerous bioapplications.

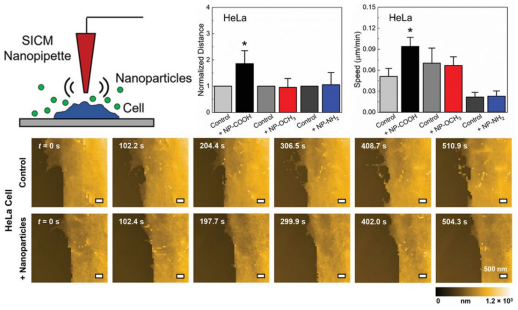

Fig. 20: Nanoparticle-cell interactions measured by HS-SICM

v) Dynamic mechanisms of CRISPR interference by *Escherichia coli* CRISPR-Cas3 (*Nature Communications,* 2022, IF: 17.694**) (Kodera, Umeda)**

Genome editing is a very useful technology not only for basic research to elucidate life phenomena but also for a wide range of other fields such as improving the efficiency of bioproduction in industry, breeding in the agricultural and fishery industries, and developing gene therapy and new drugs in the medical field. A new genome editing tool using CRISPR-Cas3 has unique features that make long-range unidirectional deletions of genome-DNA and have fewer off-target problems compared with the widely used CRISPR-Cas9 system. Despite many studies using cryo-electron microscopy and single-molecule FRET, the precise mechanism of genome editing by CRISPR-Cas3 remained elusive. In this study, in collaboration with researchers of the University of Tokyo and RIKEN, they succeeded in reconstructing the CRISPR-Cas3 system *in vitro* and clarifying the detailed mechanism of genome editing by CRISPR-Cas3. Notably, using high-speed atomic force microscopy (HS-AFM), a series of videos could be obtained of CRISPR-Cas3 in action from target search and binding to DNA degradation including the events of DNA-reeling and unreeling (Fig. 21). The dynamic information obtained from this study provides important and fundamental knowledge for the

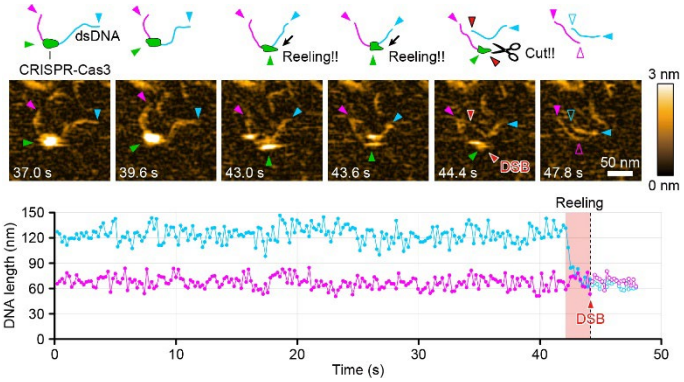

Fig. 21 Direct observation of the long-range unidirectional deletions of DNA by CRISPR-Cas3, using HS-AFM. A Cascade-Cas3 complex bound to the target site (green) reels the upstream long strand side (blue). After reeling DNA, a double-strand break (DSB) is introduced (red). Bottom graph shows a time course of the DNA lengths (short side, magenta; long side, blue).

development of CRISPR-Cas3 into a more efficient and accurate genome editing technology.

vi) Structure and functional role of a nucleolar protein PQBP5 (*Nature Communications,* 2023, IF:17.694**) (Umeda, Kodera, Ando)**

The nucleolus is a membrane-less organelle where rDNA is transcribed to pre-rRNA, which is processed and used for constructing ribosomes. PQBP5 was found to be one of the major nucleolus proteins related to PolyQ diseases but its structure and function have long been elusive. Using HS-AFM, they found that PQBP5 is an IDP with a C-terminal globule and an N-terminal IDR undergoing order-disorder transitions (Fig. 22a). RNA interacts with the IDR at its distal and proximal regions when these regions transiently form small globules (Fig. 22b, c). Super-resolution fluorescence microscopy revealed that PQBP5 forms a basket-like structure surrounded by (and partially colocalized with) other IDPs containing low complexity sequences, fibrillarin and nucleolin (Fig. 22d). Therefore, the PQBP5's basket-like structure seems to function as an anchor for assembly and reassembly of other nucleolar proteins. This was further supported by the imaging of HeLa cells subjected to osmotic stress, where the nucleolus was dissolved by dispersion of fibrillarin, nucleolin, and other proteins whereas PQBP5 remained in the nucleolus.

