February 2022

Research Center Project

Center name: Nano Life Science Institute (NanoLSI)（Within 15 words）

Host institution: Kanazawa University

Head of host institution: Koetsu Yamazaki, President of Kanazawa University（Name, Affiliation）

Center director: Takeshi Fukuma, Professor of Kanazawa University（Name, Affiliation）

Administrative director: Yoshihiro Fukumori, Professor of Kanazawa University（Name, Affiliation）

（Name, Affiliation）

1) Overall Image of Your Center

* Concisely describe your institute's identity and provide its mission statement as a WPI center.

1. Mission statement

We combine the world-class bio-scanning probe microscopy (SPM) and supramolecular chemistry techniques to develop "nano-endoscopic techniques" that allow us to directly image, analyze, and manipulate the nanodynamics of proteins and nucleic acids on the cell surface or inside the cell. Furthermore, we use these techniques and multi-scale simulation techniques in a complementary fashion to elucidate various molecular and cellular dynamics by comparing normal and cancer cells. Based on the developed techniques and knowledge gained through this process, we establish a new academic field, "nano-probe life science", which aims to fundamentally understand and control various life phenomena, including development, disease and aging.

2. Center identity

The most salient feature of NanoLSI is our world-class bio-SPM techniques for nanoscale live imaging of molecular and cellular dynamics, which offer distinctive advantages over other bio-imaging techniques. For example, electron microscopy allows one to image the static ultrastructure of proteins in vacuum, but not to directly image protein dynamics in solution. Live imaging by fluorescence microscopy visualizes the positions of fluorescence-labeled molecules, but does not allow direct imaging of dynamic changes in the protein structure or the positions of unlabeled molecules. To overcome these limitations, Ando developed the high-speed atomic force microscope (AFM) and made it possible to directly image the dynamic structural changes of unlabeled protein molecules in solution (PNAS 2001, 785 citations). In addition, Fukuma developed the liquid-environment frequency modulation AFM (RSI 2005, 313 citations) and achieved true atomic-resolution imaging even in solution. Based on this technique, he also developed three-dimensional AFM (3D-AFM) (PRL 2010, 302 citations) and made it possible to visualize the 3D distribution of water and molecular chains at sub-nanometer resolution. Based on these achievements, Ando, Fukuma and their group members have led progress in the bio-SPM research field. Therefore, we are confident that we have gathered together the world's best scientists and launched a center of excellence aiming at creating novel bio-SPM techniques for visualizing nanodynamics of molecules and cells in solution.

To accomplish our mission, NanoLSI has attracted many internationally top-class scientists with a wealth of experience in life science, supramolecular chemistry, and mathematical/computational science. The Cancer Research Institute of Kanazawa University, the only joint usage/research center in Japan solely focusing on cancer research, has amassed superb achievements in the study of cancer stem cells, tumor microenvironment, and molecularly targeted therapy, including the identification of critical molecules involved in leukemia stem cell regulation (Hirao *et al.*, Nature 2010, 454 citations). Kanazawa University has also garnered global attention for its advances in supramolecular chemistry, such as the development of novel pillar[n]arenes (Ogoshi *et al.*, JACS 2008, 1325 citations). Additionally, we have collaborations with outstanding scientists at prestigious universities, including the Adam Foster's group at Aalto University, one of the few research groups in the world with a strong track record in AFM simulation in liquids, and Carsten Beta's group at the University of Potsdam that has made great strides in the simulation of complex systems especially on cell motility. The contributions of these scientists will help us to build an in-depth understanding of life phenomena based on the results of nanoscale experiments.

Our greatest, defining strengths are our many accomplished scientists and research achievements across all core disciplines necessary for executing our mission—nanometrology, life science,

supramolecular chemistry, and mathematical/computational science. Another advantage that sets us apart is that our principal investigators (PIs) include relatively young researchers, meaning that we can continue to lead the new science of "nano-probe life science" also in the future.

2) Research Activities

2) -1 Research field

* Write in the target research field(s)
* Describe the importance of the target research field(s), including the domestic and international R&D trends in the field(s) and scientific and/or social significance.
* Describe the value of carrying out research in the field(s) as a WPI center (e.g., Japan's advantages, global impact on science and/or society, future prospects)
* If there are other centers either in Japan or overseas advancing research in fields similar to the center's field(s), please list them. (up to 5 organizations)

1. Name of the target research field: Nano-probe Life Science

We establish a novel interdisciplinary research field "nano-probe life science", where we develop and utilize nano-probe techniques for directly imaging, analyzing and manipulating dynamic behaviors of biomolecules and cells at nanoscale resolution in physiological environments. This new approach will promote the fundamental understanding of mechanisms underlying diverse life phenomena such as diseases and aging.

2. Importance of the target research field

[Domestic/global trends and key challenges in this field]

To achieve our mission as outlined above, we will seek to address the following challenges in the core disciplines of nanometrology, supramolecular chemistry, and life science.

(1) Challenges in nanometrology: Nanoscale imaging, analysis, and manipulation of molecular dynamics at the periphery and interior of cells

Advances in super-resolution fluorescence microscopy have allowed nanoscale imaging dynamics of fluorescence-labeled molecules occurring in the intra-/extracellular spaces (Betzig *et al.*, Science, 346 (2014) 439); however, this technique does not support direct imaging of the target molecules' structural changes or the position of many unlabeled molecules. Furthermore, fluorescent tags may interfere with biomolecular functions. The microscopic imaging of living cells with advanced atmospheric scanning electron microscopy and polymer membranes have begun to emerge (Takaku *et al.*, PNAS, 110 (2013) 7633), but this approach offers optimal resolution only in the tens of nanometers, and thus, is insufficient to reveal detailed molecular dynamics. Moreover, the electron beams can damage the molecules of interest. The high-speed AFM developed by Ando at NanoLSI has been used to visualize the nanodynamics of aquaporins on live bacteria having a relatively hard surface (Yamashita *et al.*, JMB 422 (2012) 300), but it has not yet been successfully used to measure molecular dynamics on the much softer surfaces of eukaryotic cells. Although scanning ion-conductance microscopy (SICM) has been used to image endocytosis and to locate ion channels and receptors on eukaryotic cell surfaces (Korchev *et al.*, Science, 327 (2010) 1653), the optimal resolution of this technique is lower than that of AFM and insufficient for imaging the nanodynamics of receptors and transducers. SICM has been applied in various techniques, including one that uses a nanopipette to inject substances into specific intracellular nano-regions, as well as sample collection and analysis (Mirkin *et al.*, PNAS 104 (2007) 11895). However, SICM has not yet been successfully used to inject molecular machines with high controllability for manipulating protein structures or functions. In the first half of the grant period, we were able to map the distributions of physical properties, such as pH and oxygen concentration, in liquids using molecular sensors (Zhang *et al.* Nat. Commun. 10 (2019) 5610). However, this technique has not been applied to the mapping of metabolites. As these advances and limitations illustrate, progress is being made in the visualization of cell-surface nanodynamics, but a breakthrough technology is needed for direct imaging of intracellular molecular dynamics. In addition, the development of techniques requiring the fusion of nanometrology with supramolecular chemistry, whether for intracellular or cell-surface applications, is a challenge that has yet to be solved.

(2) Challenges in supramolecular chemistry: Controlling the position and orientation of molecular sensors/machines to operate them on nanostructures of interest

Supramolecular chemistry has developed as a field of chemistry for creating new molecular functions through the design of selective intramolecular interactions. Recent research attention has focused on

molecular machines, which were the subject of the 2016 Nobel Prize in Chemistry. These nanoscale devices can rotate, reciprocally move, or perform other actions in response to external stimuli. Examples include a molecular elevator that moves up and down in response to pH (Stoddart *et al.*, Science 303 (2004) 1845) and molecular tweezers that open and close in response to light (Aida *et al.*, Nature 440 (2006) 512). However, these highly functional molecular sensors and machines are yet to be translated into practical applications. One barrier is the lack of technology for effectively controlling the position and orientation of these functional molecules so that they can operate on nanostructures of interest. Here, SPM techniques could help by offering abilities such as the conveyance of molecules to specific nanoscale regions using a nanopipette, or sub-nanometer-scale positional control of a molecule affixed to a probe tip. The combination of SPM and supramolecular chemistry promises to pave the way for effectively controlling and operating functional supramolecules upon intracellular and cell-surface nanostructures of interest.

(3) Challenges in life science: Achieving nanoscale understanding of cell functions and their cancer-specific abnormalities

Recent progress in whole-genome sequencing of cancer cells has led to identification of driver oncogenes in many types of cancer. Discovery of drugs that selectively inhibit function of driver gene products has established the practice and concept of a molecularly targeted therapy of cancer. Despite the progress of recent medical and pharmaceutical sciences, mechanisms of malignant progression of cancer, such as drug resistance and metastasis are still insufficiently understood. This is partly because there are no techniques for real-time imaging of nano-dynamic features of non-labeled cells and molecules involved in cancer progression. For example, it is almost impossible to understand the process leading up to drug resistance without directly imaging the changes that take place inside cells and their environment (e.g., pH, oxygen concentration, osmotic pressure, and amino acid and sugar distributions). The significance of interactions between cancer cells and the microenvironment mediated by growth factors, inflammatory cytokines, and exosomes has been increasingly recognized, but without direct imaging of the molecular and cellular dynamics, it is extremely difficult to elucidate mechanisms of adhesion, migration, and invasion peculiar to cancer cells, through understanding the changes occurring inside and on the surface of cancer cells. Thus, techniques to directly image and analyze molecular cell dynamics at nanoscale resolution are a prerequisite to elucidating the unresolved mechanisms of cell functions and their cancer-specific abnormalities.

[Scientific/social significance]

We seek to tackle the aforementioned challenges in nanometrology, supramolecular chemistry, and life science through research that expands and combines the knowledge and techniques of each of these disciplines. Instead of just evolving these fields, we will endeavor to pioneer a whole new discipline of "nano-probe life science" that will have enormous implications for the future of science. The knowledge and techniques emerging from our research will pave the way to a fundamental understanding of various biological phenomena that will allow their precise control. Such advances hold immense social significance as they could aid in conquering cancer and other intractable diseases, extending lifespan, and achieving other important health improvements.

