Abstract

We at the Membrane Cooperativity Unit are working hard to reveal how the dynamic platforms for signal transduction and the synapses for the neuronal transmission form and function in the plasma membrane. For this purpose, we take a unique approach (in addition to other more conventional approaches). Namely, we develop new and unique methods for single-molecule imaging and manipulation at nanometer precisions in living cells, with a special attention paid to high time resolutions (world's fastest single fluorescent-molecule imaging). The combinations of interesting biological targets and fundamentally important biophysical technologies characterize our research. The smooth liaison between physics/engineering and biomedicine is a key for our research.

The plasma membrane is the outermost membrane of the cell, and thus it encloses the entire cell. It is critically important for the cell - the fundamental unit of life - because it defines the space for it. The plasma membrane exchanges information, energy, and substances with the outside world, and we pay special attention to the mechanism for signal transfer from outside to inside the cell, a function generally called "signal transduction". In the signal transduction process, the plasma membrane works like a sensor + computer + effector.

The Membrane Cooperativity Unit strives to understand how the plasma membrane works at very fundamental levels, based on unique insights we obtain by applying single-molecule imaging-tracking methods. More specifically, we are now revealing the mechanisms by which the metastable molecular complexes and meso-scale membrane domains, including actin-induced membrane compartments, raft domains, and protein oligomers, form and work in concert to enable signal transduction and synapse formation/modulation in/on the plasma membrane.

Recently, we have succeeded in developing the world's fastest single-molecule sensitivity camera, pushing the frame rates to the ultimate, which is determined by the number of photons that could be emitted from a single fluorescent molecules during a single video frame: with the best dyes in the market we accomplished a 30-kHz frame rate or a time resolution of 33 μs, during which ≈70 photons are detected, providing the single molecule localization precision of ≈35 nm. This rate is 1,000 times higher than normal video rate, and 80 times faster than the second fastest, which was also achieved by us four years ago. Based on this ultrafast camera, we further developed ultrafast super-resolution microscopy, ultrafast PALM and dSTORM, which can be performed simultaneously. Generally, the data acquisition time to produce a single super-resolution image is 5-10 minutes, but the

ultrafast method we developed shortened the data acquisition time to ≈10 seconds, making it possible to observe live cells rather than fixed dead cells.

1. Staff

- Dr. Amine Betul Nuriseria Aladag, Post Doctoral Scholar
- Dr. HooiCheng Lim, Post Doctoral Scholar
- Dr. Bo Tang, Post Doctoral Scholar
- Dr. Taka-Aki Tsunoyama, Post Doctoral Scholar
- Dr. Maoji Wang, Post Doctoral Scholar
- Dr. Jun-Seok Lee, Technician
- Ms. Irina Meshcheryakova, Technician
- Mr. Ryuto Shinozaki, Technician
- Dr. Saahil Acharya, Technology Pioneering Fellow
- Dr. Peng Zhou, Technology Pioneering Fellow
- Ms. Izumi Kim, Research Intern
- Mr. Daiki Matsudo, Research Intern
- Ms. Tayyaba Ramzan, Research Intern
- Mr. Yuta Kogi, Research Assistant (Part-time)
- Mr. Souma Shimabukuro, Research Assistant (Part-time)
- Mr. Yasuyuki Shiroma, Research Assistant (Part-time)
- Mr. Yoshito Takaesu, Research Assistant (Part-time)
- Mr. Ryu Tsuha, Research Assistant (Part-time)
- Mr. Tasuku Wake, Research Assistant (Part-time)
- Ms. Miwa Matsui, Research Unit Administrator
- Dr. Akihiro Kusumi, Professor

2. Collaborations

2.1 Revealing the dynamics, structure, and function of metastable signaling molecular complexes by single-molecule imaging

- Description: Developing ultrafast 3D single-molecule imaging, and applying it to revealing the dynamics and formation mechanism of the signal transduction platform for the cancer promotion-immune evasion signals, Fcepsilon signals, focal adhesion-based signals, and GPI-anchored proteins' raft-based signals

- Type of collaboration: Joint research
- Researchers:
  - Dr. Takahiro Fujiwara, Associate Professor, Institute for Integrated Cell-Material Sciences (iCeMS), Institute of Advanced Studies, Kyoto University
  - Dr. Kenichi Suzuki, Professor, G-CHAIN, Gifu University

