



Title	Advanced Interferometry with 3-D Structured Illumination Reveals the Surface Fine Structure of Complex Biospecimens
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1 **Title**

2 Advanced Interferometry with 3-D Structured Illumination Reveals the Surface Fine Structure of
3 Complex Biospecimens
4

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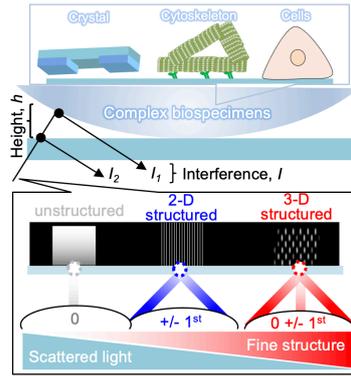
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39 **Notes**

40 The authors declare no competing financial interest.
41

42 **Abstract**

43 Interference reflection microscopy (IRM) is a powerful, label-free, technique to visualize the surface
44 structure of biospecimens. However, stray light outside a focal plane, obscures the surface fine
45 structures beyond the diffraction limit ($d_{xy} \sim 200$ nm). Here, we developed an advanced interferometry
46 approach to visualize the surface fine structure of complex biospecimens, ranging from protein
47 assemblies to single cells. Compared to 2D, our unique 3-D structure illumination introduced to IRM,
48 enabled successful visualization of fine structures and the dynamics of protein crystal growth under
49 lateral ($d_{x-y} \sim 110$ nm) and axial ($d_{x-z} \leq 5$ nm) resolutions; and dynamical adhesion of microtubule fiber
50 networks with lateral resolution ($d_{x-y} \sim 120$ nm), ten times greater than unstructured IRM ($d_{x-y} \sim 1000$
51 nm). Simultaneous reflection/fluorescence imaging provides new physical fingerprints for studying
52 complex biospecimens and biological processes such as myogenic differentiation; and highlights the
53 potential use of advanced interferometry to study key nanostructures of complex biospecimens.
54

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57 **Keywords**58 Interference reflection microscopy, Structured illumination, Crystal growth, Microtubule fiber network,
59 Cell Adhesion.60
61 **Main text**62 Interference reflection microscopy (IRM), is one of the leading, label-free, techniques for visualizing
63 nanoscopic axial topography¹⁻³ using the interference between light reflected from the surface of the
64 biospecimens (I_1) and substrate (I_2) (**Figure 1A**). Due to the interference intensity (I) in monochromatic
65 IRM images depending on height (h), thus the greatest advantage of IRM is to evaluate h at the
66 resolution of the sub-nm scale in a label-free manner³. However, conventional IRM setups adopt
67 homogenous illumination light⁴; where stray light from the internal structure of complex biospecimens,
68 obscures the surface fine structures beyond the diffraction limit ($d_{xy} \sim 200$ nm).69 Alternatively, various super-resolution “fluorescence” microscopies, such as stimulated emission
70 depletion microscopy (STED), photoactivated localization microscopy (PALM), and stochastic optical
71 reconstruction microscopy (STORM), structured illumination microscopy (SIM) have been well-
72 developed^{5, 6}. Among those techniques that rely on specialized nonlinear properties of fluorophores^{7,}
73 ⁸, SIM achieved lateral super-resolution via non-restrictions on fluorophore types using only its
74 instrumental aspect (*i.e.*, Moiré fringes). Moreover, for obtaining fluorescence images of complex
75 biospecimens such as cells, optical sectioning via 3-D structured illumination efficiently eliminates
76 stray light outside a focal plane (*e.g.*, organelles, cytoskeleton, etc.) that obscure fluorescence
77 signals⁹. However, the axial resolution of conventional SIM is approximately in the range of several
78 hundred nanometers, which is insufficient for imaging surface nanostructures of complex
79 biospecimens.80 To overcome long-standing limitations of IRM to visualize complex biospecimens with sufficient
81 lateral resolution, we combined such optical sectioning concepts of 3-D structured illumination with
82 IRM (*i.e.*, 3-D SIM-IRM). We introduced structured illumination as a light source to the IRM optical
83 setup (**Figure 1B bottom**, further details: **SI method**), and prepared three illumination patterns using
84 diffraction grating^{7, 8} (**Figure 1B top**): unstructured illumination (0th-order diffraction light), 2-D
85 structured illumination on the lateral plane (interference among 1st-order diffraction light), and 3-D
86 structured illumination along the optical axis (interference between 1st- and 0th-order diffraction light).
87 Based on the optical setups, in this work, we systematically compared the image contrast of
88 biospecimens between unstructured and 2-D/3-D structured illuminations. Further, we visualized the
89 molecular organization and dynamics of complex biospecimens, such as protein molecular
90 assemblies (crystals, cytoskeleton) and single cells (**Figure 1A**).91 Before visualizing complex biospecimens, we first systematically verified the IRM image contrast of
92 a polystyrene bead on a glass substrate under un-/2-D/3-D structured illumination (**Figure 1C**). Here,
93 the Moiré pattern (real space) for interference images was also visible, as in the case of fluorescence
94 images (**Figure S1A, S1B, magnification**), leading to the success of image reconstruction for IRM
95 images. In contrast to IRM images under unstructured illumination producing untargeted interference
96 (*i.e.*, speckles), Newton’s ring-like fringes in IRM images were clearly observed under 2-D and 3-D
97 structured illumination. **Figure 1D** shows intensity profiles according to the dotted line in **Figure 1C**.
98 The result also supports that optical sectioning via 3-D structured illuminations eliminated scattered