3. Reasons why our research is a good match for the WPI initiative

[Japan's competitive edge]

Japan's competitive edge in this research area lies in the fact that it hosts the world's foremost bio-SPM techniques and the inspired minds that created them. As noted earlier, bio-SPM is the surest path to realizing nano-endoscopic techniques. The most fundamental and critical performance factors of bio-SPM are resolution and speed, and two Japanese scientists from our institute, Fukuma and Ando, have led the world in the ongoing improvement of these factors. Having designed and built their systems from scratch, both scientists have expertise in developing SPM techniques of superior resolution and speed. Having a team of scientists of such caliber at one institute is an incredibly strong advantage.

[Appeal as global scientific/social challenges]

(1) Appeal as a scientific challenge

The origins of material properties and various phenomena can be explained in terms of nanoscale structures formed from atomic and molecular assemblies and their dynamic behaviors. Hence, understanding and controlling nanoscale structures would empower us to manipulate at will all sorts of physical properties and phenomena. This represents the ultimate aim of science—to transcend the boundaries of the established disciplines of physics, chemistry, biology, pharmacy, and medicine—and forms the core concept of nanotechnology. Ever since the pursuit of nanotechnological research was

declared a key pillar of the US strategy by President Clinton in 2000, enormous research as well as financial investments have been made in nanotechnological R&D worldwide. These efforts have blazed new paths toward humankind's understanding and control of natural phenomena at the nanoscale. In the early 2000s, the main focus was on materials and devices, but in the second half of that decade, bioscience became a greater target of exploration. Subsequently, nanoscience encompassed life sciences such as the medical and pharmaceutical sciences, evolving into one of the greatest attempts in humankind's unending quest to expand the borders of science and technology.

(2) Appeal as a social challenge

Initially, we will perform detailed comparisons of nanodynamics between normal and cancer cells using our innovative nano-probe techniques. This will lead to fundamental understanding of the mechanisms of basic cellular functions and their cancer-specific abnormalities. Achieving this goal will enable precise control of various phenomena involved in cancer, and thus make it possible to overcome this intractable disease. Clearly, our research target represents a very important social challenge globally as well as specifically for Japan, where cancer kills roughly one in every three people.

[Future prospects for this field]

We will endeavor to establish the foundation for nano-probe life science by building techniques for and accruing expertise in the imaging, analysis, and manipulation of life phenomena at the nanoscale. Further in future, those techniques and expertise promise to deepen our understanding and control not of only cancer, but also other life phenomena. Our research field is thus anticipated to pave the way for immense contributions to human health, including lifespan extension and conquering of intractable illnesses such as cancer, heart disease, neurodegenerative disease, and liver disease.

4. Japanese and overseas institutes focusing on similar fields

1) Center for Biosystems Dynamics Research (BDR), RIKEN, investigates events unfolding in the body during all stages of the life cycle to gain a comprehensive understanding of the biological functions regulating living system, and apply these findings to develop and advance medicine and diagnostics. Although their research goals are similar to ours, unlike us they mainly use optical microscopy to achieve them.
2) Institute of Transformative Bio-molecules, Nagoya University (ITbM) develops novel functional molecules to visualize and control living systems. Although they combine chemistry and biology to tackle issues in life sciences as we do, their focus is on animals and plants. In addition, they use optical microscopy for imaging.
3) Janelia Research Campus, Howard Hughes Medical Institute (HHMI) focuses on basic medicine, with novel bio-imaging techniques and neuroscience research at the core. In contrast to us, they use optical microscopy as the main imaging technique.
4) Max Planck Institute for Multidisciplinary Sciences combines basic research in the natural sciences and medicine. In contrast to us, they use NMR and optical microscopy as core imaging techniques.
5) The Center for Nanophase Materials Sciences (CNMS) in the USA researches nanotechnology in general and is globally reputed for SPM research. However, their focus is mainly on the materials and energy fields, not life sciences.

As described above, while there are numerous research institutes aiming to elucidate the principles of living systems, their main imaging tool is optical microscopy. While there are many nanotechnology research centers in the world, none of them is solely focused on the understanding of life phenomena. We, as the first in the world, established a research institute for understanding living systems using advanced nano-probe techniques and to create the novel academic field "nano-probe life science".

2)-2 Research objectives

* Describe in a clear and easy-to-understand manner the research objectives that the project seeks to achieve by the end of its grant period (in 5 years). In describing these objectives, the following points should be articulated in an easily understandable manner: The kind of research area(s) you plan to cultivate by, for example, fusing various fields. In that process, what world-level scientific and/or technological issues are you seeking to solve? What will the expected impact of the scientific advances you aim to achieve be on society in the future?
* Describe concretely your research plan to achieve these objectives and any past achievements related to your proposal.

1. Exit goals (5 years from now)

[Research goals]

Our institute is committed to achieving the following goals by the conclusion of the five-year period:

Develop nano-endoscopic techniques enabling direct imaging, analysis, and manipulation of the dynamic behavior of molecules inside and on cells.
Acquire an accurate understanding of mechanisms of basic cellular functions as well as their cancer-specific abnormalities by using the nano-endoscopic and computer simulation techniques we will develop.
Pioneer the emerging discipline of "nano-probe life science".

[Global scientific/technological challenges and their social impact]

Over the years, various techniques have been developed with the aim of gaining a fundamental understanding of the mechanisms underlying a multitude of life phenomena, including diseases and aging, by directly visualizing molecular and cellular dynamics; however, challenges remain. The resolution of these challenges will allow a fundamental understanding and manipulation/control of the mechanisms of these phenomena. This endeavor has immense significance for society, as it will contribute to human health by conquering intractable diseases and extend life expectancy.

2. Specific plans for research activities and related achievements to date

[Development of technique for measuring nanodynamics on the cell surface and interior]

(Development of nano-imaging techniques)

・**Further improvement of high-speed AFM technologies:** The power of high-speed AFM (HS-AFM) has been demonstrated by an increasing number of imaging studies on biological molecules (Curr. Opin. Chem. Biol. 51 (2019) 105). As notable examples, structural morphologies of membrane proteins (Nature 574 (2019) 132; Nature 575 (2019) 395), a liquid-like condensate for autophagy initiation (Nature 578 (2020) 301) and structural dynamics of intrinsically disordered proteins (Nat. Nanotechnol. 16 (2021) 181) have been directly visualized. However, the vast majority of biological processes have not yet been visualized by HS-AFM. In order to apply HS-AFM technologies to a wider range of biological phenomena, further improvement of HS-AFM technologies are thus necessary. After NanoLSI was established, we improved devices in the HS-AFM (i.e., cantilever (Fig. 1a), optical beam deflection system, amplitude detector (Appl. Phys. Lett. 119 (2021) 181602; Patent application number (PAN): 2021-121704) and Z-scanner (Rev. Sci. Instrum. 93 (2022) 013701; PAN: 2019-120245) to improve the scanning performance of HS-AFM. Notably, the only-trace-imaging mode that eliminates the backward scanning in X-axis was invented (Fig. 1b, Rev. Sci. Instrum. 92 (2021) 033705; PAN: 2020-199938), enhancing the speed performance of HS-AFM ~2.5 times. The combination of developed devices enables us to perform HS-AFM imaging ~10 times faster than before. As we continue to improve the scanning performance of HS-AFM, we plan to apply this faster HS-AFM to capture highly mobile biomolecules that have so far been inaccessible (i.e. actin and microtubule binding proteins, membrane proteins, DNA and RNA binding proteins, and proteins on isolated organelles and living cells). Furthermore, we have been developing the following devices to extend the functions of HS-AFM: (1) An AFM substrate with controlled concave/convex shapes that mimic the curvature of biological membranes and that induce mechanical stress on proteins placed on it (Fig. 1c, Front. Immunol. 11 (2020) 520; Issued patent: JP677310), (2) HS-AFM with nano-manipulator enabling us to manipulate a target object during HS-AFM imaging (PAN: 2019-149584), (3) HS-AFM with patch-clamp technique enabling us to simultaneously record the conformational changes and electrophysiological properties of membrane channel proteins (PAN: 2019-153689). As we continue to refine these technologies, we plan to apply them to membrane binding proteins, cytoskeletal proteins and membrane channel proteins, to extract further insight into the functional mechanism of these molecules.

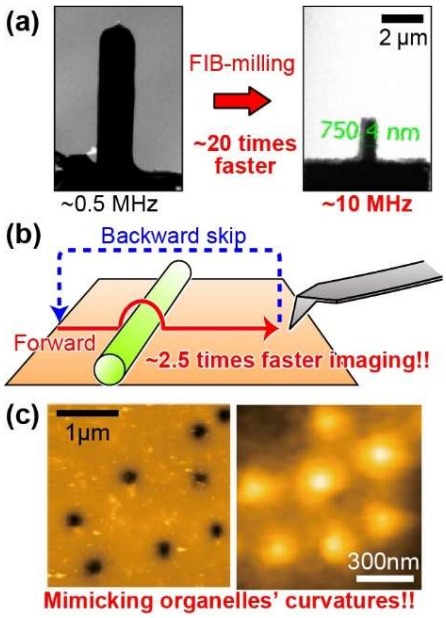

Fig. 1: (a) Ultra-small cantilever milled by FIB. (b) Only-trace-imaging mode. (c) Concave/convex substrate.

・**Measurement of nanodynamics on cell surfaces:** In the cell membrane, various proteins such as receptors and channel proteins are present that allow intra/extracellular signaling and substance transport and are often closely related to cancer development and progression. However, conventional techniques cannot satisfactorily visualize protein dynamics on live-cell surfaces at the nano level. To overcome this limitation, we are developing AFM and SICM techniques for visualizing molecular scale nanodynamics on

a living cell surface.