2.2 Unraveling the molecular-species selective macroscopic diffusion barriers in the axonal initial segment in the neuron using ultrafast single-molecule imaging

- Description: By applying ultrafast single-molecule imaging and ultrafast single-molecule localization microscopy developed by us, we try to unravel the large-scale molecular-species selective diffusion barriers in the axonal initial segment in the neuron
- Type of collaboration: Joint research
- Researchers:
  - Dr. Takahiro Fujiwara, Associate Professor and Ms. Hiroko Hijikata, Technician, Institute for Integrated Cell-Material Sciences (iCeMS), Institute of Advanced Studies, Kyoto University
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2.3 Elucidating the functions of plasma membrane compartmentalization

- Description: Elucidating how the signal transduction functions of the plasma membrane is regulated using the actin-based compartmentalization of the plasma membrane, using ultrafast single-molecule imaging-tracking and super-resolution microscopy
- Type of collaboration: Joint research
- Researchers:
  - Dr. Pakorn Tony Kanchanawong, Associate Professor, Mechanobiology Institute, The National University of Singapore

2.4 Development of deep-learning methods for single-molecule imaging experiments and analysis

- Description: Developing AI-based methods for performing single-molecule imaging and for analyzing single-molecule imaging data
- Type of collaboration: Joint research
- Researchers:

- Dr. Kazuhiro Hotta, Professor, Mechanobiology Institute, Department of Electrical and Electronic Engineering, Faculty of Engineering, Meijo University

2.5 Revealing the mechanisms for the synapse formation and long-term potentiation by combining super-resolution microscopy and single-molecule imaging

- Description: To discover the mechanisms for functional and structural synaptic plasticity underlying learning and memory, by the combined use of super-resolution microscopy and single-molecule imaging
- Type of collaboration: Joint research
- Researchers:
  - Dr. Michisuke Yuzaki, Professor and Dr. Tetsuko Fukuda, Researcher, Graduate School of Medicine, Keio University
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2.6 Revealing the cellular signaling platforms formed by regulated liquid-liquid phase separation (LLPS)

- Description: To reveal the mechanisms by which nano-micron-sized liquid-like signaling platforms form and facilitate signaling in the cell and between the cells
- Type of collaboration: Joint research
- Researchers:
  - Dr. Dragomir Milovanovic, Lab Head, Dr. Christian Hoffmann, Postdoctoral Fellow, and Mr. Gerard Aguilar Perez, PhD Student, DZNE; German Center for Neurodegenerative Diseases

3. Activities and Findings

3.1 Development of ultrafast camera-based single fluorescent-molecule imaging for cell biology

The spatial resolution of fluorescence microscopy has recently been greatly enhanced. However, improvements in temporal resolution have been limited, despite their importance for examining living cells. Here, we developed an ultrafast camera system that enables the highest time resolutions in single fluorescent-molecule imaging to date, which were photon-limited by fluorophore photophysics: 33 and 100 µs with single-molecule localization precisions of 34 and 20 nm, respectively, for Cy3, the optimal fluorophore we identified. Using theoretical frameworks developed for the analysis of single-molecule trajectories in the plasma membrane (PM), this camera successfully detected fast hop diffusion of membrane molecules in the PM, previously detectable only in the apical PM using less preferable 40-nm gold probes, thus helping to elucidate

the principles governing the PM organization and molecular dynamics. Furthermore, as described in the companion paper, this camera allows simultaneous data acquisitions for PALM/dSTORM at as fast as 1 kHz, with 29/19 nm localization precisions in the 640 × 640 pixel view-field.

3.2 Development of ultrafast single-molecule-localization super-resolution microscopy (PALM/dSTORM) and its application to revealing focal adhesion nano-architecture and molecular dynamics

Using our newly developed ultrafast camera described in the companion paper, we reduced the data acquisition periods required for photoactivation/photoconversion localization microscopy (PALM, using mEos3.2) and direct stochastic reconstruction microscopy (dSTORM, using HMSiR) by a factor of ≈30 compared with standard methods, for much greater view-fields, with localization precisions of 29 and 19 nm, respectively, thus opening up previously inaccessible spatiotemporal scales to cell biology research. Simultaneous two-color PALM-dSTORM and PALM-ultrafast (10 kHz) single fluorescentmolecule imaging-tracking has been realized. They revealed the dynamic nanoorganization of the focal adhesion (FA), leading to the compartmentalized archipelago FA model, consisting of FA-protein islands with broad diversities in size (13–100 nm; mean island diameter ≈30 nm), protein copy numbers, compositions, and stoichiometries, which dot the partitioned fluid membrane (74-nm compartments in the FA vs. 109-nm compartments outside the FA). Integrins are recruited to these islands by hop diffusion. The FA-protein islands form loose ≈320 nm clusters and function as units for recruiting FA proteins.

