High-Definition X-Ray Imaging of Small Gecko Skin Surface Protuberances for Digitization and 3D Printing

David W. Green,* Stephen T. Kelly, Kenneth Ka-Ho Lee, Gregory S. Watson, Jolanta A. Watson, and Han Sung Jung*

A detailed analysis of zoological and botanical engineering structures reveals remarkable ideas for developing new healthcare technologies. One eclectic example is the surface microprotrusions of Gecko lizard skins, featuring nanoscale tips that engage in bacterial repulsion and killing. However, building biomimetic synthetic nanostructures in long range for technological use is hampered by the lack of concentrated digital information for 3D nano-fabrication. For instance, standard micro-computed tomography (µCT), confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM) imaging failed to replicate gecko spinules at the nanoscale. The authors now show that the latest generation of high-power X-ray microscopy (Zeiss X-Radia 810) builds high contrast images of the gecko skin surface, and following 3D digital conversion the individual spinules, in the large array, are perfectly delineated at 150 nm and clearly featured the 50 nm nanotips crucial for bacterial cell rupturing. Consequently, the authors generate stenographic (STL) digital file formats loaded with the instructions for 3D printing and the design blueprints for soft lithography replication. A further reconstruction of the digital data is undertaken into a 3D solid object using MeshLabs, yielding biologically realistic, virtual-world spinule arrays. In this form, the authors are able to edit the spinules for optimal killing performance against bacterial cells.

1. Introduction

Biomimetic and bioinspired engineering strategies have become substantially more sophisticated and accurate in duplicating zoological structures. This evolving trend toward higher complexity biomimicry will boost clinical relevancy and applicability of future materials. The latest fabrication machine tools, namely, 3D bioprinting, soft lithography, ion beam lithography (IBL) and microcontact imprinting are providing precise and accurate reproductions of nature-derived materials and structures, with high fidelity, at the microscale. These tools thus increase the possibilities for constructing functional biomedical technologies at greater levels of biomimicry than before such that the copy contains composites of materials, a complex interface, and nanoscale organization. In microphysics and chemistry, organisms operate whole systems at small scales. For example, biological attachment mechanisms only work because of the small-scale components (the scaling effect).[1,2] However, many standard tools for microfabrication are unable to reproduce copies with resolution below 100 µm. Furthermore, the translation of biological structures at the nanoscale remains problematic. However, laser-assisted printing of small material droplets[3] and beamline lithography now yield sub-micrometer surface structures at 100–50 nm resolutions and achieve aspect ratios of 10–20.[4]
In fact, the potential of X-ray lithography (XRL) can go below 50 nm (moreover, such techniques fabricate structures with very high aspect ratios of 10). Importantly, XRL can achieve intricate structures by tilting the subject and applying multiple exposures of X-ray onto the base material. Two-photon lithography techniques are able to generate structures with “near-diffraction-limit” resolutions. Future strategies to increase complexity and achieve <50 nm resolution structures would rely on combining nanoimprint lithography (NIL), X-ray lithography (XRL) and two photon absorption (TPA) on the single material template.[4]

However, one of the most important barriers in manufacturing copies of nature-derived biomaterials (from zoological and botanical models) designs is capturing high-quality digital images (with voxels small enough to distinguish the intricate nanoscale forms and features) to reveal the structures and morphologies at small scales, particularly <100 μm, at the surface and deep inside the objects. Internalized or embedded structures are a prevalent part of biomaterial design for interlocking form and function in its environment.[5] They improve fracture toughness, “bridges” between different high contrast materials, ease of deformation and elasticity, actuation to external influences and breaking or folding lines, etc. These physical and mechanical features embedded in materials are uniquely challenging to engineer and the majority of biomaterials do not capture such necessary complexity. Bioimaging is touching on resolving these components for design and to eventually incorporate in printed replicates. However, the techniques have no capacity to create hierarchical, finely structured compound biomaterials.

