The Responses of Human Cancer Cells on Functionalized Silicon Nanostructures

Chung-Yao Yang and J. Andrew Yeh

***Abstract*—This study describes an easy method to fabricate and functionalize silicon nanostructures for investigating cell-substrate interaction between silicon nanostructures and human cancer cell lines (breast and lung cancers). These newly developed nanotextured silicon nanostructures with different chemical modifications can give us more knowledge of the interactions between cells and surrounding environment *in vitro*. In this study, the chemical etching is used to fabricate the silicon nanostructures for mass production. Also, two silane-based chemicals can be self-assembled on the surfaces via high quality vapor deposition technique. We have found that breast and lung cancer cells showed different morphogenesis when cells adhered on planar and nanotexured silicon surfaces. These results showed that the cell behaviors including cell adhesion and spreading would be affected by the combination of physical properties come from surface topology as well as the chemical properties such as the hydrophobicity of adhered surfaces. The cell pattern also can be controlled via these functionalized patterned silicon nanostructures. This study showed potential applications in tissue engineering or basic cell biology research such as manipulating cell development and how cell migrate on these nanotextures by using these nanotextured materials.**

***Index Terms*—Silicon nanostructures, cell pattern, cancer cell, cell-substrate interactions**

I. Introduction

CELL behavior is highly dependent on the surrounding environment, for example, cell interaction between two contiguous cells, or cell contact with the extracellular matrix (ECM). There are various pathways/mechanisms that regulate how a cell senses and replies to the surrounding environments. These include cell-based properties (e.g., integrin-ligand interactions), physical cues (e.g., topographical influences of the surface) and chemical cues (e.g., effects of surface chemistry). The ability to understand and, further, control or modulate cell-substrate interactions (or cell-material interfaces) through transmembrane receptors such as integrin has provided us a more comprehensive understanding of cellular responses associated with the physiologically/pathologically relevant microenvironment wherein single cells attach and spread, coherently linking to cell structural properties and responses. Furthermore, maintaining deregulation and homeostasis which can lead to disease are important under these microenvironmental conditions [1]. For example, the formation of new blood vessels from pre-existing vessels, which called angiogenesis, will secret growth factors and supply required nutrients for tumor growing, and provide tumor metastasis and expansion later. Numerous researches show that cell-cell interaction in cancer cell lines may be correlated with the regulation of angiogenesis [2], [3]. It is crucial to explain how the molecular pathways of angiogenesis resulting in cancer disease, and this knowledge can be used to design suitable drugs for more efficient therapies. There is still a challenge to be solved which is the signaling pathway of focal adhesion based on single protein assembly in the research field of cell adhesion. In particular, the mechanism of cell adhesion and the size/shape of focal adhesion cluster are still mysteries in cell biology. Using micro-/nano-technology combined tools to manipulate these processes in focal adhesion would allow us to regulate cell adhesion in the future.

How to arrange cells with a specific location *in vitro* is one of the major issues in biomedical-relevant research, which can closely mimic physiological environments *in vivo* [4]–[6]. Nano-fabricated surfaces with several nanometer scale textures have potentially provided physical-based biocompatible tools to manipulate the arrangement of various cells. Patterning with growth factors or cell ECMs takes advantage of particular biomolecular effects. Surface topographical patterning (physical cues) or patterning of regions with various chemicals (chemical cues) are likely the most widely used manners. The most commonly used method among these properties for surface patterning is hydrophobicity and hydrophilicity of the surface. Typically, one region is hydrophobic, and the other is more hydrophilic than another region. Furthermore, the combination of nanotopographical effects and proteins/chemicals effects displayed that either of which could regulate gene expression/cell adhesion dependently or independently. Therefore, numerous studies have been used nanoengineered methods in combination with coating specific bio-molecules on surfaces to manipulate cell-material interaction *in vitro*.