SICM can image the surface physical properties of specimens, such as topography, surface charge density, and viscoelasticity, in ionic solutions without mechanical probe-sample contact. This unique capability is advantageous for measuring fragile cells, but its maximum imaging rate and spatial resolution are far lower than those desired in biological studies. To solve this problem, we have been continuously improving the spatiotemporal resolution of our SICM system such that it now performs the highest spatiotemporal resolution of all existing SICM systems (Rev. Sci. Instrum. 2019). We established a method for measuring the geometry of an SICM probe tip with a sub-nm resolution (Fig. 2a, Anal. Chem. 2020), allowing us to perform the qualitative mechanical mapping of living cells by SICM. These developments enabled simultaneous visualization of the dynamic change in topography and mechanical properties of living cancer cells (Fig. 2b) and revealed the variation of mechanical properties of cancer cells depending on cancer driver genes (Fig. 2c, Biomaterials 2022). In the next five years, we will further improve the spatiotemporal resolution of SICM to extend the capability of SICM from the sub-cellular to inter-cellular level to investigate and characterize cell-cell interactions and communications. To this end, we have been developing a method to further sharpen the SICM probe tip and designing an ultra-low-noise current amplifier with a wide-bandwidth performance. In addition, we plan to develop a machine-learning-based denoising technique of a transient signal response from the SICM probe to exceed the usual low-noise limit of the current amplifier. By combining these developments, we will investigate structural dynamics of plasma membranes related to various cellular processes associated with changes in physical properties, such as mechanical and electrical properties, at nanometer-scale resolution.

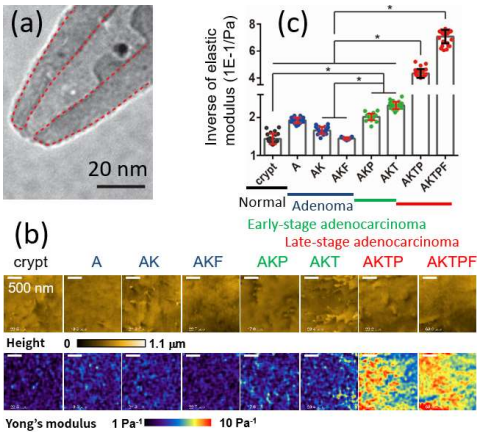

Fig. 2: (a) High-resolution SICM probe. (b) Height and stiffness maps of cancer cells with different cancer driver genes. (c) Averaged stiffness of measured cells.

AFM enables observation of intact samples with sub-nm resolution, but there have been no reports of observations of living animal cell surfaces with a sub-100 nm resolution. The reason for this is that the cell surface is very soft and easily fluctuates during AFM observation. To overcome this problem, we have developed a novel method for cell surface AFM imaging using Micro Porous Silicon Nitride Membrane (MPM), a Si_3N_4 membrane with 100-200 nm thickness and 1-5 μm diameter holes. In this method, we culture cells on the MPM and image the bottom cell surface through the holes. As the membrane around the hole is supported by the MPM, the membrane fluctuation and molecular diffusion are suppressed. As a result, protrusions of less than 10 nm in diameter were successfully imaged on the living colon cancer cell surface. Furthermore, to recognize specific molecules in the AFM images, we superimposed the AFM images onto the images obtained using the stimulated emission depletion (STED) microscope and confirmed that the localization of the E-cadherin molecule observed in the STED images corresponded to the protruding structures on the cell surface observed in the AFM images. In the next five years, we will improve the spatiotemporal resolution by exploring various force detection schemes and enhancing the response speed of various AFM components. We also aim to explore methods to recognize specific molecules by AFM. Possible candidates include labeling with a structure that can be recognized by AFM, and functionalizing a tip with a molecular sensor. We plan to apply the method so developed to visualize clustering behavior of MET receptors on a living cell surface for understanding its correlation with cancer drug resistance.

・**Visualization of intracellular nanodynamics (nano-endoscopic observation):** The behavior of proteins and nucleic acids on the surfaces and in the interiors of organelles such as nuclei and mitochondria plays important roles in signal transduction through vacuoles and in physiological processes such as transport. Direct visualization of these phenomena at the nano level is unachievable by conventional techniques. To explore this uncharted realm, we are developing a nano-endoscopic imaging technique based on the 3D-AFM technique of Fukuma. In hydration-structure measurement by 3D-AFM, the probe is scanned in a 3D space such that the probe penetrates the hydration structure and the vertical force applied to the probe apex is recorded to visualize 3D water distribution. Similarly, if we can penetrate the membrane at the cell surface with an acceptable perturbation to cellular activities using a very thin and long probe, we should be able to scan the probe in a 3D space including the cell interior, for visualizing

the nanodynamics occurring in the scanned region (Fig. 3a).

So far, we established a method to fabricate a long nanoprobe (Fig. 3b, Sci. Rep. 2021) and succeeded in imaging the whole cell structure (Fig. 3c), 3D configurations of the actin fibers (Fig. 3d), and 2D nanodynamics of the inner scaffold of the bottom plasma membrane (Sci. Adv. 2021). Importantly, our fluorometric assay reveals that such imaging does not produce detectable changes in cell viability. Unlike previous AFM techniques using ultrasonic waves or elastic responses, this method allows an AFM probe to directly access the target intra-cellular components so that we can exploit the full range of AFM capabilities such as high-resolution imaging, nanomechanical mapping, and molecular recognition. These features should greatly expand the range of intra-cellular structures and properties observable in a living cell.

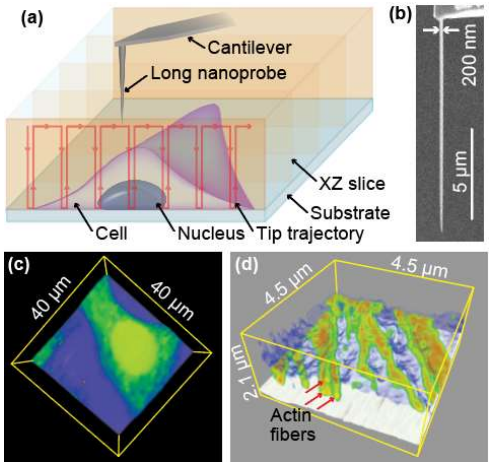

Fig. 3: (a) Principle of Nanoendoscopy. (b) Long nanoprobe. (c) Living HeLa cell. (d) Actin fibers in the live fibroblast cell.

In the next five years, we will continue to optimize the design and fabrication method of long nanoprobes, combining nanoendoscopy with various optical microscopy techniques, and developing analysis techniques for the 2D/3D intra-cellular images. We also aim to improve the imaging speed and spatial resolution to visualize molecular-scale dynamics, and to expand its capability from imaging to mechanical property measurements. Meanwhile, we aim to apply this method for investigating mechanisms of various intra-cellular phenomena, including nucleus stiffness changes induced by cancer progression or virus infection, formation and disassembling of a focal adhesion, and different cell compaction behaviors induced by E-cadherin variation.

(Development of nano-endoscopic analysis and manipulation techniques)

Based on the nano-endoscopic imaging technique, nano-endoscopic analysis and manipulation techniques will be developed.

・**Injection and sampling of substances using a nanopipette:** If substances can be injected into specific near-surface or intracellular nano-regions, nanodynamics induced by the injection can be measured directly using nano-imaging techniques. To date, there are only a limited number of research groups who have successfully injected substances into intra/extracellular nano-regions using nanopipettes and examined the reactions by fluorescence microscopy. The Korchev group is one of these groups. We combine this technique with the nano-endoscopic imaging technique to enable direct visualization of nanodynamics that cannot be imaged by fluorescence microscopy. Moreover, sampling from specific nano-regions, which will allow high-precision analysis of the molecules and ions present, has recently become possible. The Takahashi group has already achieved local sampling of cell cytoplasm for mRNA analysis. The combination of these leading nanopipette and nano-imaging techniques will make nano-endoscopic analysis possible.

We established a chemical injection system using nanopipettes over the last five years. The previous applications of this nanopipette-based chemical delivery or stimulation were limited to the cell surface. In the next 5 years, we will focus on developing organelle (mitochondria, ER, Golgi body) level local stimulation technology to examine organelle-organelle interaction. We also investigate cell migration by continuous release of chemical substances to cells over several hours using AI-based cell recognition and nanopipette position control. Using the local sampling nanopipette technology, we also focus on organelle collection to analyze mRNA within the organelle. We have already established a single organelle collection method with a SICM-fluorescence microscope hybrid system, and we will continue to develop the technology for analyzing the collected organelles. Tools to quantify the sample will also be exploited through combination with a chemical biology approach.

・**Analysis of nano-distribution of physical properties using a molecular sensor:** Intra/extracellular pH and oxygen concentration are closely related not only to cancer development but also the function of drugs and drug delivery systems. Conventional techniques including fluorescence imaging are not necessarily sufficient for analyzing nanoscale distributions of these physical properties. We aim to solve this problem by combining bio-SPM and supramolecular chemistry techniques. We will develop molecular sensors that change their structures in response to pH or oxygen concentration in solution using our expertise in supramolecular chemistry. By attaching the sensor to the end of a nanopipette for SICM, distributions can be detected based on changes in the ion current passing through the pipette. By combining this nanopipette technique with 3D-AFM, the probe apex can be precisely

positioned to a specific nano-region.

Until now, we have integrated a range of novel nanoprobe-biosensors for oxygen gradients, ROS species, ATP, and pH with our advanced SICM and applied them to map intracellular and extracellular chemical species with higher spatial and temporal resolution. For example, we have constructed label-free zwitterionic-like cross-linked glucose oxidase and poly-L-lysine pH-sensitive nanoprobes at the tip of dual-barrel nanopipettes (Fig. 4a-b). These SICM feedback-controlled pH-nanoprobes allowed SICM topography and 3D extracellular pH mapping of living breast cancer MCF7 cells with pH sensitivity better than 0.01 units, fast response times down to ~2 ms, and higher spatial resolution of ~50 nm (Fig. 4c). In another example, carbon-filled nanoprobes with a radius as small as 2 nm were functionalized with Pt as ROS-nanoprobe biosensors, which were applied to detect intracellular ROS of melanoma and melanocytes with high sensitivity (Fig. 4d-g) (Nat. Commun. 2019). We have also developed a new biosensor for quantitative detection of 1-methylnicotinamide (1-MNA), which is produced by the cancer-associated nicotinamide N-methyltransferase (NNMT), even in crude biological samples (Commun. Chem. 2020). The sensitivity of the biosensor was improved through polymer conjugation. In addition, we have started to develop new sensor molecules that can bind to lactate, oligosaccharides, and ions with different charges (Fe^{2+} and Fe^{3+} ions) or measure the temperature to probe the environment of cancer cells using nanoprobe technology.