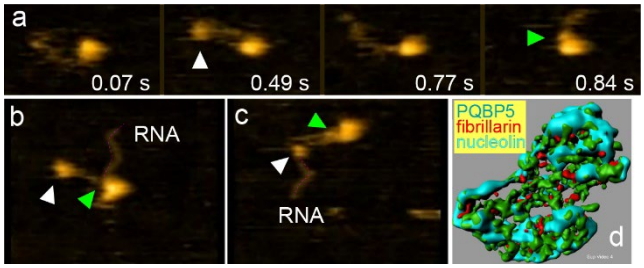

Fig. 22: HS-AFM images of PQBP5 alone (a), PQBP5 bound to RNA (b, c), and localization of three nucleolar proteins (d).

vii) Dynamics of target DNA binding and cleavage by Staphylococcus aureus Cas9 as revealed by high-speed atomic force microscopy (*ACS Nano,* 2023, IF:18.027**) (Puppulin, Sumino, Marchesi, Flechsig, Kodera, Shibata)**

Programmable DNA binding and cleavage by CRISPR-Cas9 has revolutionized life sciences. However, the off-target cleavage observed in DNA sequences with some homology to the target still represents the major limitation for more widespread use of Cas9 in biology and medicine. For this reason, complete understanding of the dynamics of DNA binding, interrogation and cleavage by Cas9 is crucial to improve the efficiency of genome editing. Here, they use HS-AFM to investigate *Staphylococcus aureus* Cas9 (SaCas9) and its dynamics of DNA binding and cleavage. Upon binding to single-guide RNA (sgRNA), SaCas9 forms a close bilobed structure that also transiently and flexibly adopts an open configuration. The SaCas9-mediated DNA cleavage is characterized by release of cleaved DNA and immediate dissociation (Fig. 23). The direct visualization of the process by sequential topographic images suggests that SaCas9-sgRNA binds to the target sequence first, while the following binding of the PAM is accompanied by local DNA bending and formation of the stable complex. Collectively, their HS-AFM data reveal a potential and unexpected behavior of SaCas9 during the search for DNA targets.

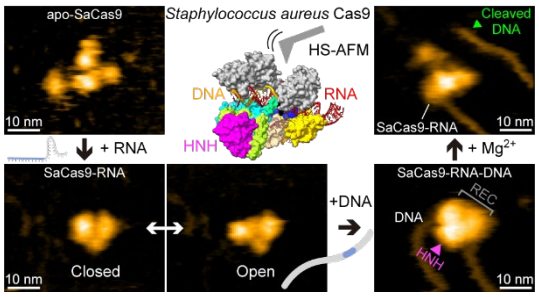

Fig. 23: HS-AFM images of SaCas9 in action on mica surface.

2. Generating Fused Disciplines

* Describe the content of measures taken by the center to advance research by fusing disciplines. For example, measures that facilitate doing joint research by researchers in differing fields. If any, describe the interdisciplinary research/fused discipline that have resulted from your efforts to generate fused disciplines. You may refer to the research results described concretely in "1. Advancing Research of the Highest Global Level."

We have taken the following measures to create an interdisciplinary research domain consisting of four fields, i.e. nanometrology, life science, supramolecular chemistry, and mathematical computational science.

Top-down approach

From the second half of FY2020, we have continued to carry out interdisciplinary research that addresses the three priority research themes set out by the NanoLSI Future Planning Board. The three priority research themes are as follows:

- Intracellular imaging and cell monitoring after treatments for observation (coordinators, PIs Fukuma and Wong);
- Local stimulation/manipulation and chemical mapping (inside cells and cell surface) (coordinators, PIs Hirao and Maeda);
- Cell surface imaging and cell-cell communication (coordinators, PIs Oshima and Hanayama).

For each priority research theme, research expenses of 4 million yen were provided from the on-campus COE research funds from the University Headquarters, and personnel expenses of 6 million yen were provided from the WPI subsidy.