The most common surface modification scheme for materials is reaction of alkylchlorosilanes with water and/or surface hydroxyl groups in a solution-based process to form a thin alkylsilane film. This process highly depends on

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reaction temperature, silanol species in solution, and nature of the solvent. Additionally, liquid-based coating process is a time-consuming process and may influence the topography of nano-scale features due to the unsolved molecules in solution. Vapor-phase coating processes via volatile fluorinated alkylsilanes has been demonstrated [7], [8], which offers several advantages, compared to liquid-based approaches, which inherently have several major issues including (i) diffusion limited transport of reagents into specific areas, (ii) incomplete wetting of high-aspect ratio structures, (iii) control of dissolved water in non-aqueous solvents, (iv) uncertain reaction in solution, and (v) disposal of solvent waste. Vapor-phase process provides distinct advantages such as (i) efficient transport into high-aspect ratio structures, (ii) no solvent waste, (iii) good control of reagent, and (iv) provide convenient, in situ cleaning prior to deposition. According to these reasons, cell behavior on nanostructures with/without chemistries is unclear because the liquid phase chemistries may change the topography of nanostructures. In this study, we used photolithography and chemical etching to fabricate a platform for investigating and manipulating cancer cells. In addition, monolayers also could be self-assembled through vapor-phase coating process within one hour, which is faster than the liquid-based coating process. Silicon nanostructures also can be easily integrated into IC process for high throughput production.

For investigating cell-substrate/cell-cell interactions of human cancer cells on silicon nanostructures, we provided an easy-to-handle and photolithography-based method to imitate the *in vivo* environment and functionalize the surfaces with different functional group chemicals with respect to cell-substrate interaction. This nano-fabrication method has previously applied for producing anti-reflective solar cells [9]. In addition, the immunofluorescence of cytoskeleton was also investigated using epi-fluorescence microscope. The result shows that the cell shape and adhesion were correlated to the wettability of the surface, and the similar investigation was proposed in fibroblast cells. Through this technique, the effect of nano-scale topographical features similar to the ECM can help researchers to more accurately understand the cell behavior *in vivo* environment.

II. Materials and Methods

A. *Surface Treatment of Silicon Nanostructures*

Patterned silicon nanostructures in this study are carried out using photolithography to define the desired pattern and then $AgNO_3$ chemical etching on single-crystal silicon wafers to make nanostructures (Figure 1). The mechanism of $AgNO_3$ chemical etching is based on the reaction of oxidation reduction between silver atom and silicon. The following organo-chloro-silane chemicals were used for surface modification of the substrates: 3-Aminopropyltrimethoxysilane (APTMS) to form a hydrophilic surface and perfluorodecyltrichlorosilane (FDTS) to form a hydrophobic surface. These two chemicals are all purchased from Sigma. The processes of reactions are described as follows. First, a remote oxygen plasma treatment is used for surface hydroxylation. Second, silanol groups are formed from the chemical precursors. Finally, a self-assembled molecular layer on the surface is formed between silanol and hydroxyl reaction in the vacuum chamber. The times for surface treatment are 30 minutes (FDTS) and 2 hours (APTMS). After surface modification, the photoresist was removed by acetone to expose the silicon

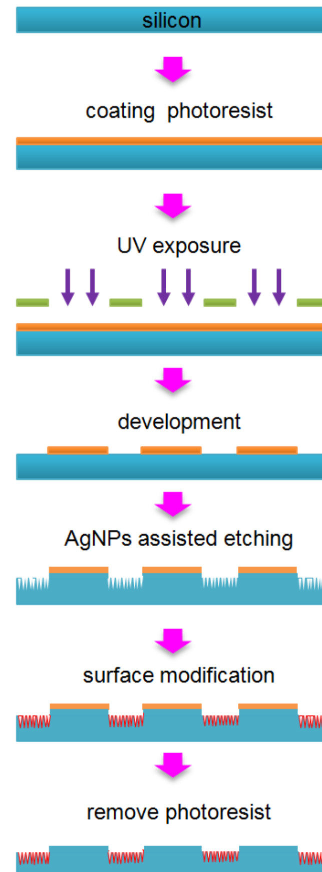

Fig. 1. The schematic of fabrication process for functionalized patterned silicon nanostructures.

layer. In this study, we used two kinds of silicon surface, one is functionalized silicon nanostructures with pattern, and the other one is patterned silicon nanostructures. After the photoresist was removed in acetone, the functionalized patterned silicon nanostructures were completed for investigating cell-cell/substrate interaction.