3.3 Revealing the mechanism for synaptic vesicle sequestering and dynamics: control by synapsin condensation

Neuronal transmission relies on the regulated secretion of neurotransmitters, which are packed in synaptic vesicles (SVs). Hundreds of SVs accumulate at synaptic boutons. Despite being held together, SVs are highly mobile, so that they can be recruited to the plasma membrane for their rapid release during neuronal activity. However, how such confinement of SVs corroborates with their motility remains unclear. To bridge this gap, we employ ultrafast singlemolecule tracking (SMT) in the reconstituted systemof native SVs and in living neurons. SVs and synapsin 1, the most highly abundant synaptic protein, form condensates with liquid-like properties. In these condensates, synapsin 1 movement is slowed in both at short (i.e., 60-nm) and long (i.e., several hundred-nm) ranges, suggesting that the SV-synapsin 1 interaction raises the overall packing of the condensate. Furthermore, two-color SMT and superresolution imaging in living axons demonstrate that synapsin 1 drives the accumulation of SVs in boutons. Even the short intrinsically-disordered fragment of synapsin 1 was sufficient to restore the native SV motility pattern in synapsin triple knock-out animals. Thus, synapsin 1 condensation is sufficient to guarantee reliable confinement and motility of SVs, allowing for the formation of mesoscale domains of SVs at synapses in vivo.

4. Publications

4.1 Journals

Original Articles

1. C. Hoffmann*, J. Rentsch*, T.A. Tsunoyama* (*equal contribution), A. Chhabra, G.P. Aguilar, R. Chowdhury, A. Korobeinikov, F. Trnka, S.H. Ali, M. Ganzella, G. Giannone, S.O. Rizzoli, A. Kusumi, H. Ewers, D. Milovanovic. Synapsin condensation controls synaptic vesicle sequestering and dynamics. **Nat. Commun.** 14:6730. (2023) doi: 10.1038/s41467-023-42372-6.

2. T. K. Fujiwara, T. A. Tsunoyama, S. Takeuchi, Z. Kalay, Y. Nagai, T. Kalkbrenner, Y. L. Nemoto, L. H. Chen, A. C. E. Shibata, K. Iwasawa, K. P. Ritchie, K. G. N. Suzuki, and A. Kusumi. Ultrafast single-molecule imaging reveals focal adhesion nano-architecture and molecular dynamics. **J. Cell Biol.** 222: e202110162 (2023). doi: 10.1083/jcb.202110162
   Selected as one of the 11 papers of the JCB's "The Year in Cell Biology: 2023"
   Featured in "SPOTLIGHT" of the journal, written by Huang and Kanchanawong

3. T. K. Fujiwara, S. Takeuchi, Z. Kalay, Y. Nagai, T. A. Tsunoyama, T. Kalkbrenner, K. Iwasawa, K. P. Ritchie, K. G. N. Suzuki, and A. Kusumi. Development of ultrafast camera-based single fluorescent- molecule imaging for cell biology. **J. Cell Biol.** 222: e202110160. (2023). doi: 10.1083/jcb.202110160
   Selected as one of the 11 papers of the JCB's "The Year in Cell Biology: 2023"
   Featured in "SPOTLIGHT" of the journal, written by Huang and Kanchanawong

Review Articles

1. S. Mayor, A. Bhat, and A. Kusumi. A survey of models of cell membranes: toward a new understanding of membrane organization. **Cold Spring Harbor Perspective Biology** (2023). doi: 10.1101/cshperspect.a041394

2. A. Kusumi, T. A. Tsunoyama, B. Tang, K. M. Hirosawa, N. Morone, T. K. Fujiwara, and K. G. N. Suzuki. Cholesterol- and actin-centered view of the plasma membrane: updating the Singer-Nicolson fluid-mosaic model to commemorate its 50th anniversary. **Mol. Biol. Cell** 34, plx1-15 (2023). doi: 10.1091/mbc.E20-12-0809