Bottom-up approach

In order to support interdisciplinary research by teams consisting of young researchers, a bottom-up interdisciplinary research promotion grant was set up from the WPI subsidy, and a total of 19.7 million yen was provided for 13 research projects via application and selection. Of the 13 research projects supported, one is a research project led by a graduate student in the Doctoral Level Section of Integrated Course, Division of Nano Life Science. For each research project, the PI corresponding to the research project acts as a supervisor and at the beginning of the fiscal year following the support, a research report meeting attended by all PIs and Professors is held to give advice on future research development.

T-meetings

In order to promote interdisciplinary research, T-meetings were held 38 times in FY2022 by combining two research teams out of those (23 teams in total) individually led by 16 PIs, one associate PI and 6 Jr. PIs from different disciplines to allow them to introduce their research. Twelve of these T-meetings were conducted in combination with a research team led by an overseas PI and a team by a resident PI or a Jr. PI in NanoLSI.

3. Realizing an International Research Environment

* Describe what's been accomplished in the efforts to raise the center's recognition as a genuine globally visible research institute, along with innovative efforts proactively being taken in accordance with the development stage of the center, including the following points, for example:
- Efforts being developed based on the analysis of number and state of world-leading, frontline researchers (in Appendix 2); exchanges with overseas entities (in Appendix 4); number and state of visiting researchers (in Appendix 5)
- Proactive efforts to raise the level of the center's international recognition
- Efforts to make the center into one that attracts excellent young researchers from around the world (such as efforts fostering young researchers and contributing to advancing their career paths)

Total number of papers by PIs, top 1% papers, top 10% papers, internationally co-authored papers in 2017-2022

During the six years from 2017, when NanoLSI was established, to 2022, the total number of papers authored by PIs was 697, of which 317 (45.5%) were internationally co-authored. Of the total 697 papers, twenty-eight were in the top 1% (3.8%) and 219 in the top 10% (29.5%) in terms of citations. The number of top 1% papers based on field-weighted Citation Impact corrections was 7 (0.9%), and the number of top 10% papers was 99 (13.3%).

In 2022, the total number of papers published by 16 PIs was 106, of which 45 (42.5%) were internationally co-authored. Of the total 106 papers, two were in the top 1% (1.9%), and 26 were in the top 10% (24.5%). The number of top 1% papers based on field-weighted Citation Impact corrections was 0 (0%), and the number of top 10% papers, 9 (8.5%).

Number of co-authored papers with overseas PIs in 2017-2022

The total number of papers co-authored by one of the four overseas PIs with resident researchers in NanoLSI was 24 in the six years from 2017 to 2022. Of these, 9 papers were published with Prof.

Yuri Korchev (nanometrology) of Imperial College London, UK, an overseas satellite, and 4 with Prof. Mark MacLachlan (supramolecular chemistry), the University of British Columbia, Canada, also an overseas satellite. In addition, a total of 12 papers were published with Prof. Adam Foster (computational science) of Aalto University, Finland, and one with Prof. Alexander Mikhailov (former overseas PI, computational science) of the Fritz Haber Institute of the Max Planck Society, Germany, or with his successor, Prof. Carsten Beta (computational science and biophysics), the University of Potsdam, Germany. Of the total of 24 papers, two were published with both Prof. MacLachlan and Prof. Foster.

Seven co-authored papers were published in 2022 alone: 3 with Prof. Korchev, one with Prof. MacLachlan, one with Prof. Foster, and 2 were published with both Prof. MacLachlan and Prof. Foster.

Outreach programs for external researchers in FY2017-FY2022

The Bio-SPM Summer School and Bio-SPM Collaborative Research Program aim to invite external researchers to disseminate the scanning probe microscope (Bio-SPM) technology of NanoLSI, leading to joint research. In FY2022, when the Bio-SPM Summer School was held in August, it was difficult for overseas researchers to come to Japan due to COVID-19, so only one overseas researcher from one country participated. On the other hand, 17 Japanese researchers participated. In the Bio-SPM Collaborative Research Program, a total of 5 joint research projects with overseas researchers from 5 countries were conducted in FY2022. As a cumulative result from FY2017 to FY2022, a total of 30 overseas researchers from 16 countries participated in the Bio-SPM Summer School. In addition, a total of 65 Japanese researchers participated. It should be noted that in FY2020 and FY2021, overseas researchers were not invited due to COVID-19. In the Bio-SPM Collaborative Research Program, a total of 22 joint research projects with overseas researchers from 14 countries were conducted from FY2017 to FY2022.