Surface architecture in 3D is amenable to existing microfabrication and nanofabrication techniques and there are developments where hierarchical structures are made. For example, four-beam laser interference lithography is harnessed to generate ordered “multiscale” surface embedded structures and to introduce anisotropy and isotropy. Significantly, the method is simpler and more efficient (patterning in minutes) than comparable ones and makes large-area patterned materials—many of the attributes needed for effective translation.[6,7]

One of the main components for the speedy microfabrication of artificial materials from natural role models of every kind is very precise, high-quality imaging. A considerable proportion of recent developments in biological imaging, related to improving absorption in soft tissues, is directed at highlighting physiological functions in snapshots and in real-time. Moreover, quantitative imaging is an important requirement to measure phenomena such as hypoxia and vascular branching. Majorly, the emphasis on imaging zoological nature is structural biomaterials with high density, being amorphous, the crystallinity, which have much higher contrast than soft tissue. Currently, X-ray microComputer Tomography (muCT) is a stock technique of imaging biological materials. Synchrotron radiation X-rays are making a large impact in image analyses of cells, sub-cellular structures, protein aggregates, and single biomolecules. This concentrates on these hard X-ray phase contrasts, with coherence diffraction phenomena, because it is 500× stronger than absorption phenomena from conventional optics in imaging. Synchrotron beamlines can deliver 1011 photons mm−2, which can damage delicate biomaterials, especially a few cell layers thick.

The image creates a virtual world template of biological objects. High-quality imaging is necessary for significant progress in tissue engineering,[8] which has depended on immunohistological and microscopic viewing. Accordingly, 3D imaging produces realistic volumizing of tissue structures and architectures and provides information on function. Such 3D imaging has been enhanced with ultrasound (spatial resolution to a few100 μm), photoacoustic microscopy (<1 μm and slightly below),[9] magnetic resonance imaging (MRI) (spatial resolution to a few100 μm), optical imaging, and X-ray imaging (1.65 μm).[8]

MicroCT is employed routinely to image and model biological objects, structures, and materials in computer graphics. It is used to create 3D digital models of anatomical structures, organs, and compound tissues. The scanned data are rendered into 3D reconstructions. Models of this kind are regularly used to create individualized medical implants and devices for patients, view anatomical structures from any viewpoint or angle, and measure physical properties and sizes with high accuracy. 3D printed products are in use for hip replacements, shoulder implants, knee implants, etc. The 3D imagery is used for surgical diagnosis, planning of operations, optimization, and practicing virtual surgical operations. Printed models provide realistic solid objects of the patient’s unique anatomy, in disease or trauma, for expert examination and education at any time. They give the surgeon an accurate future sense where to make speedy and exact surgical interventions and excavations.

Zoological materials and structures have been investigated in order to mine for attributes in solving key problems of survival and function, as well as making viable trade-offs between conflicting events, which together have analogies in biomedicine. Material solutions abound in zoology for all possible physical, mechanical, and chemistry based problems and trade-offs. Direct copying or partial copying of the function significant features is a strong option for exploiting design richness and diversity in the zoological world. Existing microfabrication and nanofabrication systems present an excellent opportunity for manufacturing high-quality replicas with pinpoint precision. Printing machines can produce copies of natural microstructures, for instance, sharkskin. In a seminal study by Wen et al., sharkskin (Isurus oxyrinchus) printouts were fabricated from X-ray images of a segment of the natural model material.[10] The X-ray microscope (XRM) datasets, at a 12× magnification, were fed into a computer-aided controlled 3D printer, employing Solidworks (3D CAD Software) volumizing software, to create high fidelity arrays of dermal denticles with individual dimensions of 1.5 mm. These reproductions demonstrated hydrodynamic properties similar to the native sharkskin and presented a flexible sharkskin patterned membrane aimed at studying the hydrodynamic properties in detail and for large-scale, high throughput translation.

Micromodel printing in 3D has approached limits between 1 and 100 nm using a series of technologies. Piezoactuators and galvanometers provide the high degree of control necessary to fabricate optic devices such as hemispherical 1 μm micro-lenses. However, there are no existing examples where the nanometric design of zoological materials has been reproduced using printing processes. Biological materials are intrinsically
hierarchical transcending the nanoscale, microscale, and macroscale. High-resolution 3D microprinting machines, implementing 2-photon laser beam technology printing machines (e.g., the Photonic Professional GT) have been engineered to mimic and duplicate intricate microscale/sub-micrometer biological structures, including the periodic arrays of crown-like hairs of *Salvinia molesta*,[11] nanostructured tarantula hairs with multilayered (300 nm thick, spaced 450 nm apart)[12] cylinders to recreate the structural blue coloration of the tarantula spider (*Araneae: Theraphosidae*), and microneedles (100 × 200 µm²) replicating the mosquito proboscis.[13] This technology is based on laser lithography techniques and levels of resolution approach 0.2 µm.