3. K. G. N. Suzuki and A. Kusumi. Refinement of Singer-Nicolson fluid-mosaic model by microscopy imaging: Lipid rafts and actin-induced membrane compartmentalization. **Biochim. Biophys. Acta - Biomembranes** 1865, 184093 (2023). doi:10.1016/j.bbamem.2022.184093

4.2 Books and other one-time publications

Nothing to report

4.3 Oral and Poster Presentations

Invited Presentations

1. A. Kusumi. Development of the ultrafast camera system for single-molecule imaging and discovery of metastable nano-liquid signaling platforms on the cell membrane. OIST Workshop: Recent Trends in Microrheology and Microfluidics. Okinawa. January 12, 2023.

2. A. Kusumi. Single-molecule imaging studies of postsynaptic receptor turnover on the PSD protein condensates. 15th NeuroWissenschaftliche Gesellshaft (German Neuroscience Society) Meeting. Symposium 23: Phase separation in neuronal (patho)physiology, Göttingen, Germany. March 24, 2023.

3. A. Kusumi. Development of ultrafast camera-based single fluorescent-molecule imaging for cell biology. OPTICA Biophotonics Congress: Optics in the Life Sciences. Vancouver, Canada. April 24-27, 2023.

4. A. Kusumi. Development of ultrafast single fluorescent-molecule imaging and discovery of the metastable nano-liquid signaling hub on the plasma membrane. ***Keynote Opening Lecture.*** Minisymposium on developments and applications of advanced fluorescence technologies. China Medical University, Taichung, Taiwan. June 13, 2023.

5. A. Kusumi. Nano-liquid platform for receptor signal integration which promotes tumor growth. The 75th Annual Meeting of Japanese Cell Biology Society (Cell Biology 2023). Symposium S-D2-A001 Dynamic landscape of membranes in motion. Nara. June 29, 2023

6. A. Kusumi. SynGAP condensate formation and recruitment of PSD95 and receptors: Single-molecule imaging studies. Symposium (S17 T#4) Biomolecular condensates in synaptic function and neurodegenerative diseases. ISN-ESN 2023 - International Society for Neurochemistry. Porto, Portugal. August 10, 2023.

Invited Presentations: Departmental Seminars

7. PBIO (Physiology & Biophysics) & Chemistry Joint Seminar, University of Washington, Seattle, U. S. A. "Development of the ultrafast camera system for single fluorescent-molecule imaging and detection of hop diffusion of membrane molecules in the plasma membrane." April 21, 2023

8. School of Biomedical Engineering, University of British Columbia, Vancouver, Canada. "Development of the ultrafast camera system for single fluorescent-molecule imaging and discovery of metastable nano-liquid signaling platforms on the cell membrane" April 25, 2023

9. German Center for Neurodegenerative Diseases (DZNE; Deutsches Zentrum für Neurodegenerative Erkrankungen), Berlin, Germany. "Development of ultrafast camera-based single fluorescent-molecule imaging and unravelling of synaptic receptor turnover on SynGAP condensates." August 14, 2023

Oral Presentations

1. S. Acharya. SynGAP LLPS condensates as the basic platform for recruiting PSD95 and receptor oligomers for generating neuronal excitatory synapses. Japan Neuroscience Society Meeting. Sendai. August 1-4, 2023.

2. T. Tsunoyama. Nano-liquid platform on the plasma membrane that integrates receptor signals for cancer promotion. The 61st Annual Meeting of the Biophysical Society of Japan. Nagoya. November 14-16, 2023.

3. T. Tsunoyama. Liquid-like nanoscale signaling platform on the plasma membrane that integrates receptor signals leading to cancer promotion. The 68th Annual Meeting of Biophysical Society. Pennsylvania, USA. February 11, 2024.

Poster Presentations

1. S. Acharya. SynGAP LLPS Condensates as the basic platform for recruiting PSD95 and receptor oligomers for generating neuronal excitatory synapses. The 61st Annual Meeting of the Biophysical Society of Japan. Nagoya. November 14-16, 2023.

5. Intellectual Property Rights and Other Specific Achievements

PCT Application
Application Number: PCT/JP2023/022333
Application Date: 15 June 2023
Title of Application：Peptide drugs for suppressing tolerance development by blocking homo- and hetero-dimerization of opioid receptors

6. Meetings and Events

Nothing to report.

7. Other

Nothing to report.