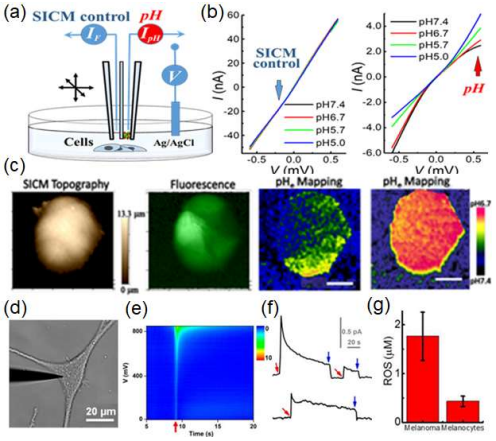

Fig. 4: (a) Schematic demonstrating simultaneously SICM imaging and extracellular pH sensing. (b) Nanoprobe shows pH sensitivity in pH-barrel but not in SICM-barrel. (c) SICM & fluorescence imaging and pHe mapping of $CD44^{GFP}$ MCF7 cells. (d) Optical image of ROS-nanoprobe intracellular in place in a cell. (e) Voltammograms of penetrating ROS-nanoprobe in melanoma. (f) Current traces of ROS-nanoprobe inside (red arrows) and outside (blue arrows) a melanoma. (g) ROS levels measured with ROS-nanoprobe inside melanoma and melanocytes.

In the next five years, we will continue to improve the sensitivity and selectivity of the developed sensors by optimizing the chemical structures and we will also newly design selective sensors for chiral molecules such as L- and D-amino acids, which will be introduced into the nanoprobes. We also aim to further improve the sensitivity and selectivity by integrating many sensors into a polymer film with a controlled mesh size formed at the nanopipette apex. Furthermore, we will develop tailor-made enzyme-modified nanopipettes and nanoelectrodes functionalized with chemical sensors based on a supramolecular chemistry approach. For example, we will use a layer-by-layer technique to modify a nanopipette with the charged enzymes. In addition, we will use metal-organic frameworks (MOF) to form a molecular-level membrane at the tip of the SICM nanopipette to trap enzymes and improve the sensitivity and temporal resolution.

・**Nano-manipulation using molecular machines:** While the development of SPM imaging techniques is progressing, tools to provide a stimulus to the target specimens at a nanoscale locality, the so-called "nano-manipulation method", are also indispensable and in high demand. For this purpose, we employ a system using molecular machines that can be manipulated by various external stimuli, such as changes in temperature, electric potential or light irradiation. More specifically, we use two approaches to allow these molecular machines act on targeted places inside or outside cells.

The first is to utilize molecular machines that can interact with the target proteins, organelles, and cells directly and physically. For instance, the supramolecular chemistry group have synthesized light-operated molecular machines in which their structures are altered by light illumination. These machines provide the physical perturbation against cellular components having sophisticated structure such as cell membranes, DNA, and organelles. Coupling with the SPM technology in NanoLSI, the spatiotemporal dynamics of these components in response to perturbation is captured in real time, enabling studies on unexplored mechanisms in biology. Supramolecular chemistry researchers have already synthesized the photo-triggered lipid (azo-tab), which will be examined in cell membranes and further extended to the other targets using HS-AFM. In addition, we aim to attach a molecular machine to the end of a SPM probe (SICM or AFM). In this case, it should be emphasized that the orientation of the molecular machine in the targeted intra/extracellular site can be controlled, allowing it to act on a specific site with nano level precision. For example, an exosome plays a critical role as a machine for regulating cellular functions. Hanayama's group is working on how antigen presentation by exosomes differs from that of other immune cells. By immobilizing exosomes derived from immune cells or engineered exosomes expressing immune molecules on nanopipettes to allow them to act locally on cells and investigate their responses, they are trying to

understand the mechanisms of immune response via exosomes at the nanoscale. Thus, in addition to using machines produced from synthetic chemistry, the "natural" molecular machines that exist in living systems are also employed for this concept.

In a second approach, the molecular machine, especially a capsule type, is used to alter the concentration gradient of bioactive molecules close to the SPM observation area at the nanoscale. The tools for creating nanoscale concentration gradients of small molecules that bind to target proteins and cellular components are accelerating SPM imaging studies to allow the real-time dynamics of biological events to be captured at the nanoscale. To achieve this, supramolecular chemists produce various types of capsules enabling the on-demand release of the internal bioactive molecule by external stimuli. For the next five years, using molecular machines including those that have already been developed (JACS 130 (2008) 5022; Nature Chem. 6 (2014) 429; JACS 139 (2017) 4631), these practical and useful tools will be provided to SPM users who are addressing several biological questions.

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

・**Modeling and simulation of Bio-SPM experiments:** In general, the accuracy and reliability of a newly-developed measurement method should be verified before it is used for practical applications. Such verifications typically involve comparison with results obtained by an existing method and/or measurement of model samples with known properties. However, both of these approaches are often difficult for an atomic- or molecular-scale measurement technique. As an alternative solution, simulation techniques are becoming increasingly important owing to rapid advances in mathematical/computational sciences. However, very few research groups have experience in reproducing SPM measurements at the atomic/molecular level in silico and verifying the principles behind them. At NanoLSI, we are developing methods for modeling, simulation and AI-based analysis to understand the principles of various Bio-SPM experiments.

In the last five years, we have expanded our machine learning infrastructure to predict electrostatic potentials and hydration structures, while adapting multichannel experimental data as input and taking the first steps in autonomous AI instrument operation. We have also extended our molecular modeling tools to the study of complex two-dimensional materials (Nature 2020). In particular, we explored the sensitivity of characteristic electronic structure signatures to the properties of the tip and how the analysis could further be applied to organic systems (Adv. Funct. Mater. 2021). To advance the quantitative interpretation of resolution-limited AFM experiments, we have developed the BioAFMviewer software platform, which integrates simulated AFM scanning and optimized fitting of atomic protein structures to experimental images (PLoS Comput. Biol. 2020). In addition, we have developed a method for simulating the dynamic-mode AFM imaging of surfaces of biopolymer assemblies such as chromosomes (J. Phys. Chem. C 2020).

In the next five years, we will perform simulation of cell membrane penetration with various long nanoprobes for understanding the optimal conditions for non-invasive nanoendoscopy experiments (Hall, Foster). We will also perform simulation of 3D-AFM imaging of simple 3D model structures made of carbon nanotubes and chromosomes for understanding the imaging mechanisms (Sumikama, Fukuma, Foster). Furthermore, we will develop a contact mechanics model to estimate tension applied to an intra-cellular fiber from a force versus distance curve measured by directly indenting it by a nanoprobe (Okuda).

・**Understanding life phenomena:** Regardless of the measurement technique, we obtain information on the structure and properties of targets through probe–target interaction. Therefore, unless we fully understand the nature of the probe and interaction, even a clear atomic-resolution AFM image may not provide a complete understanding of the structure of the target. Simulation and AI-based analysis techniques help in bridging this gap between the measured data and the real-space model. At NanoLSI, we are developing such methods of analysis for understanding nanoscale mechanisms of biological phenomena from Bio-SPM data.

In the last five years, we performed mathematical modelling and simulations to understand pioneering interactive HS-AFM experiments of ATP-less walking of the myosin V molecular motor and provide an explanation in terms of conformational dynamics and energetics. As a result, we clarified that the classical model emphasizing the importance of ATP-induced chemo-mechanical motions, has to be reexamined. We also developed a method of analysis for HS-AFM movies based on statistical mechanics. The novel method was then applied to HS-AFM movies filming repeating associations and dissociations of scorpion toxin to a K^+ channel (Fig. 5) (Sci. Adv. 2019). Combining the results of the analysis with the discrete state Markov model, the binding dynamics of the toxin to the channel were found to occur solely via the induced-fit pathway, and the states of the channel at each moment in the HS-AFM movies were successfully classified as a high- or low-affinity state to the toxin.

In the next five years, we will develop a method for classifying AFM images of different drug-resistant cancer cells using a machine learning approach for single-cell level cancer diagnosis (Foster). We will also develop a method for producing a 3D and 4D model of protein dynamics from experimentally observed HS-AFM images using data assimilation technique (Flechsig). This enables a deep molecular understanding of biological processes beyond what is accessible from Bio-SPM experiments. In addition, we will develop a method to produce a model of X-shaped human chromosomes from their 3D-AFM image for understanding the internal structures of chromosomes (Sumikama). Furthermore, we continue to work on simulations of protein dynamics observed by HS-AFM for understanding their mechanisms. We will apply molecular simulations combined with simulation AFM to active membrane transporters (ABC transporters), 2D protein crystals (Annexin V), and components of basal membranes (laminin networks) (Flechsig). Examples in progress include 1) further zooming in on the dynamics of association between phospholipase A_2 and cell membranes using molecular dynamics simulations (Sumikama), and 2) applying statistical mechanics to the biomolecular dynamics of CaMKII and Na^+ channels (Sumikama).

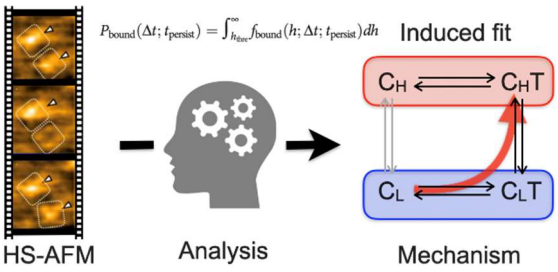

Fig 5: HS-AFM movie and mechanism deduced from analysis of the movie based on statistical mechanics

[Nano-level understanding of the mechanisms of basic cellular functions using expertise in cancer research]

(Nano-level understanding of basic cellular functions using expertise in cancer research and innovative nano-probe techniques)

At NanoLSI, we will image, analyze and manipulate nanodynamics of unlabeled nucleic acids, metabolites, proteins and organelles in normal and cancer cells using nanoendoscopic technologies. This will allow us to understand the mechanisms of basic cellular functions and their cancer-specific abnormalities.