Notwithstanding the ability to generate droplets in the picometer size range, at levels equaling 6–10 pL in 3D (normal inkjet printers work with 60–70 pL droplets), opens up the possibility of drop printing intricate nanostructures and nanoprotuberances, such as hair, spinules, and pillars with high fidelity and in high definition.

Natural gecko skin is a multilaminar architecture and because it can be arranged planar, it is highly accessible to imaging and transfer to a technological product by microduplication techniques. We can consider three important strategies for the translation, fabrication, and production of a synthetic gecko skin copy for technology use (Figure 1). The first translation step is a choice between placing together the entire design criteria ready for mathematical modeling or computer modeling (A), undertaking high order imaging to carry through digital based replication procedures (B), physical biotemplating to straightforward create a solid biomimetic object (C). In a translation procedure (Figure 1A), the premeasured design features (i.e., from scanning electron microscopy (SEM) images) are computerized to control lithography and other micro/nanoprinting machines. This approach allows the design to be modulated and to hone the structure and individualize the rupturing mechanics on a larger range of cell morphologies and to increase the killing rates. In a translation project (Figure 1B), a digital image is generated, which provides the building blocks (pixels) for reconstruction and 3D printing. According to approach (Figure 1C), the skin is reproduced by casting and molding with synthetic materials, which in the case of gecko skin has been efficiently and effectively demonstrated before.[15,16]

It is often imperative that fabrication incorporates nanometric features of the design. The field of fabrication of small systems is young and there are few published examples of 3D printing featuring and duplicating nanoscale designs. One reason is the droplet scale and the other relates to the image detail. In this study, we show that with high power X-ray imaging it is possible to generate high-quality digital images of very intricate features and that are suitable for the programming of 3D printing.

We have applied high power X-ray imaging capabilities to image nanoscale spinules on the surface of ultrathin, shed gecko skin (*Lucasium sp.*) and their synthetic biotemplated

---

**Figure 1.** The different options available among biomimetic translation of zoological materials into micro and nanofabricated products for healthcare purposes. The gecko skin model can be copied using simple biotemplating methods to produce a negative stamp for microcontact printing. Alternatively, the design criteria of the model are entered into a computer design software package to produce a virtual model that is suitable for 3D printing. A third strategy and the topic of this study are to generate high-quality digitized images of the gecko skin at nanoscale resolution. The raw digital data are converted into a machine-printing computer-aided design (CAD) software file (StereoLithography STL. File format), which represents real shapes with a framework/mesh of tesselated triangles conforming tightly to the surfaces of 3D objects) in exactly the same way CT images are used to print 3D copies. Advances in 3D printing make it feasible to fabricate materials with nanometric features such as hairs and spines according to design criteria among natural analogs. The route for translation with gecko skin is highlighted in pink.
replicas made for biotechnology applications. Spinules are small surface protrusions that merge spine and hair morphologies. Skin surface protrusions, such as the small spines and longer setae, are expressed across all species of gecko-like lizards,[14–16] projecting large variations in length (2–5 \(\mu m\)) and spinule morphology (erect versus curved, bulbous versus straight). Spinule morphology is related to natural history and the position on the body surface. These have arisen in evolution for the self-cleaning of contaminants on the skin surface, in both dry and wet environments. Moreover, it has been discovered that the spinule arrays have a repellent property against surface associating bacteria. Infact, the spinules rupture bacterial cells on contact. At least four mechanisms (puncture, stretching, entanglement, compression) have been interpreted from SEM snapshots of the dynamic interactions between bacteria and spinules, predominantly at the nano-tips.[15,16] Furthermore, synthetic replicas projecting the key bactericidal design criteria of the spinules, identified by possession of nanotips, flexible mechanical properties, spacing less than the bacteria cell size, have shown similar antibacterial and bactericidal effects. High power imaging of the real skin and the replicas is necessary to resolve and clarify the true nature of the mechanisms involved and the fidelity of copies made by biotemplating and lithography. Moreover, high-resolution imaging will open-up a route toward the creation of 3D gecko printouts.

The gecko skin was specifically selected to demonstrate the capacity to digitally image some of the smallest zoological morphostructures for 3D printing with intricate structures that have previously shown to strongly repel and destroy surface associating microbes (including bacteria, yeasts, etc.). Thus, providing us with the possibility to prepare technological relevant structures for healthcare applications in microbial cell control and repellency at surfaces.