B. *Cell Culture*

Human breast cancer cells (MDA-MB-231 and MCF-7) and human lung cancer cells (A549 and HARA) were cultured in tissue culture T75 flasks with Dulbecco's Modified Eagle Medium (Invitrogen molecular probes, CA) for MDA-MB-231, A549 and HARA, and Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen molecular probes, CA) for MCF-7, both supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine in a humidified 5% CO_2 incubator at 37 ℃. Cells were replated on the samples placed in cultured plates for 4 and 24 hours to perform cell-substrate interaction and fluorescent staining experiments. The samples were

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sterilized via steam process before cell culture.

C. Scanning Electron Microscopy

The cell morphology of above mentioned cells on the planar silicon and silicon nanostructures was examined with high-resolution field-emission SEM — Ultra 55 (Carl Zeiss SMT AG, German). Before SEM imaging, cells were fixed in a fixative (2.5% glutaraldehyde with 0.1 M PBS) at 4℃ for 30 minutes. After using 0.1 M PBS washed twice, the samples were post-fixed with 1% osmium tetroxide from 4 ℃ to room temperature for 30 minutes. Fixed samples were washed twice with PBS again, dehydrated through a grade ethanol series (50%, 70%, 80%, 90% and 100% for 10 minutes each), and then dried by CO_2 dryer CPD-406 (BAL-TEC, Germany). Finally, the samples were sputtered with gold at 15 mA for 3 minutes and examined with SEM.

D. Epi-fluorescence Microscopy

Immunofluorescent staining was performed as follows: briefly, human cancer cells were washed using PBS after cells adhered onto the surfaces for 4 and 24 hours. Then, fresh 4% paraformaldehyde in 0.1M PB was used to fix cells at room temperature for 15 minutes. After PBS wash, the cell membranes were permeabilized by immersing substrates into 0.3% Tween 20 (Sigma-Aldrich, USA) in PBS for 5 minutes. Rhodamine phalloidin (Invitrogen molecular probes, CA) and DAPI were used for staining F-actin and nuclei, respectively. All fluorescence images were imaged via an epi-fluorescence microscope (Zeiss Axio Observer, German), under 10X eyepiece and 20X objective lens.

III. Results and Discussion

A. Surface Characteristics of Silicon Nanostructures

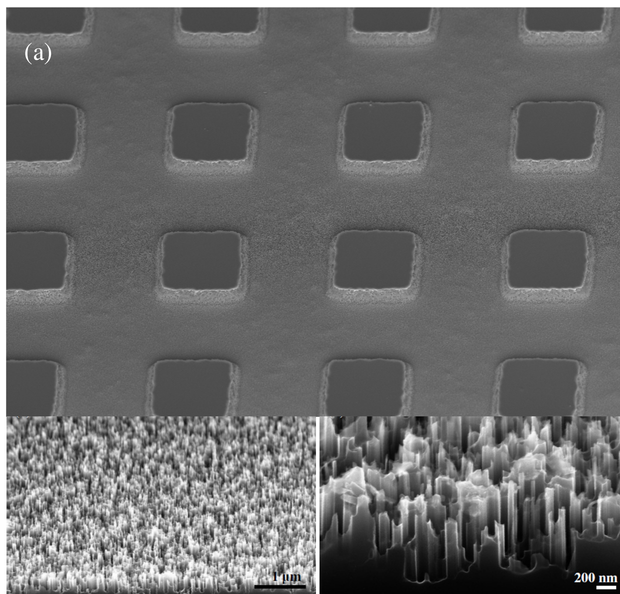

Fig. 2. SEM images of pattern silicon nanostructures: (a) Planar silicon and silicon nanostructures. (b) 50,000 X and (c) 100,000 X.

The surface morphology of silicon nanostructures was employed via scanning electron microscopy as shown in Figure 2. The nanostructures can be fabricated uniformly at wafer-level scale using 4 and 6 inches wafers. The size of silicon nanostructures is about 75 nm in width and 600 nm in depth, which corresponds to an aspect ratio of 8. The depth of silicon nanostructures also can be adjusted through the different etching time.