The control of intracellular transport is vital for cell growth and differentiation. Nuclear pore complexes (NPCs) are multi-protein turnstiles that regulate nucleo-cytoplasmic traffic. Wong's group found that NPC protein TPR regulated autophagy induction in a brain tumor (Autophagy 2021). Recently, we further visualized the native NPC and succeeded in the observation of single filaments inside the inner ring of nuclear pore by HS-AFM (Biomaterials 2020) and chromatinization of DNA (J Phys Chem Lett 2021). Moreover, we approached the question as to how various viruses invade cells and hijack the NPC functions to replicate themselves. To answer this, we have started to reveal the conformational dynamics of influenza protein hemagglutinin (HA) (Nano Lett 2020) and SARS -CoV-2 spike proteins (J Extracell Vesicles 2021) via HS-AFM. We are actively collaborating with several groups within the NanoLSI, with the support of various organoids, exosome models and nano-endoscopy and HS-AFM analysis. Thus, we will try to elucidate the nanoscale mechanisms of intracellular transport (from the cell membrane to the nucleus), which will contribute to future development of diagnostic and therapeutic strategies against viral pandemics.

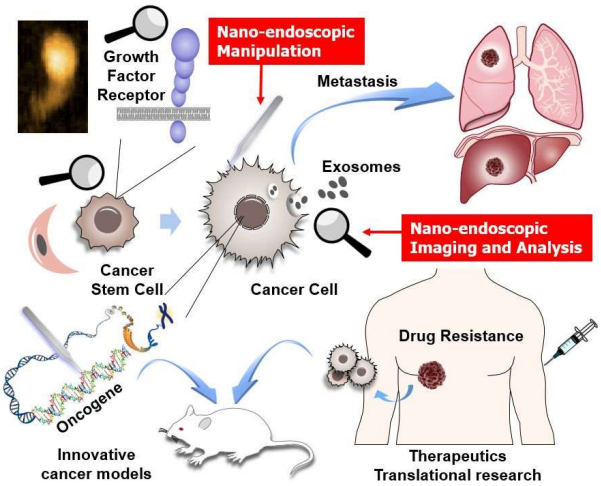

Fig. 6: Comprehensive research on cancer progression using nano-endoscopic techniques.

Exosomes are small extracellular vesicles containing lipids, proteins, RNAs, etc. and participate in cell-cell communications, which contribute to variety of cellular functions and phenomena. Hanayama's group found how cancer cell-derived exosomes promote cancer metastasis, angiogenesis, and immune-evasion, and thereby participate in the formation of a microenvironment that promotes cancer progression (Carcinogenesis 2020; Int J Cancer 2021; Cell Death Dis 2021; Front Oncol 2021). To get more detailed information on the characteristics of cancer cell-derived exosomes, we collaborated with Fukuma's group to perform 3D-AFM force mapping, which unexpectedly revealed a malignancy-dependent increase in exosome stiffness due to changes in the amount of elastic fiber-related proteins (Nanoscale 2021). This finding has led to the proposal of a new hypothesis that malignant cancer cells can become more flexible

and easier to metastasize through the release of these proteins by exosomes. In the next five years, we will analyze exosomes before their secretion from the cancer cells and after uptake in the microenvironment cells. This will be performed locally at the single particle level using nanopipettes to track characteristic molecular changes to elucidate how cancer cell-specific exosomal functions are loaded in the donor cells and released in the recipient cells.

Epitranscriptomic regulation modulates RNA processing, affecting protein expression and function. Aberrant A-to-I RNA editing is relevant with the initiation and progression of cancer. Nakajima's group found that the inhibition of ADAR catalyzing RNA editing can suppress cancer cell proliferation, and can increase the effects of anti-cancer drugs (Pharmacol Ther 2018). Nakajima has also discovered new roles for drug-metabolizing enzymes controlling pharmacokinetics and toxicity of anti-cancer drugs (Annu Rev Pharmacol Toxicol 2022). Since there are no small molecular compounds to inhibit ADAR, Nakajima's group is working on the development of aptamer molecules, a new modality that is increasingly attracting attention, which potently and specifically inhibit ADAR. We recently succeeded in visualizing the molecular dynamics of the binding of the aptamer to ADAR by HS-AFM. We will evaluate the effects of the aptamer on cancer cell viability and invasion ability as well as cell morphology by using SICM.

In the cancer research field, recent technical progress with tumor-derived organoid cultures allowed us to create sophisticated preclinical cancer models that recapitulate biological processes of human cancer in mice. Oshima’s group has succeeded in establishing gastric and colon cancer models by using mouse and human cancer-derived organoids (Cancer Res 2018; PNAS 2019; Nat Commun 2020). Using these model systems, we discovered a novel mechanism of polyclonal metastasis (Nat Commun 2021), and showed defined nanoscale physical properties of the organoid cell surface by using scanning ion conductance microscope (SICM) (Biomaterials 2022). Using these organoid models and SICM analysis, we will try to identify the nanoscale mechanisms of malignant progression of cancer cells, which will accelerate future development of unique diagnostic and therapeutic strategies against cancer.

Stem cells are defined as cells that have the ability to perpetuate an undifferentiated status through self-renewal, and develop into mature cells through differentiation. Hirao’s group aims to elucidate the essential nature of stem cells, and has identified that inhibition of cell cycle transition, removable of reactive oxygen species, and the activation of nutrient starvation signals are important for regulation of the stemness and self-renewal of hematopoietic and leukemia stem cells (Nat Med 2006; Cell Stem Cell 2007; Nature 2010). Therefore, precise measurement of the redox state and the distribution of nutrients such as glucose and amino acids in cells will enable us to elucidate essential stem cell properties. In this WPI program, we have revealed several critical cell fate determinants for normal and malignant hematopoiesis (Cell Stem Cell 2018; Nat Immunol 2019). Among them, we discovered critical metabolites for drug resistance of malignant cells. Based on these findings, we promoted transdisciplinary projects for the development of a visualization system for small chemical compounds with SPM technology (Commun Chem 2020), leading to a deep understanding of the pathophysiological roles of metabolites. We will develop a novel imaging system for metabolites with sensors developed at NanoLSI, for mapping the extra- or intra-cellular distribution of the metabolite at the nanoscale, leading to a deep understanding of the pathological roles of cancer-specific metabolites. In addition, comparative analyses between normal and cancer stem cells would lead to identification of cancer stem cell-specific metabolic abnormality.

Molecular-targeted cancer therapy has changed the concepts and practice of cancer treatment. However, even when molecular-targeted therapies yield a favorable response, relapse due to drug resistance is a major problem. Yano’s group has discovered that activation of receptor tyrosine kinases, such as AXL and IGF-1R, leads to the emergence of drug tolerant cells that underly resistance (Nat Commun 2019; Nat Commun 2020). In addition, epithelial-to-mesenchymal transition is an independent mechanism of drug resistance (Cancer Res 2019). Moreover, measurement of the surface profile, elastic modules, and adsorption of living tumor cells by AFM revealed that tumor cells with an epithelial-to-mesenchymal transition associated with drug resistance had a smoother surface compared with drug sensitive tumor cells. We will establish cell classification methods using machine learning, which enables the diagnosis of drug resistant tumor cells at a single cell level by AFM.

Cell membrane signal reception and cellular responses are fundamental processes of tissue organization and homeostasis. Growth factors and their transmembrane receptors play definitive roles in development, regeneration, and cancer progression. Matsumoto‘s group discovered high-performance cyclic peptides that bind to HGF (hepatocyte growth factor) (Nat Chem Biol 2019) and its transmembrane receptor MET (Nat Commun 2015; Sci Rep 2018). We applied these peptides to 1) analysis of molecular dynamics by HS-AFM (Nat Chem Biol 2019), 2) molecular recognition using a peptide-conjugated tip in HS-AFM (ACS Appl Mater Interfaces 2021), and 3) creation of designer therapeutic proteins (Nat Commun 2021; iScience 2021). We will verify: 1) dynamic mechanisms of HGF-MET interaction and MET activation

on lipid bilayer and living cells by high-speed/high-resolution AFM, 2) the creation and application of designer proteins by Lasso-Graft technology for diagnosis and therapeutics, and 3) molecular imaging of cancer metastatic niche formation.

Thus, by collaborations with experts in cell biology, cancer biology and nanotechnology, we hope to build a path to future developments in life science and a new era of cancer biology.

2)-3 Project management

* Describe the center's research organization (including its research, support and administrative components) and your concept for building and staffing the organization.
* Describe your concrete plan for achieving the center's final staffing goal, including steps and timetables.
* If the center will form linkage with other institutions, domestic and/or foreign, by establishing satellite functions, provide the name(s) of the partner institution(s), and describe their roles, personnel composition and structure, and collaborative framework with the center project (e.g., contracts to be concluded, schemes for resource transfer).
* If the center will form linkage with other institutions, domestic and/or foreign, without establishing satellite functions, provide the names of the partner institutions and describe their roles and linkages within the center project.
* List in Appendix the principal investigators who are expected to join the center.