2. Results

The gecko skin and resin replica were imaged under a range of highly-accessible imaging techniques, found in most well equipped research centers, at increasing depth of scales, starting with an analogue viewing by fluorescence light microscopy (FLM) (Leica fluorescence microscope (DM5500) (Figure 2A), digitized (\(\mu CT\)) (Figure 2B), analogue SEM, so as to provide a base contrast for digital rendering work (Figure 2C,D), and finally (AFM), which can detect nanoscale features on planar surfaces (Figure 2E (natural) and Figure 2F(i) (resin copy)) to reveal and resolve the surface details of the gecko design and any new features at the skin surface. Thin sections of the shed gecko skin were stained with a mixture of fluorescent cell dyes, in phosphate buffered saline (PBS), to highlight the anatomical layers including the exterior keratinous oberhauchten layer with its protruding spinules, as viewed in cross-section. As viewed under 1000x magnification, the spinules can be just resolved (Figure 2A). In \(\mu CT\) image projections, the level of detail is confined to the microscopic scales of the skin (Figure 2B). AFM produced digital 3D reconstructions of natural gecko skin in details that were reminiscent of the spinule array as observed in SEM. However, the distal morphology failed to represent the tapering and the fine nanotips (Figure 2E,F(i)).

Spinules that cover the body, except the toepads, are evidenced as survival adaptations for self-cleaning and their shape and size correspond strongly to the prevalence of dirt particles in the habitat.[17] In the laboratory, cultures of the spinule arrays have shown significant ability to prevent microbial attachment, of selected oral pathogens and \(E.\ coli\), and the prevention of life-threatening biofilms[16] (Figure 2F(i) smooth surface versus Figure 2F(ii) natural gecko spinule array).

Two types of image synthesis, confocal laser scanning microscopy (CLSM) (slit confocal) and synchrotron X-ray microscopy, were investigated as candidates to generate digital data sets desirable for 3D printing. High fidelity digital images are convertible into shadow data for guidance and control of robotic printing devices. It is conventional to create images by \(\mu CT\), but this does not formulate structures and morphologies at nanometric scales (<1 \(\mu m\)) (Figures 1 and 2). The resolving power of conventional \(\mu CT\) is detectable at the minimum of 1 \(\mu m\) and a voxel size of 2 \(\mu m\).[18]

Resolution is defined by the beam width, which is 50 nm. When imaging soft tissues of gecko skin, \(\mu CT\) gives a poor contrast of soft anatomical structures.[19] However, increased computer analyzing techniques and power to measure spectral contrast and refraction properties offer to resolve details in higher contrast at least in microscale dimensions. Furthermore, \(\mu CT\) has proven a valuable tool for visualizing arthropod neurological anatomies and brain structures of \(Drosophila melanogaster, Apis mellifera,\) and \(Tribolium castaneum\).[19]

2.1. Confocal Laser Scanning Microscopy Imaging of Gecko Skin Surface

An alternative method explored was CLSM at 1000 times magnification and at varied 10x–15x digital zoom where more details are revealed. We avoided zoom above 20x, where pixels are increased in size; leading to increased spatial resolution. Confocal imaging resolves at 0.5 \(\mu m\). According to the Nyquist stability criterion, then 2–3 pixels are needed to resolve the smallest nanoobject given the maximum possible resolution of the microscope. The experiment was organized to optimize the pinhole size (1AU), pixel resolution, and z-plane sampling (at an average of 35–50 Z-stacks constructing the 3D compound image equal to 100–70 nm slices spanning the gecko skin), above sampling time, signal intensity, and gain/offset values. The samples were stained with common cell and tissue fluorescence dyes (Dextran-FITC, CMFDA and FITC conjugated Alginade), which impregnated the surfaces of the spinules and the base plate. Spinules were coated down to their base; however, the tips remained somewhat poorly delineated and were frequently missing from the scanned image. Moreover, the natural curvature of the spinules is absent from the laser-scanned images.

Natural (shed) gecko skin samples and gecko skin replicas that were imaged under CLSM, showed impressive rendition of the spinule arrays in organization and morphology (Figure 3A–C). The entire shaft of the spinules together with the correct shape and morphology was tightly replicated as highlighted. Image datasets
from confocal imaging were transformed using a meshing and volumizing software system to create a 3D reconstruction (Figure 3D) (Mimics Innovation Suite 20.0, Materialise Mimics software).

The silica resin copies did not easily adsorb the dye causing weakly visible fluorescence images, whereas polystyrene copies emitted a strong fluorescence signal at the same intensity. Moreover, this was virtually identical to the natural gecko skin.
spinule arrays in the height (3–4 µm), shaft thickness (200 nm), and 500 nm spacing (Figure 3A). The important limits of confocal laser imaging relate to the number of photons entering the specimen, photodamage of the specimen, and the counting statistics of the computing software.