B. Cell Morphology on Planar Silicon and Silicon Nanostructures

To characterize how human cancer cells interact with planar and nanotextured substrates, the cells were cultured on 5-mm^2 planar silicon and silicon nanostructures with or without APTMS/FDTS on top. After re-plating for four hours, the morphological appearances of the adhered human cancer cells were imaged by SEM (Figure 3). For human breast cancer cells, the MDA-MB-231 cells on the planar silicon substrates appeared similar to those on the normal culture dishes (Figure 3a). Intriguingly, on the silicon nanostructures shown in Figure 3b, the MDA-MB- 231 cells stretched out and extended some radial nanospikes from the cell edges, although they were prominently visualized as having a rounded-up shape. The MCF-7 cells showed similar results with MDA-MB-231, but exhibited more spreading ability than MDA-MB-231 cells due to the MCF-7 cells can proliferate more on silicon nanostructures. For human lung cancer cells, the A549 and HARA cells both showed

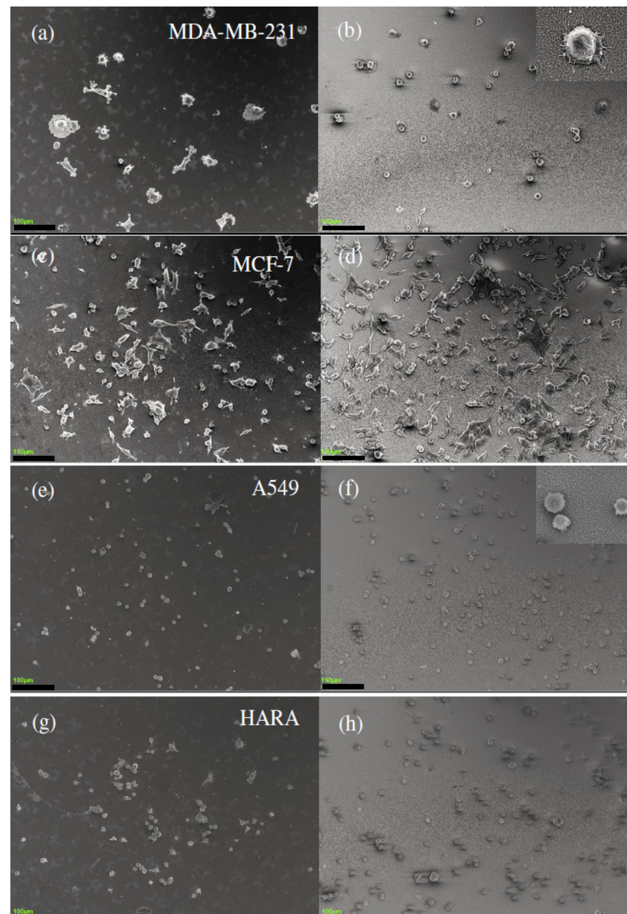

Fig. 3. Cell morphology of various cancer cells cultured on planar silicon (left image) and silicon nanostructures (right image) after 4 hours cultured durations: human breast cancer cells (a-b) MDA-MB-231, (c-d) MCF7; human lung cancer cells (e-f) A549, and (g-h) HARA. The scale bars are all 100 μm.

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rounded-up shape on planar silicon and silicon nanostructures. However, the cells cultured on planar ones showed more spreading morphology than on silicon nanostructures but with less radial nanospikes on silicon nanostructures. The morphology shows the human breast cancer cells can spread more than human lung cancer cells after 4 hours cultured durations. This may indicate that the human lung cancer cells needed more cultured duration than human breast cancer cells to spread.