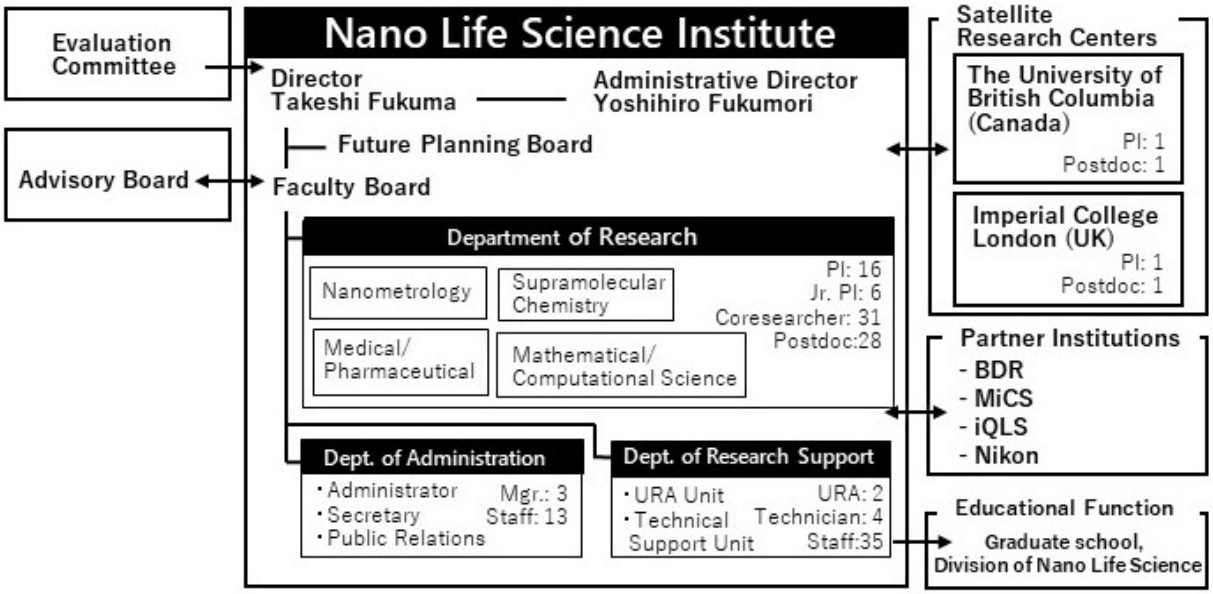

Fig. 7: Organization of NanoLSI

NanoLSI members

For the second five years of the WPI grant period, the Center will be comprised of around 80 persons for research, around 17 persons for administration, and around 30 persons for support. Sixteen or more principal investigators including 5 or more foreign PIs will be appointed. These PIs will build a sound foundation for NanoLSI in advancing research.

Satellite institutions

Satellite Research Centers will be maintained in Europe (the UK) and North America (Canada) in order to accelerate international joint research, train human resources through exchanges of young scientists, and improve international visibility.

Center in Europe: Imperial College London, London, UK: Professor Korchev as PI

Center in North America: University of British Columbia, Vancouver, Canada: Professor MacLachlan as PI

Partner institutions

Contract-based agreements on joint research, information exchange, mutual support for human resources development, and joint use of facilities and equipment will be sustained with the following institutions:

i. RIKEN Center for Biosystems Dynamics Research (BDR)
ii. Microbiology Research Center for Sustainability, University of Tsukuba (MiCS)
iii. Institute for Quantum Life Science, National Institutes for Quantum Science and Technology (iQLS)

iv. Nikon Solutions CO., LTD.

Educational functions

The graduate school, "Division of Nano Life Science," fosters excellent graduate students who will carry out their own research independently in an excellent research environment with the latest laboratory equipment at NanoLSI. The supervisors for this graduate school are all world-class researchers who belong to NanoLSI. In addition, most of the PIs will give lectures to undergraduate students in various departments, helping NanoLSI to recruit them to the graduate school division. Furthermore, NanoLSI will start a new lecture course by Jr. PIs and other young researchers for undergraduate students.

Approach for benefitting from diversity

NanoLSI will employ another female PI, while increasing research staff by at least two female assistant or associate professors every year. For the fixed-term assistant professors, our basic policy was to employ young foreign researchers, yet NanoLSI has decided to extend this to include Japanese female researchers. At the end of FY2023, the proportion of female researchers will be over 20%, while keeping the proportion of foreign researchers near 40%.

a) Principal investigators (full professors, associate professors or other researchers of comparable standing)

	At beginning of project	At end of FY 2021	Final goal (Date: March, 2023)
Researchers from within the host institution	12	11	12
Foreign researchers invited from abroad	4	4	4
Researchers invited from other Japanese institutions	0	1	1
Total principal investigators	16	16	17

b) Total members

	At beginning of project		At end of FY 2021		Final goal (Date: March, 2024)	
	Number of persons	%	Number of persons	%	Number of persons	%
Researchers	49		81		81	
Overseas researchers	8	16	31	38	31	38
Female researchers	6	12	15	19	17	21
Principal investigators	16		16		17	
Overseas PIs	5	31	5	31	5	29
Female PIs	1	6	1	6	2	12
Other researchers	33		65		64	
Overseas researchers	3	9	26	40	26	41
Female researchers	5	15	14	22	15	23
Research support staff	8		41		29	
Administrative staff	13		17		18	

Total number of people who form the "core" of the research center	70		139		128		

2)-4 Securing research funding

Past record

* Give the total amount of research funding (e.g., competitive funding) secured by the principal investigators who will join the center project. Itemize by fiscal year (FY2017-2021).

FY	2017	2018	2019	2020	2021
Amount	702	644	686	626	596

(Million Yen)

Prospects after establishment of the center

* Based on the past record, describe the concrete prospects for securing resources that match or exceed the WPI project grant.
* Calculate the total amount of research funding (e.g. competitive funding) based on the percentage of time that the researchers will devote to research activities at the center vis-à-vis the total time they spend on research activities. Be sure that the prospects (FY2022-2026) are realistically based on the past record.

From FY2017 to FY2020, the external research funds secured by all NanoLSI researchers consisting of PIs, NanoLSI full-time researchers other than PIs, and associated researchers who work at NanoLSI and other departments of KU are shown below. In each year, the amount of external funds secured exceeded the WPI subsidies received.

	Number of NanoLSI researchers	Total amount of external funds	Allocation amount from the host institution
FY2017	50	¥886,045,607	¥573,713,736
FY2018	72	¥866,977,892	¥629,295,011
FY2019	74	¥1,044,068,024	¥701,070,766
FY2020	83	¥1,047,075,206	¥711,349,232
FY2021	81	¥1,174,472,752	¥844,208,468

The funding track record of NanoLSI is so far quite strong and its continuation to secure the research funding of around one billion yen can be realistically expected.

3) Interdisciplinary Research

* Describe why interdisciplinary research is necessary and important in the target field(s) and what new field(s) can be expected to be created by way of this project. Describe your concrete strategy for advancing such interdisciplinary research.

1. Necessity and significance of interdisciplinary research

We aim to gain a fundamental understanding of the mechanisms of basic cellular functions at the atomic or submolecular levels by comparing nanodynamics inside normal and cancer cells. To this end, we will expand and combine our world-leading bio-SPM and supramolecular chemistry techniques to take a dramatic technological leap toward the creation of nano-endoscopic techniques for direct imaging, analysis, and manipulation of nanodynamics of proteins, nucleic acids, and other molecules inside and on the surface of cells, as well as nano-distributions of pH and oxygen concentration. Naturally, this work will require collaborations with life scientists in the medical and pharmaceutical sciences. Moreover, partnering with simulation experts will be essential for accurately understanding atomic and molecular dynamics from experimental results. As this illustrates,

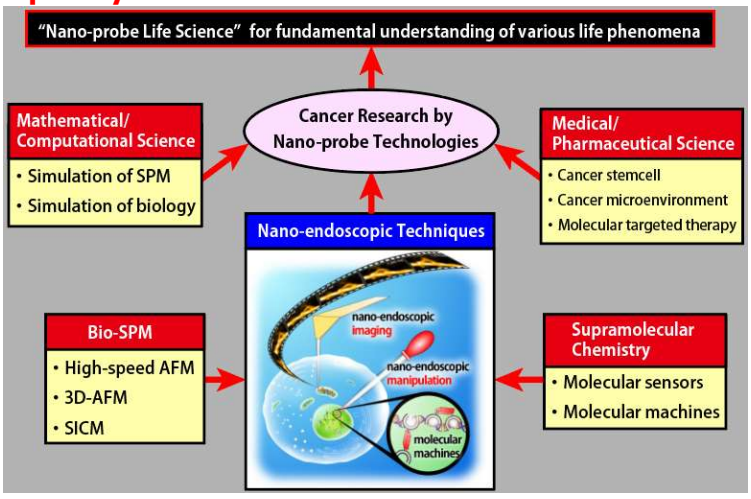

Fig. 8: Strategy for interdisciplinary research at NanoLSI.

the path toward our goal will require an all-out effort that draws upon the techniques and knowledge of bio-SPM, supramolecular chemistry, medical and pharmaceutical sciences, and mathematical and computational sciences. This work will lead to the establishment of the new interdisciplinary science field of "nano-probe life science".

2. Strategy for interdisciplinary research

In our research, there are two key challenges that are tackled by combining expertise in multiple disciplines. The strategies for achieving this goal are outlined below.

[Development of nano-endoscopic techniques through the combination of bio-SPM and supramolecular chemistry techniques]

We are developing nano-endoscopic analysis for visualizing the nano-distribution of pH, oxygen concentration, and other properties that significantly influence molecular and cellular dynamics, using SPM probes fitted with highly environmental-responsive molecular sensors. Simultaneously, we are seeking to achieve nano-endoscopic manipulation to stimulate nanodynamics using probes fitted with functional molecules, offering a high-level of control of functionality. Furthermore, we are developing a mode of combined measurement whereby molecular machines are delivered to nano-regions and a nano-probe will be used for live imaging of the molecular and cellular dynamics induced by stimuli from the molecular machines. Additionally, we are developing long nanoprobes functionalized with anti-fouling molecules for reproducible and non-invasive nano-endoscopic imaging. In this way, we combine the expertise in the two disciplines, nanometrology and supramolecular chemistry.