2.2. High-Power X-Ray Imaging of the Gecko Skin Surface

The Zeiss Xradia 810 ultrasystem was capable of imaging natural gecko skin, nondestructively at 150 nm resolutions. Samples of natural gecko skin and replicas were mounted on polyimide films (Figure 4A(i,ii,iii)) and imaged under phase contrast (Figure 4B,C). The Zernike phase contrast optics enabled high-contrast images to be generated from the biological materials of the gecko skin and resinous polymers, which show low attenuation (Figures 4 and 5). The shapes, morphology, distribution, and dimensions of spinules were very easily rendered and observed with exceptional detail as shown in cross-section (Figure 4D versus Figure 4E). The copies translated with high fidelity although there were areas in which spinules agglomerated, or had fallen horizontally as shown in face view (Figure 4F versus Figure 4G). Dragonfly software was then used to reconstruct the surface structures in 3D virtual space. The spinules covering natural gecko skin were clearly delineated and defined with hard edges. The density and spacing (500 nm) were identical to SEM images of the spinule array. The hair shape and dimensions of the spinules were accurately represented and included the tips with similar angles of curvature (Figure 4A–E).

2.3. Expansion of Gecko skin Patterned Replicas for Greater Participation in Technology

The final link in the chain of translation has to be the large area duplication of the gecko skin small structures defined by exact precision and accuracy in a chosen polymer. For the purpose of speed and efficiency, it is all but necessary to employ automated machinery for the direct fabrication of the structures over large areas of synthetic polymer (e.g., polycaprolactone or polystyrene) membranes and films and potentially fused onto textiles. This is because conventional biotemplating introduces errors in the outline shape, morphology, and standing position of synthetic spinules. While the structures were accurately replicated at high fidelity, in terms of positioning and general shape (Figure 5 versus Figure 6), the precision of curvature and tip formation varied slightly. Approximately 5–10% of the surface provided inaccurate replication between consecutive copies and zonally across each individual sample (Figure 5A–C versus Figure 6A–C). As observed in the siloxane resin copies (Figure 6), there were focal areas of the duplicated spinule array that are largely populated with fallen spinules.
Figure 4. Sample preparation, scanning parameters, and immediate results of image analysis. A) The mounted sample was cut into small shapes (i, ii) and glued on top of a pin (iii). B) The actual X-ray generated image of the mounted gecko copy (i) side view; (ii) face view. C) The X-ray generated image of the mounted natural skin (i) face view; (ii) side view. D) High powered side view of the natural gecko skin showing the spinules in alignment. E) High powered side view of the replica gecko skin showing the spinules in alignment. F, G) The full-scale X-ray microscope image of the natural spine array. F) 3D image rendition of the natural gecko skin. G) 3D image rendition of the resin replica gecko skin (the table segment displays the optimized X-ray microscope scanning parameters).

Figure 5. Digital representation of the natural gecko skin (shed during molting) in three different plane slices across the individual sample. A) The combined individual plane segments spanning the sample. B) The low field of view section through the natural gecko skin. A) The obliquely positioned low field of view section through the natural gecko skin; B) A face view of the gecko skin in thin section; C) The final volumised 3D reconstruction of natural gecko skin, which is concave.

Figure 6. Digital representation of the gecko Resin copy in three different plane sections across the individual sample. A) The combined individual plane segments spanning the sample. B) The low field of view section through the replica (resin) gecko skin. C) The obliquely positioned low field of view section through the replica (resin) gecko skin. A) The obliquely positioned low field of view section through the replica (resin) gecko skin; B) A face view of the replica (resin) gecko skin in thin section; C) The final volumised 3D reconstruction of natural gecko skin, which is partially flat and concave.
areas (sheets of materials in m²) is not feasible by the biotemplating method, since each individual duplicate would typically measure a maximum of 15–20 cm (length) × 10 cm (width), if a single shed gecko pelt of *Lucasium sp.* was actually imprinted in one whole piece. Moreover, the 3D images of spinule arrays accrued from the X-ray scan data, using DragonPro software compare favorably, in terms of gross morphology, with life-like SEM snapshots (Figure 2C,D).