C. Cell Cytoskeleton on Functionalized Planar Silicon and Silicon Nanostructures

The effects of nano-topography (physical cues) or protein coating (chemical cues) on focal adhesion formation and cytoskeleton orientation have been related to the type and adhering nature of cells in response to particular extracellular microenvironments. According to the distinguishing morphologies, as mentioned above, we then examined the actin cytoskeleton organization of cancer cells that had adhered to the chemi-/physi-cally modified (APTMS-coated and nanotextured modified) substrates via cytoskeleton staining using Rhodamine phalloidin. As shown in Figure 4a and b, after APTMS (amino group) modified, the MDA-MB-231 cells showed more spreading morphology either on planar silicon or silicon nanostructures than without treatment (see Figure 3a and b). For MCF-7 cells, the projected cell areas on planar ones are slightly larger than on silicon nanostructures and this phenomenon is consistent with Figure 3c and d. For A549 cells, they showed more spreading morphology on APTMS-coated planar silicon than on pristine planar silicon (Figure 3e). Also, the cells showed more radial nanospikes on APTMS-coated silicon nanostructures than on pristine silicon nanostructures (Figure 3f). For HARA cells, they showed similar results when cultured on either APTMS-coated or pristine ones. Interestingly, after 24 hours cultured durations, the HARA cells cultured on APTMS-coated silicon nanostructures showed more radial nanospikes than on APTMS-coated planar silicon (data not shown). In the present study, cell spreading onto the APTMS-coated silicon nanostructures was found to be enhanced in comparison with the pristine ones. The physical characteristics of the surface used for the silicon nanostructures that affect the contact guidance are currently under investigation. Nevertheless, the nanoscale surface of the silicon nanostructures may provide an *in vivo*-like microenvironment for better realizing the interactions between cells and ECMs.

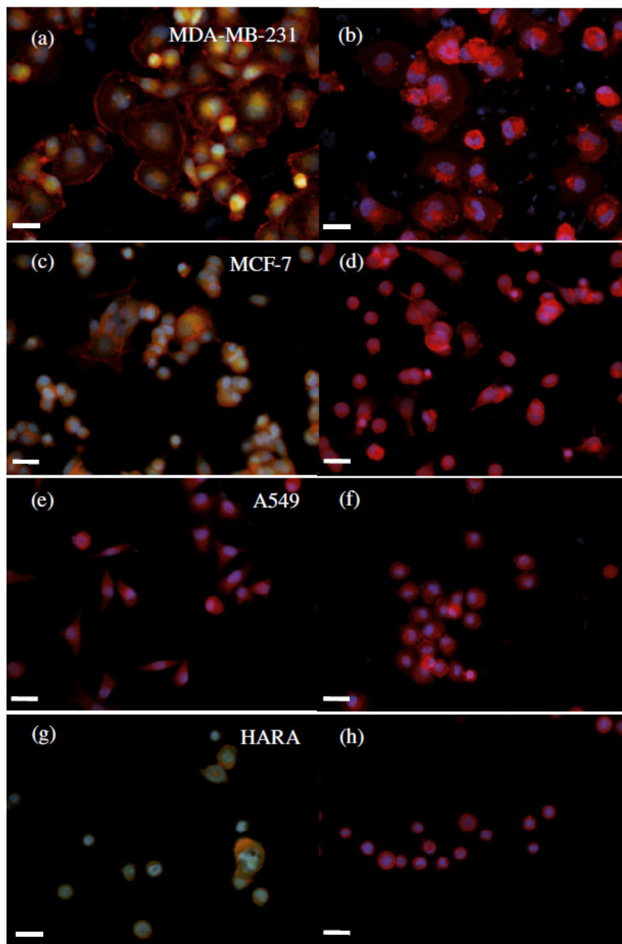

Fig. 4. Cell cytoskeleton of various cancer cells cultured on APTMS-coated planar silicon (left image) and APTMS-coated silicon nanostructures (right image) after 4 hours cultured durations: human breast cancer cells (a-b) MDA-MB-231, (c-d) MCF7; human lung cancer cells (e-f) A549, and (g-h) HARA. The scale bars are all 25 μm.

D. Cell Patterning on Functionalized Patterned Silicon Nanostructures

Figure 5a shows the SEM image of functionalized patterned silicon nanostructures that combined with planar silicon without treatment and silicon nanostructures with chemical modification (FDTS). According to previous results, we concluded the cancer cells preferred to adhere on the planar silicon more than on silicon nanostructures. Therefore, we can use this difference to re-arrange cells into specific direction or patterns (e.g., different geometrical pattern or specific letters, data not shown). As shown in Figure 5b-e, the various human cancer cells were successfully re-arranged into the pattern that shown in Figure 5a.