[Understanding of mechanisms of basic cellular functions and their cancer-specific abnormalities using nano-endoscopic and simulation techniques]

The success of nanometrology depends on not only the performance of the instruments but also the operating skills of their users and sample preparation conditions. In NanoLSI, SPM researchers are working on the optimization of the operating conditions while medical/pharmaceutical researchers are optimizing the sample preparation procedures. In the meanwhile, simulations necessary for proper interpretation of the measured results are performed by the following two researchers having different expertise in mathematical/computational sciences: Foster, specialized in the atomistic simulation of molecular dynamics and in *ab initio* calculation (Phys. Rev. Lett. 102 (2009) 126807; Phys. Rev. Lett. 93 (2004) 187202), and Beta, focusing on modeling and simulation of complicated biological dynamics such as cell motility (PNAS 117 (2020) 6330; Sci. Adv. 6 (2020) eaaz6153). The comparative examination of our experimental/simulation results and development of a nanoscale understanding of cellular functions and their cancer-specific abnormalities will involve pooling the knowledge of SPM, medical/pharmaceutical science, and mathematical/computational science researchers and repeated discussions.

[Formation of international alliance for "nanoprobe life science"]

We will establish an international alliance for the new research field "nanoprobe life science" to achieve the following three goals.

(Complementary use of various bio-imaging techniques)

Although nanoprobe technology is unique and powerful, it is not the only tool for bio-imaging. There are many other technologies that are developing very rapidly. The complementary use of these different technologies will be a key to achieve fundamental understandings of various life phenomena. To achieve this goal, we have formed an international alliance with other life science institutes having world-leading bio-imaging technologies. Tight collaborations through this alliance network will also allow us to keep monitoring the progress of other bio-imaging technologies and to adjust the plan for the development of our nanoprobe technologies. In this way, we will ensure that our technologies always stay at the frontier of bio-imaging.

(Complementary use of various expertise in molecular cell biology)

At NanoLSI, we have gathered various experts in each important cancer research area as well as in basic medical and pharmaceutical science. However, fundamental understanding of basic cellular functions requires a broad range of expertise so that we may need to expand the range of our expertise. To this end, we continue to seek for a possibility to expand the international alliance network. For particularly important research areas, we have already recruited talented researchers to join us as Junior PIs, and will continue to do so when it is necessary. The formation of international alliance should help us to meet and find the right people to join our research team.

(Widespread use of nanoprobe technologies for life science research)

The ultimate goal of our institute is to achieve nano-level understandings on various life phenomena using advanced nanoprobe technologies. Considering the wide variety of life phenomena, this goal cannot be achieved by our institute alone. Thus, we aim to open our nanoprobe technologies to all life science researchers in the world. However, newly developed methods and instruments are not necessarily usable by non-experts and often need improvements in usability and applicability. Thus, we are initially opening our technologies to the limited members in the international alliance so that we can obtained detailed reports from them. Based on the feedback, we will improve our technologies to establish them as common tools for nanoprobe life science research.

4) International Research Environment

4)-1 System for advancing international research

* Describe your concrete plan for building an international research center including the makeup of its foreign researchers, establishment of oversea satellites, and provision of researcher exchanges. Please include a timetable for this plan.
* Describe concretely your strategy for staffing foreign researchers (e.g., postdoc positions) through open international solicitations. Describe the procedures you will use to do so.

Retaining five or more overseas PIs and two overseas satellites

Of the 16 current PIs at NanoLSI, 5 are from overseas. One of the five is a full-time researcher at NanoLSI, while the other four stay at NanoLSI for one month or more a year while being employed permanently at an overseas research institution. In order to promote joint research with these four overseas PIs, one assistant professor in NanoLSI is assigned to each of them. In addition, one research associate in charge of joint research is assigned to each of the two laboratories designated as overseas satellites at Imperial College London, UK and the University of British Columbia, Canada with the financial support of NanoLSI. This framework of five overseas PIs and two satellites will be retained for the second five years of the WPI grant period.

Measures to solicit more robust activities by the foreign PIs at NanoLSI.

All four foreign PIs employed at an overseas institution will secure research space and personnel in the NanoLSI building and will be engaged in collaborative research with domestic PIs and other NanoLSI researchers. Some of the results obtained by joint research will be published and featured on the NanoLSI WEB site. To continue these activities under the COVID-19 outbreak, NanoLSI will hold regular online inter-group meetings (T-meetings) between an overseas PI and a domestic PI. In this way, NanoLSI will promote publications of the achievements obtained and continue to explore new collaborative research projects.

Employment of overseas postdoctoral researchers to be given high priority

When recruiting postdoctoral researchers internationally, the host institution, KU, has a policy of hiring them as specially appointed assistant professors whose three-year term can be renewed. In accordance with this policy, NanoLSI conducts recruitment of postdoctoral researchers through international announcements, and almost all overseas postdoctoral researchers are hired as specially appointed assistant professors. The number of postdoctoral researchers hired at the end of FY2021 is 28, and the proportion of those from overseas is 24, i.e., 86% of 28. NanoLSI will retain the policy to employ overseas young researchers with priority. All NanoLSI recruitments will be done through open international solicitation.

Outreach programs for external researchers and development of joint research

NanoLSI will place emphasis on outreach programs for external and overseas researchers. The purpose is to introduce and disseminate NanoLSI's scanning probe microscope (Bio-SPM) technology to external researchers in the life sciences, which will lead to joint research. They include the Bio-SPM Summer School for young researchers for one week, the Bio-SPM Collaborative Research for mid-career researchers for about two weeks, and the NanoLSI Visiting Fellows Program for senior researchers and their research groups for one month. Participants will be selected on the basis of calls for applications. NanoLSI will retain these outreach programs to invite external and overseas researchers to NanoLSI with its world-level in Bio-SPM technologies. Despite the COVID 19 outbreak, some remote joint research will be attempted.

4) -2 Establishment of international research environment

* Describe your concrete strategy for establishing an international research environment and administration system, and the support system to be provided for researchers from overseas.
* Describe your strategy, procedure and timing for periodically holding international research conferences or symposiums (at least once a year).

* Describe your measures to ensure that top-caliber researchers from around the world can work comfortably in carrying out their research within a competitive international environment.

Sharing of Bio-SPM equipment

One of the features of NanoLSI in terms of equipment is the deployment of 60 or more Bio-SPMs and 6 EMs. These instruments are not only used by overseas researchers belonging to NanoLSI, but also by many overseas researchers who visit NanoLSI through various programs for shared equipment.

Deployment of advanced and multi-lingual technicians

In order to support the maintenance of these Bio-SPM instruments and to assist overseas researchers who use them under this open facility system, one technician who specializes in AFM and speaks three languages, English, Chinese, and Japanese, and one technician who is fluent in English and specializes in EM will be continuously deployed exclusively for NanoLSI. In addition, one Ph. D. technician and one technician with a master's degree will be continuously employed to maintain NanoLSI equipment and assist visiting researchers. This deployment of four advanced technicians will be retained.

Evaluation-dependent annual salary system

NanoLSI will continuously adopt an evaluation-dependent annual salary system in order to ensure an internationally competitive salary level. The evaluation will be conducted by the Center Director on the basis of various evaluation criteria, i.e. research performance and contribution to various activities such as promotion of transdisciplinary research, outreach programs and development of Bio-SPM technologies related to establishing NanoLSI.

Multi-layered financial support for young overseas researchers

NanoLSI will provide multi-layered support for young researchers to initiate and conduct interdisciplinary research and to obtain external grants. First, when a young researcher is employed at NanoLSI, they will be provided with a startup budget of 1 million yen. After spending the startup budget, a basic research budget of half a million yen will be provided every year. In addition to this budgetary support, Transdisciplinary Research Promotion Grants of 0.5 to 2 million yen per year will be provided after joint review by PIs of young researchers' interdisciplinary research proposals.

URAs' support

In order for young researchers to obtain external research funds, a URA is assigned to NanoLSI who supports the preparation of application documents through close consultation. With support by the URA through individual consultation and elaboration/confirmation of the application form of overseas researchers, 14 young overseas researchers have so far got their own KAKENHI as of February 2022. For planning and operating outreach projects for researchers such as the Bio-SPM Summer School, another URA is assigned to be responsible for prior contact with participating overseas researchers and support after visiting NanoLSI. The deployment of two URAs for the above functions will be retained.

Administrative support in English

In order to maximize the research focus of overseas researchers belonging to NanoLSI, the administrative staff members assigned to the NanoLSI will provide support in English. The support is wide-ranging, including explanation of the terms of the employment contract, personnel rules and the Japanese tax system, and accompanying the researchers during resident registration, driver's license acquisition or renewal, accommodation search and interpreting during signing of the contract with a real estate agent, interpreting at the time of private car purchase, interpreting when renting a parking lot, interpreting when opening a bank account, applying for a credit card, etc. In addition, support is provided for the families of overseas researchers, including accompanying and interpreting during hospital visits, for finding a nursery school, and introduction to community activity circles.

International research meetings

International research meetings such as the NanoLSI Symposium held each year have been held 22 times from FY2017 to FY2021, either sponsored by NanoLSI only or co-sponsored by NanoLSI and other research institute(s). Regarding cooperation with the other WPI centers, iCeMS and NanoLSI have agreed to organize a joint international symposium once a year. NanoLSI will organize its own international symposium once a year and keep on holding several international research meetings per year even online despite COVID-19.

5) Center Management

5) -1 Operational management

* Describe the role of the center director.
* Describe the role of the administrative director.

* Concretely describe your concept for establishing an administrative organization.
* Concretely describe the center's decision-making system.
* Concretely describe how authority is allocated between the center director and the host institution.
* Concretely describe how the center will adopt a rigorous system for evaluating research and will introduce a system for merit-based compensation (e.g. annual salary scheme). Please describe your procedures and timing for operationalizing these systems.

Role of Center Director: Creating a new field of academic research

The aim of the Center is to create a new interdisciplinary research field called Nanoprobe Life Science, which integrates nanometrology, life science, supramolecular chemistry, and computational science in order to elucidate the mechanisms underlying a variety of vital phenomena. To achieve this goal, the Center Director will draw on his own background as a leader in various research fields to develop a vision for this interdisciplinary research. The Center Director will have authority to determine strategies and all other matters necessary for achieving this vision as well as deciding on the Center's direction.