As a result, the printed and imprinted spinulated surfaces are able to produce polymer surfaces with much greater technological applicability for healthcare, nonwetting textiles materials, and optical surfaces. The large area (m²) duplication compels itself toward automation with high rates (mm² min⁻¹) of gecko pattern transfer. This can be done via two outstanding soft lithography techniques with a capacity to generate high aspect ratio structures (>10) in the nanorange of the spinule tips (50–75 nm). The two techniques are IBL and NIL. An alternatively feasible microfabrication, nanofabrication method is 3D inkjet printing. Projection of nanoscale objects by 3D inkjet printing is untested and reaches the physical limits of the printing process, because droplet sizes must be picolitre volumes or lower and the degrees of freedom of movement in the 3D x,y,z cartesian planes are semi-restricted by the robotic acuity and flexibility. Printing in this manner is surface area restricted, but generates the structure with high precision and accuracy required for effective antimicrobial function.

### 2.4. 3D Volumization and Solid Object Projection Using MeshLab Software

In the final part of the study, in preparation for projecting the digital information into models for 3D printing, we generated digital reconstructions of the natural skin with full volumization settings, which turned them into on-screen solid objects. SEM images of the gecko skin provide the true structure and morphology of the gecko skin surfaces and their protruding structures. For the purpose of this study, we assumed that highly accurate replication of gecko spinules is necessary to perform the technical function of rupturing “surface associating” microbial cells of certain sizes, shapes, and architecture. Therefore, we assessed the successful translation from real-world origin to the solid model object in reference to the SEM images (Figure 2C,D and Figures 7B and 8B). Clearly missing was the reticulated floor supporting the spinules (compare Figure 2C,D and Figures 7B and 8B with Figure 9A–F). However, the spinules were reminiscent of the “real-world” SEM images in the majority of functional aspects (i.e., presence of tips, aspect ratio, the degree of curvature, tapering shape, and erect posture). For instance, they possessed stout bases that tapered into thin tips that also followed a curvature. The spinule array patterning was preserved, as was density of spinules when compared directly with SEM viewed spinule morphology.

All duplicated vertices were unified to generate the first volumized meshwork projection and a degree of smoothing when compared directly with SEM viewed spinule morphology. Moreover, quantitative comparisons were undertaken using the editing and measurement software, between the natural skin sample and the copy to gauge the copying fidelity. Arbitrary measurements of spacing and spinule height between natural and replicated gecko skin show high similarities according to the programmed measurement tool. Spinule height for the natural skin averages 1.5 versus 1.38 for the copied version, and interspinule spacing measuring 0.5 measuring units closer among the natural skin spinule array. Moreover, the natural skin required a meshwork projection that comprised of 3.8 million vertices and 7.7 million faces (Figure 9B,C), whereas the copy required 1.9 vertices and 2.4 faces (Figure 9E,F), to properly reconstruct it in a digital space. Thus, the digital translation from X-ray data to stenography (STL) file editing software is arguably the most detailed and accurate pathway for the highest fidelity copying, and particularly for copies where a high level of accuracy is an absolute necessity for maximum functioning. However, we would require a suitable microfabrication machine that can physically print designs of this scale and intricate detail.
Following the rendering process, the spinules reduced in thickness and the tip became more pronounced. Moreover, this powerful rendering software, therefore, allows redesign of the spinule morphology and this could be important in tailoring functions for controlling microbial cells beyond the original natural design. The nanotips, curvature, and spacing are key design criteria involved in the bacterial destruction.\cite{14}

3. Discussion and Conclusions

Imaging of zoological and botanical specimens, their materials, and structures are needed in increasingly small scales to decipher the inventive principles of design via trade-offs that drive evolution. This is more relevant at the nanometric scale, which defines incredible properties at the macroscale and organism scales of functionality. Synthetic mimicry of zoological and botanic objects by nanoengineering remains exceedingly difficult because of miniaturization involved, hierarchical organization, compound materials, and morphological complexity. However, methods in laser lithography and IBL have recently highlighted, in multiple instances, the feasibility of sculpting soft polymer interfaces with genuine biomimetic nanostructures made of pits, hair-like structures, and posts. In applying these techniques, the design criteria of the natural models are measured and transcribed into controlled spatial movements of the ion beam or laser beam coupled with the timing of application.

Accurate imaging of biological structural complexity at the nanoscale is necessary for conversion and translation through microfabrication routes. We show that high-powered X-ray imaging can generate high quality images at nanoscale resolutions approaching 50 nm, and other methods of image quality, such as full reference metrics comparing them to SEM image of the spinules. The digital information collected contains sufficient pixels, and therefore bytes of data, to resolve the spinules within the computer aided design (CAD) software. This is perfectly acceptable to program the operation of robotic printing tools.