The NanoLSI Visiting Fellows Program aims to invite PI-level life science researchers and their subordinate researchers from overseas to conduct researcher exchanges and joint research and to establish an organizational cooperative relationship between NanoLSI and the relevant research institutions. In this Visiting Fellows Program, one research group led by Dr. Borja Ibarra Urruela, IMDEA, Spain was invited in FY2022. In addition, the invitation of one research group has been carried forward to FY2023. A total of 3 research groups were invited from FY2017 to FY2022.

Support for young overseas researchers to acquire research funds in FY2017 to FY2022

With individual support from the full-time URA of NanoLSI, a total of 18 young overseas researchers have acquired a total of 25 KAKENHI grants from FY2017 to FY2022. In FY2022 alone, young overseas researchers belonging to NanoLSI submitted 9 new applications for KAKENHI, of which 3 were approved.

Mobility and career path for young researchers

As of the end of FY2022, the number of postdoctoral researchers (including fixed-term assistant professors) was 28 out of 82 researchers in total in NanoLSI, and 21 out of 28 were overseas researchers. Eight postdoctoral researchers (including fixed-term assistant professors) left NanoLSI during FY2022. Of these, one has acquired a tenured associate professorship, two have tenure-track assistant professorships, and one a research fellow position. The remaining two have been applying for researcher positions.

Results of overseas researcher visits in FY2022

Society has been recovering from the impact of COVID-19, and the ban on visa-free travel to Japan was lifted from the second half of FY2022. In conjunction with this, visits of overseas researchers to NanoLSI have also been recovering. In FY2022, 39 researchers from 17 countries visited NanoLSI for a total of 1011 person-days.

4. Making Organizational Reforms

* Describe the system reforms made to the center's research operation and administrative organization, along with their background and results.
* If innovated system reforms generated by the center have had a ripple effect on other departments of the host institutions or on other research institutions, clearly describe in what ways.
* Describe the center's operation and the host institution's commitment to the system reforms.

Continued implementation of successful reforms of NanoLSI

NanoLSI established successful cases of system reform in the first half of the WPI subsidy period. These are; research professorships for concentrating on research, the rigorous evaluation-based salary system, the tenure-track junior PI program, integrated management of NanoLSI and the Graduate School "Division of Nano Life Science," English-based administration, planning and setting up various research meetings by researchers and administrative staff working together to promote

interdisciplinary research, outreach programs to promote joint research with external researchers, and the implementation of the PDCA cycle based on external evaluation (WPI Program Committee evaluation). These successful reforms will be maintained and continued for 5 years in the latter half of the subsidy period.

Expected ripple effect on the host institution

In FY2023, Kanazawa University will begin construction of a new research facility, the "Facility for Future Co-Creation" (provisional name), which is scheduled to be completed by the end of the fiscal year. The facility will be based on the fundamental strategy of NanoLSI, i.e. to further develop the excellence of specific research areas where it has strengths, to reinforce joint research to promote interdisciplinary research and to gather together researchers under one roof, and also adding industry-academia collaboration and social implementation. Thus, we aim to create new social value by strategically and in an integrated manner reinforcing the process of social implementation of various discoveries resulting from Kanazawa University's distinctive research strengths. At the same time, the basic concept of the facility is to function as a place for interaction where researchers/staff and organizations related to the facility gather and where researchers can fully demonstrate their individuality and capabilities. It is planned to make full use of NanoLSI's experience and success stories will be used in the development and execution of mechanisms and programs to promote exchanges in different fields.

5. Efforts to Secure the Center's Future Development over the Mid- to Long-term

* Address the following items, which are essential to mid- to long-term center development:
- Future prospects with regard to the research plan, research organization and PI composition; prospects for fostering and securing of next-generation researchers
- Prospects for securing resources such as permanent positions and revenues; plan and/or implementation for defining the center's role and/or positioning the center within the host institution's institutional structure
- Measures to sustain the center as a world premier international research center after program funding ends
- Host institution's organizational reforms carried out for the center's autonomous administration simultaneously with the creation of the center.