Role of the Administrative Director: Achieving the Center Director's vision

Based on the Center Director's strong initiative, the Administrative Director will work in cooperation with other administrative staff to realize the Center Director's vision of NanoLSI management in terms of facilities and equipment, competitive fund-raising, outreach activities to both researchers and the public, cooperation with overseas satellites and other research institutes, assistance for Jr. PIs, employment and fostering of young researchers, holding international symposia and other research meetings, etc. The Administrative Director will also work on coordination between NanoLSI and the university headquarters including execution of the host institution's commitment and system reforms.

Concept for establishing an administrative organization

Kanazawa University positions the success of NanoLSI as one of the most important aspects of university management and prioritizes the placement of competent administrative staff in NanoLSI. At present, 17 administrative staff including administrative director are assigned to the NanoLSI administrative office, 13 of whom can perform their duties in English. NanoLSI administrative staff members actively participate in planning and organization of international symposia, promotion of public relations, improvement of the research environment, and livelihood support for overseas researchers. They contribute to strengthening NanoLSI by maximizing the research focus of the center's researchers.

Decision-making system

At the beginning of FY2020, NanoLSI was officially positioned in the statutes of Kanazawa University as an independent institute of the university. This revision of the statutes has endorsed the Center Director's discretionary power in terms of the personnel arrangements, budget execution, and representation of NanoLSI. Alongside the Center Director's discretionary power, the Future Planning Board consisting of the Center Director, the Administrative Director and four PIs has a steering role. This board will assess the progress of NanoLSI regularly and discuss medium- to long-term issues. The Administrative Director, the Center Director's secretary and other administrative staff hold a meeting each week, reporting the progress of major projects and activity plans. The Center is operated by transmitting the instructions of the Center Director to each of the following working groups: alliance formation, open facilities, transdisciplinary research promotion, research outreach, researcher development, and facilities management. To ensure that all members of NanoLSI are well informed about the Center's policies, a faculty board consisting of PIs and professors is held every month and minutes in English and meeting materials are shared with all staff.

Allocation of authority between the Center Director and the host institution

The statutes of Kanazawa University, the host institution, authorize the Center Director to have complete authority over decisions related to the Center's research strategy, as well as the acquisition and allocation of budgetary resources, personnel, space, and other research resources as well as all other matters of NanoLSI. To this end, Kanazawa University conforms to its commitment and will proactively reassess existing regulations and develop system reforms. Regarding cooperation and the sharing of roles between the host institution and NanoLSI, a regular meeting with the President, Vice President in charge of general affairs, finance and facilities, the Center Director, and the Administrative Director is held every month. Here, reports on the activities of NanoLSI are presented, intentions are unified on how to deal with important matters in the operation of the Center, and the sharing of roles between Kanazawa University and NanoLSI in dealing with these issues is decided.

Research evaluation and internationally competitive salary scheme

NanoLSI will continuously apply a research evaluation dependent annual salary scheme in order to ensure an internationally competitive salary level. The Center Director evaluates the annual research progress reports submitted by all NanoLSI researchers, and the evaluation score is multiplied maximally threefold by the NanoLSI researcher allowance to determine the annual salary for the following year.

5) -2 Research environment

* Concretely describe how equipment and facilities, including laboratory space, will be provided in a manner appropriate for a top world-level research center. Include your procedure and timing.
* Concretely describe how the center will provide an environment in which researchers can work comfortably on their research by being exempted from duties other than research and related educational activities, and how they will be provided adequate staff support to handle paperwork and other administrative functions. Include your procedure and timing.
* Concretely describe how the center will arrange for its researchers to participate in the education of graduate students.

NanoLSI Research Building and Bio-SPM equipment

The host institution, Kanazawa University, completed the new NanoLSI Research Building in November 2020. The new building has a total floor area of 6840 m^2, consisting of a basement and 4 floors above ground. Researchers in the fields of nanometrology, life science, supramolecular chemistry, and computational science form a research core under one roof. The structural features of the new building include the installation of a dry area (empty moat) that protects the entire building from external vibrations propagating on the ground, and the installation of floating floors that prevent vibrations transmitted from inside the building at key points. Due to these features, high-precision anti-vibration measures are now possible that could not be realized at existing facilities. The host institution will provide financial support of 10 million yen per year to maintain the facilities.

Features of the facility include 60 scanning probe microscopes (SPM) (48 atomic force microscopes, AFM, and 12 scanning ion conductance microscopes, SICM) and 6 electron microscopes (one transmission electron microscope, TEM, and five scanning electron microscopes, SEM). Most of these microscopes are located in the basement, where there is less vibration, forming the research floor that is the hallmark of NanoLSI. The electron microscopes are used for observing samples; SEMs are used for processing AFM probes, and TEM is used for measuring the diameter of nanopipettes for SICMs. In addition to SPMs and EMs, the research environment has been enhanced by an animal room, a treatment room, and three P2-level laboratories. Furthermore, a shared chemistry laboratory has been established with a total of 16 draft chambers with high-speed variable air volume control (Variable Air Volume) with excellent energy-saving performance.

Research-focused system for NanoLSI full-time researchers

The host institution has a unique research focus system for competent researchers. A Research Professorship will be given to full-time NanoLSI researchers to exempt them from non-NanoLSI duties and focus on their own research with maximal effort.

URAs and administrative staff support for NanoLSI researchers

For planning and operating outreach projects for researchers such as the Bio-SPM Summer School, URAs are assigned to be responsible for prior contact with participating overseas researchers and support after visiting NanoLSI. For international symposia and other research meetings, some administrative staff take on the responsibility for planning and organization. In addition, the NanoLSI administrative staff cover all administrative paperwork and other administrative jobs in order to maximize the research focus of NanoLSI researchers including those from overseas. The NanoLSI researchers are released from organizational work for outreach programs, international symposia and other research meetings as well as administrative details.

Graduate school "Division of Nano Life Science"

For fostering future generation researchers of NanoLSI, the Graduate School of Frontier Science Initiative, Division of Nano Life Science was established in FY2020 as an education unit paired with NanoLSI; it is now in operation. This graduate school attracts more excellent students from Japan and overseas than the fixed enrollment capacity of 6 students per year. To promote interdisciplinary research in the Division of Nano Life Science, all 27 full-time researchers of NanoLSI who have an educational assignment are engaged as educators in the Division of Nano Life Science. In addition, these graduate students in the division can join training courses organized by NanoLSI such as the Bio-SPM summer school and the transdisciplinary research promotion grants. As for financial support, a master's student in the division will be provided with 130,000 yen per month (breakdown: scholarship ¥50,000 + RA salary ¥80,000). A doctorate student receives ¥180,000 per month (breakdown: scholarship ¥100,000 + RA salary ¥80,000). These scholarships are provided from Kanazawa University's own funds. In addition, the governmental scholarships given to Kanazawa University such as the WISE Program and the Fellowship Program for Fostering Top Scientists in Fused Disciplines place a priority on the graduate students in the Division.

5) -3 Establishing the center in sync with organizational restructuring

* Concretely describe the host institution's organizational reform that will be synchronized with the establishment of the center.
* Describe measures that will be taken by the host institution to sustain the center's operation after the WPI funding ends. Also describe how the host institution will promote the centers autonomy after WPI funding ends and how it will over the mid-to-long term restructure its existing organization in ways that give the center a permanent place within its organization.

Kanazawa University's organizational restructuring

The host institution, Kanazawa University, states in its third mid-term objectives/mid-term plans (FY2016-FY2021) that the university further promotes strong research such as nanotechnology using innovative atomic force microscopy technology, innovative material development using supramolecular technology and research on cancer metastasis and drug resistance mechanisms, in a systematic and intensive manner. At the same time, KU states that the research implementation mechanism will be strengthened with the aim of establishing research centers for interdisciplinary research.

Based on the stated objectives and approaches, the university reorganized its existing research institute, the Cancer Research Institute, and subsequently established NanoLSI in FY2017, the Nanomaterials Research Institute in FY2018, the Advanced Manufacturing Technology Institute in FY2019 and Advanced Mobility Research Institute in FY2021. Thus a group of multi-disciplinary research institutes has been established which are independent from conventional departments of the university. While the university aims to provide a research base to each of these five research institutes in their own multi-disciplinary academic field, NanoLSI is particularly positioned as a pioneering, path-breaking institute.

Measures taken by the host institution to sustain the Center's operation after the WPI funding ends

It is essential to secure tenured posts for NanoLSI researchers to sustain and develop NanoLSI as an internationally top-level research institute over a long period of time. In the FY2021 site visit for WPI interim evaluation, President Yamazaki of Kanazawa University stated the intention to deploy twenty-two tenured or tenure track researchers to NanoLSI together with their tenured posts. These promised deployments were completed in FY2021. NanoLSI and the university headquarters have agreed to keep on these tenured posts for researchers belonging to NanoLSI while they are involved in graduate or undergraduate education in relevant research fields. These personnel measures will build a permanent and sound foundation for NanoLSI in advancing research for many years.

Based on the agreement of President Yamazaki and the Center Director, 6 active young researchers, Jr. PIs have been employed. Jr. PIs are key players for promoting interdisciplinary research and are treated as independent researchers in the same way as PIs with a start-up budget of 10 million yen and personnel expenses for one subordinate young researcher. The final performance reviews for 6 Jr. PIs will be carried out in FY2023 and FY2024. Six tenure positions for Jr. PIs have been secured at the President's discretion.

President Yamazaki has also agreed to sustain the administrative office and staff both in quality and quantity where all NanoLSI operations are carried out in English, such as research support, daily life support, and various communications from the office. As of the FY2021 interim evaluation, 18 administrative staff are assigned to the NanoLSI Administrative Office, 14 of whom can perform their duties in English. Around 18 deployments of administrative staff will be kept on for the second five years of WPI support period and beyond.

Securing of the Center's autonomy after the WPI funding period and beyond

At the beginning of FY2020, NanoLSI was officially positioned in the statutes of Kanazawa University as an independent institute of the university. With this revision of the statutes, the personnel rights, budget execution rights, and representation rights of the Center Director of NanoLSI have been established both in real and institutional terms.