Technically, the Zeiss Xradia 810 Ultra generated high-quality images of gecko skin and the artificial copies of gecko skin, at a resolution of 150 nm in low field of view (LFOV) setting. This imaging system provided a 10x increase in resolution compared to CT scanned image results. The CLSM super-resolution, at best, resolved structures measuring 300–400 nm. Equipped to the image in the Zernicke phase contrast mode, allowed for high contrast images of shed gecko skin, which is highly attenuated and sensitive to damage by particle radiation. However, the high power X-rays exposure did not damage the shed gecko skin, which is less than 1 µm thick and is prone to tearing and rupture. The shape and dimensions of spinules were clearly delineated. However, among the copied version of spinules, these had a tendency to agglomerate and there were noticeable regions of the surface containing fallen spinules. Furthermore, the DragonPro rendering software expressed the power of examining and modeling the gecko skin spinules and for taking snapshots of the direct and indirect interactions between different bacterial cells and the spinules under different conditions. We could generate virtual cross-sections, delay the object at any conceivable angle and Cartesian plane, and zoom to different levels of scale.

The raw data sets generated in this straightforward imaging study were easily converted into a stenography file format, which applies a triangular meshwork to the X-ray scanned gecko surface spinules. The application of the stenography formulated (STL) data into a processing and editing terminal (Meshworks open source STL conversion software) allowing us to project the solid objects in 3D and manipulate and prepare them for 3D printing. The spinules were replicated in virtual world with sufficient precision and accuracy to ensure they function properly in microbial killing efficacy. For instance, the spacing was uniform and the upright spinules conformed to the exact morphology of the real-world spinule among the key design criteria.

X-ray microscopy is an important, strategic tool for biomimetic exchanges and translation between advanced
4. Experimental Section

The Natural Shed Gecko Skin: Shed gecko skin was collected in natural habitats of the *Lucasium sp.* in southern Queensland, Australia as previously described with the necessary ethical and ecological approval.[15,17]

The Synthetic Resin Skin: Resin copies were produced according to the method of Vucko et al.,[17] which involved a simple, stepwise casting and molding transaction on the surface of live geckos, to imprint fully inflated spinules, using 2-types of siloxane polymer substrates.

Fluorescent Compound Microscopy: Ultrathin histological sections of gecko skin, in cross-section, were prepared in wax. The sections were prepared as normally for histological staining. Sections were
to a solution of PBS solution containing one of the following fluorescent dyes: TO-PRO-3 (a nucleic acid binding stain from Thermo-Fisher Scientific), CellTracker Deep Red and CellTracker Green CMFDA (Thermo-Fisher Scientific), Dextran-FITC (Fluorescein-isothiocyanate-Dextran, Sigma-Aldrich), and alginate-fluorescein (AL-500, low viscosity 100–300cP, Creative PEGworks). Images were created using a Leica microscope (MD5500D; Leica, camera: DFC495; Leica, Lens: HCX PL APO 409; Leica).

The X-Ray Microscope: The X-radia 810 ultra 3D X-ray microscope, used in this study, comprises a high brightness X-ray source with a high-efficiency condenser. Zernike phase contrast optics was employed and focused onto a high-efficiency X-ray detector (CCD camera) and extending into a precision tomography system. The Xrada 810 Ultra increased the throughput of the nanoscale, 3D X-ray imaging by up to a factor of 10 compared to normal X-ray microscopic imaging. The XRM operated at 5.4 keV, at low energy levels, delivered high contrast and image quality for medium to low Z samples.

Sample Preparation for X-Radia System: Cutting triangular sections from the polyamide tape mounted samples with a razor blade prepared the gecko skin samples. The triangular sections were glued to the end of a steel pin and imaged near the tip of the formed triangle, (see Figure 3B).

AFM and Imaging of the Gecko Skin: Use of AFM can lead to digital imaging of nanoscale structures occurring on a flat plane. AFM was applied to the natural skin to see if it was possible to generate a digital model of the spineule array mirroring, exactly the morphology from SEM, which is the most realistic representation of the spineule array (see Figure 2). We used a NF mode with a data width and height of 256 pixels and a 30 µm X and Y scan, at 0.3 Hz, with tapping amplitude of 51.64 nm at a Frequency of 271 Hz.