Research plan, research organization and PI composition

Regarding the NanoLSI research and development plan, 6 nanotechnology and 7 life sciences roadmaps have been updated, clarifying the challenges to be addressed. Concerning the research organization, the structure with 16 PIs is maintained, and by positioning Prof. Ando (age 72), who is no longer a PI, as a Distinguished Professor of Kanazawa University, we institutionalized a university-wide mechanism to allow distinguished professors, including Prof. Ando, to continue their research until the age of 75. In addition, the Center Director, the Administrative Director and four PIs who are the core members of NanoLSI operations hold an intensive discussion at the Future Planning Board positioned as the steering committee of NanoLSI, which maintains the balance between top-down and bottom-up operations.

Fostering and securing of next-generation researchers

See Appendix 3-1 FY2022 Records of Center Activities "Special mention".

Career path after graduation

As of April, 2023, the first two students (both Japanese) have graduated from the Doctoral Level Section of Integrated Course, Division of Nano Life Science. They obtained positions as researchers at a private company (Sumitomo Chemical Co., Ltd., Kureha Co., Ltd.). It is expected that three more students will graduate as of October, 2023. All three are foreign, wishing to get researcher positions at an academic institution such as a university. A follow-up survey of their career paths will be conducted.

Positioning NanoLSI within the host institution

NanoLSI is positioned as an independent research institute in the statutes of Kanazawa University. In addition, in the university statutes, it is clearly stated that "special measures can be applied to the operation of the Nano Life Science Institute in order to promote the establishment of an independent research entity," and it is assured that NanoLSI is maintained and continued as a world-class research entity.

The host institution's commitment to NanoLSI after the WPI grant ends

In the report at the FY2022 WPI Program Committee Meeting, President Wada, Kanazawa University, announced that even after the end of the WPI subsidy period, almost the same commitment as now will continue regarding provision of budgets, preferential treatment on personnel affairs, and infrastructure maintenance and development.

Amount of external fund acquisition in FY2022

The total amount of external funds acquired in FY2022 by 82 NanoLSI researchers was 1,356

million Yen (1,208 million Yen in the previous fiscal year) (see Appendix 3-1 5. “Securing external research funding” for details).

6. Others

* Describe what was accomplished in the center’s outreach activities last year and how the activities have contributed to enhancing the center’s “globally visibility.” In Appendix 6, describe concretely the contents of these outreach activities. In Appendix 7, describe media reports or coverage, if any, of the activities.

* In addition to the above 1-5 viewpoints, if there is anything else that deserves mention regarding the center project’s progress, note it.

Press release of research outcomes

In FY2022, 21 press releases concerning research outcomes were issued, of which 17 were also issued in English. Among the research outcomes publicized, an article by PI Matsumoto, “Designing receptor agonists with enhanced pharmacokinetics by grafting macrocyclic peptides into fragment crystallizable regions,” was published in a very high-ranking journal, Nature Biomedical Engineering (IF: 29.234)/vol. 7/164-176 (2023). In addition, an article by Jr. PI Arai, “Modulation of Local Cellar Activities using a Photothermal Dye-Based Subcellular-Sized Heat Spot,” was published in ACS Nano (IF: 18.027)/2022.16.6/9004-9018.

Media coverage

NHK Kanazawa and NHK BS1 broadcast the program “Open the future! Ishikawa's Forefront of Science,” featuring the high-speed atomic force microscope developed by Prof. Ando. Research of NanoLSI was introduced and featured in the April 2023 enlarged issue "WPI Special Vol. 3" of the monthly magazine, Junior Aera with support of JSPS/WPI Program Center. In 2022, a new podcast was launched, introducing the personalities and research content of 15 NanoLSI researchers.

Visitors to NanoLSI

In FY2022, a total of 8 groups from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) visited NanoLSI, including a visit by Hideyuki Tanaka, Senior Vice Minister. In addition, a total of 12 groups from other ministries and universities visited NanoLSI and meaningful discussions were held on the roles and know-how of administrative departments in the formation of research entities.