Surface patterning provides a valuable tool for investigating neuronal behavior. ECM proteins and cell-cell adhesion molecules (CAM) play essential roles in the differentiation and development of neurons. Experimental results using materials with chemical cues patterns (e.g., ECM and CAM) have provided unique understanding into the roles of ECM composition, cell shape and cell-substrate interaction on major cell functions, such as viability, neurite outgrowth and migration. The response of neurons on these patterned surfaces may help researchers in the design of functional scaffolds and nerve guidance for reconstruction and regulation of the nervous system. Cell patterning techniques have been paid more and more attention in the study of intrinsic characteristics of non-neuronal cells in the last few years. This is because cell micropatterning can control not only the location of the cells on a surface, but also the shape, attaching area, and number of contacting cells of each single cell. Those parameters have been found to be quite important in cell survival, proliferation, differentiation, cell migration, cytokinesis, and cell polarity. In addition, keeping single cells separated by cell micropatterning

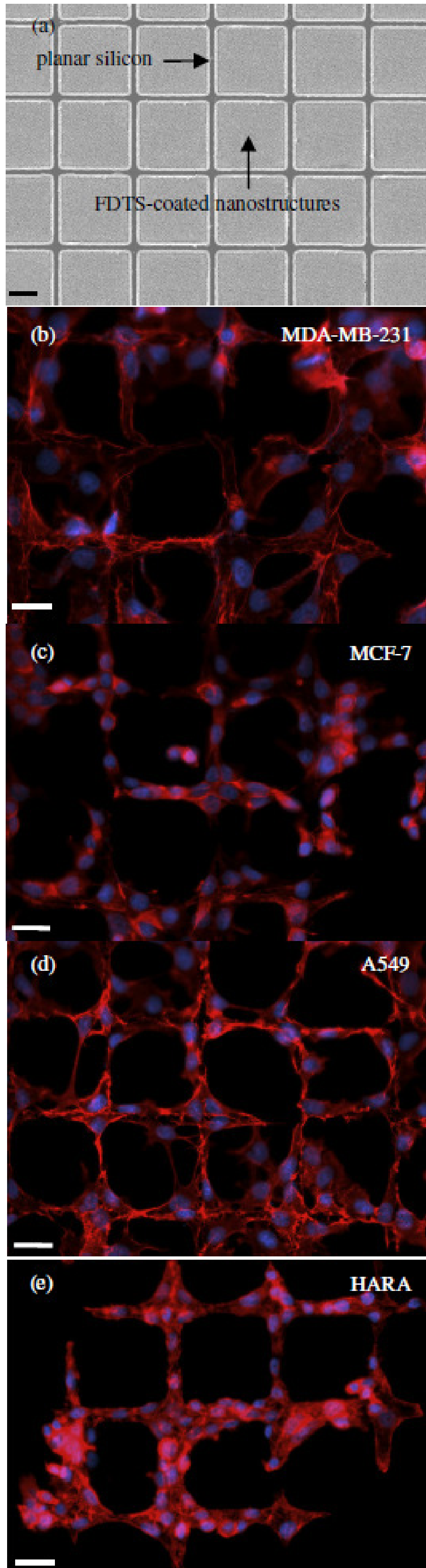

Fig. 5. Human cancer cells patterning on functionalized patterned silicon nanostructures. (a) SEM image of functionalized patterned silicon nanostructures. Cell cytoskeleton of various cancer cells after 36 hours cultured durations: (a) MDA-MB-231, (b) MCF7, (c) A549, and (d) HARA cells. The scale bars are all 25 µm.

techniques has been used to analyze variations in gene expression kinetics among individual cells. Single cell patterning can be achieved through using this patterned silicon nanostructures (data not shown). On the basis of these achievements, cell micropatterning has currently become one of the most important methods for cell biology research.

IV. Conclusion

The metastasis of cancer cells is an inherently task due to our limited abilities to control the cellular environment at micro- and nanoscale resolution. In this study, we proposed an easy method to fabricate a platform combined with nano-topography and chemical coating for manipulating cell development. This technology can be applied to investigate the adhesion and growth of cancer cells on nano-scale surfaces and the development of systematized cell cultures for applications such as drug delivery, cancer cell biology and tissue engineering. This ability to pattern surface chemistry and topographic features at sub-cellular scales will enable new insights in cell biology.

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