Microcomputed Tomography Imaging of Gecko Duplicate: µCT imaging was carried out on natural shed skin segments and the resin (siloxane) replica using the SkyScan 1076 High Resolution computed tomography (µCT) (SkyScan, Bruker, Belgium). 3D composite images were created using Bruker-microCT CT-Analyser v. 1.13 software.

Laser Confocal Microscopy: Gecko samples were scanned, digitized, and imaged using the Zeiss LSM-800 laser scanning confocal microscope, aided by LSM imaging and image processing software. Specimens were viewed either under FITC or TRITC frequencies in the Z-stack mode to generate 3D images spanning across 4 µm of the gecko skin basal layer and spineule protuberances.

Sample Preparation for Laser Confocal Imaging: Shed gecko skins, representing the skin’s uppermost beta keratin, Oberhauchten layer, see refs. [16,17] for further gecko skin details, and resin replicas made by casted and molded biotemplates[17] were soaked in a PBS solution containing one of the following fluorescent dyes: TO-PRO-3 (a nucleic acid binding stain from Thermo-Fisher Scientific), CellTracker Deep Red and CellTracker Green CMFDA (Thermo-Fisher Scientific), Dextran-FITC (Fluorescein-isothiocyanate-Dextran, Sigma-Aldrich), and alginate-fluorescein (AL-500, low viscosity 100–300cP, Creative PEGworks). To overcome the high hydrophobicity and thus ensure that the dye penetrated every spineule surface, down toward the base, the samples were forcibly immersed in the PBS solutions to propel the solution in between the spinules (see Figure 3).

Sample Image Production from Laser Confocal Microscope Data: Zeiss LSM-5 image browser software was initially used to frame the image space, orientation, magnification, and display characteristics. Completed images were transferred to Picasa software for the final modulization of spineule array images.

FM: Shed gecko skin was placed onto a thin layer of distilled water covering a glass microscope slide and dried for 30 min leading to strong adhesion to the slide. Distilled water containing a mixture of Dextran-FITC, CMFDA, and FITC conjugated Alginate fluorescein dye at concentration designed for cell staining was added dropwise onto the gecko skin. To overcome the innate hydrophobicity of the skin surface, a coverslip was pressed onto the dye solution firmly ensuring the liquid to enter the interspinule spaces and removing the tight pockets of air.

SEM: Shed gecko skin was placed onto a thin layer of distilled water covering an SEM stub. The water provided turgor pressure for the spinules and facilitated adhesion of the skin to the glass slide. The gecko skin topped SEM stub was dried in an oven at 40° for 30 min. No fixative was applied, as this could change the shape and orientation of the spinules.

Sample Imaging Using Dragonfly Pro Visualization Software: Individual natural gecko skin and a resin copy were viewed in the LFOV mode and rendered with 64 nm voxels produced after twelve and half hours total scan with high-powered X-rays through Zernike phase-contrast. Dragonfly Pro software by ORS showed reconstructed into various 2D slices. The software enabled surface screening and selection of any part of the sample for direct high powered visualization, and to quantify the morphologies of the visualized structures and morphologies on display.

Sample Image Analysis with Dragonfly Pro Software: The digital images can be reconstructed in a number of ways; however, here the images were formed into 2D slices. This can be achieved in various planes and positions. A quadrant generated and selected equaled a specific orthogonal virtual slice. In 1–4 times zoom mode, the virtual slices could be resolved at 150 nm.

3D Solid Object Reconstruction of the Spineule Array Using MeshLabs (2016.12) Software: The raw digital data amassed via X-ray imaging was converted into stenography (STL) Meshworking file formats and then loaded into a meshwork reading and volumizing open-source software program, MeshLabs (2016.12) based on the GitHub code. Meshlabs software is an open source project and was downloaded from http://www.meshlab.net. This allowed us to project the spineule based X-ray image data into a 3D virtual reality. In addition, this package provided the editing and processing interface to fabricate a solid object of the Gecko spineule array pattern. Moreover, the software enabled initial projections of the 3D solid object were to be analyzed, cleaned, fully rendered, and textured. Furthermore, the software could facilitate the redesign and modification of spineule morphology and architecture for further optimization.

Acknowledgements

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI14C3266). This research was financially supported by grants from the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (NRF-2017M3A9B3061833).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

3D bioimaging, CLSM, gecko skin, nanoscale imaging, X-ray microscopy

Received: February 5, 2018
Revised: February 7, 2018
Published online: May 2, 2018