Approach to high school students

Concerning interactions of the super science high schools designated by MEXT of Ishikawa Prefecture, students from Kanazawa Izumigaoka High School gave research presentations in English and young NanoLSI researchers commented and advised on these research presentations. In addition, NanoLSI and Cancer Research Institute (CRI), Kanazawa University, jointly planned and implemented an outreach program for high school students. This “Cancer Research Early Exposure Program” is a program in which researchers from NanoLSI and CRI serve as instructors and are aimed at excellent high school students who aspire to become cancer researchers and allowed them to experience practical experiments. In 2022, a total of 39 high school students participated in the elective program of 11 events during 4 days. In launching this program, CRI and NanoLSI jointly carried out crowdfunding and received donations of 3.14 million yen from 156 people.

7. Center’s Response to Results of Last Year’s Follow-up

* Transcribe the item from the “Actions required and recommendations” section in the site visit report and the Follow-up report, then note how the center has responded to them.

* If you have already provided this information, indicate where in the report.

Comment *"Every effort that NanoLSI is currently making appears to be in good shape and is strongly encouraged to continue. It is very good to hear the strong commitment of support from Prof. Wada, the new president of Kanazawa University. Center Director Fukuma has slightly changed gears to a more top-down governance so as to support mission-oriented research subjects, which will be important to achieve the Center’s final goals. At the same time, bottom-up research activities emerging from young researchers will also be extremely useful in producing unexpected cutting-edges in "Nanoprobe Life Science." The leadership of the Director in balancing the top-down and bottom-up trajectories will be very much appreciated. Strategic approaches to involve more early-stage students and female researchers should be continued. In the future, it will also be good to see industry collaborations and perhaps an expansion to non-biological sciences."*

Thank you very much for the favorable and encouraging comments. We agree that balancing the top-down and bottom-up approaches is extremely important. Thus, we will continue to keep

monitoring and adjusting this. For example, we started the top-down type transdisciplinary research promotion grant (TDRP-G) in FY2021 in addition to the bottom-up type. This top-down grant has successfully promoted biological applications of the newly developed live-cell SPM techniques. Meanwhile, we removed the selection rule to prioritize the proposals in line with the WPI missions for the bottom-up grant in FY2022. This change has promoted the exploration of a wide range of transdisciplinary research collaborations. In this way, we promote mission-oriented projects through the top-down approach while pioneering new possibilities through the bottom-up approach.

We will also continue to enhance the involvement of undergraduate students and female researchers. As for students, the PIs have lecture courses in undergraduate schools of various disciplines, including physics, engineering, chemistry, biology, and medical and pharmaceutical sciences. Thus, many undergraduate students in these schools join NanoLSI research groups every year. In addition, we recently set up a new lecture course on nano life science by Jr. PIs for undergraduate students in the biological science and technology school. In this way, we aim to increase the number of students interested in this field and make it easier for them to join our institute. As for female researchers, we have successfully increased the female proportion from 10% to 20% in the past three years using various strategies. For example, we started to encourage hiring not only foreign but also Japanese female researchers. As far as we can maintain the foreign researcher proportion over 30%, we will continue this policy to further increase the proportion of female scientists.

SPM is a fundamental technology applicable to various research areas. Thus, we have been exploring its applications not only to basic life science but also to industrial collaborations and non-biological sciences. Examples of recent industrial collaborations include studies on metal corrosion by an in-liquid potential measurement technique (*JPCC* **2023**) and polymer brush and surfactants by 3D-AFM (*ACS ANM* **2021**, *ACS AMI* **2022**). Meanwhile, in this WPI project, we have developed various live-cell bio-SPM techniques and pioneered their applications in molecular cell biology and medical sciences, which should lead to future collaborations with private companies in the life science area.

So far, we have established an excellent environment for transdisciplinary research among nanometrology, supramolecular chemistry, life science and computational science. While this WPI project aims to combine all four disciplines to perform "nanoprobe life science" research, this environment has also motivated us to explore non-biological transdisciplinary research. For example, SPM, chemistry and computational science groups investigated nanoscale structures and functions of biomaterials (*Small Methods* **2022**, *Sci. Adv.* **2022**) and helical polymers (*JACS* **2021**, *Chem. Commun.* **2021**). Taking advantage of this environment and the bottom-up TDRP-G system, we will continue to explore a wide range of research subjects.