99 light from untargeted planes, thereby enhancing the contrast with low noises. Second, we verified the
100 qualitative lateral resolution of obtained IRM images via a fast Fourier transform (FFT) analysis for
101 **Figure 1C**. FFT space images showed a flower-like structure in frequency space (**Figure 1E**) which
102 was also seen in the case of our fluorescence SIM data (**Figure S1B, bottom**) and other group's
103 ones¹⁰ implying the lateral resolution reaching the super-resolution regime. We also analyzed the FFT
104 power spectrum (**Figure 1F**) of the intensity profile along the dotted line in **Figure 1E**. In the case of
105 unstructured illumination used, FFT power exponentially decays to reach a plateau, which is the
106 potential noise level (mean power = 90 ± 7). It should be noted that the FFT power under unstructured
107 illumination does not be beyond the diffraction limit ($f = 5 \mu\text{m}^{-1}$, corresponding to $d_{xy} \sim 200 \text{ nm}$) which
108 is defined by the abbe equation. In contrast, FFT power under 3-D structured illumination is above
109 noise level till the $f = 8.5 \mu\text{m}^{-1}$ ($d_{xy} = 118 \text{ nm}$) which is surely beyond the diffraction limit. Thus, these
110 FFT power spectrum analyses suggest 3-D SIM-IRM reaches the super-resolution regime. It should
111 be noted that the FFT power of typical fluorescence SIM¹⁰ also reaches the background noise at a
112 comparable frequency of 3-D SIM-IRM (data not shown). To evaluate whether IRM's theoretical bases
113 for height reconstruction remain in 3-D SIM-IRM, we reconstructed height profiles according to the
114 dotted line in **Figure 1C**. Here, interference intensity generally can be written as **Eq. (1)**:

$$115 \quad I = I_1 + I_2 + 2\sqrt{I_1 I_2} \cos(4\pi n h / \lambda + \pi)$$

116 and previous works derive practical equations to convert I to h assuming quasi-normal incidence³ as
117 **Eq. (2)**:

$$118 \quad \frac{2I - (I_{\max} + I_{\min})}{-(I_{\max} + I_{\min})} = \cos\left(\frac{4\pi n}{\lambda} h\right)$$

119 where I is the measured intensity, I_{\max} and I_{\min} are the maximum and minimum intensities, respectively.
120 λ is the wavelength of light, n is the refractive index of the medium (~ 1.333). Reconstructed height
121 profiles under unstructured illumination led to severe rough surfaces (**Figure 1G**) which is influenced
122 by the untargeted speckle pattern in **Figure 1D**. In contrast, the reconstructed height profile of the
123 polystyrene bead under 3-D structured illumination is in good agreement with its predicted height
124 profile ($\Phi = 100 \mu\text{m}$) up to $0.15 \mu\text{m}$ from the surface. It should be noted that the most crucial point is
125 to visualize the newton fringes with enhanced contrast, and the success in reconstructing height
126 profiles of beads. Based on these results, we revealed that 3-D structured illumination eliminated
127 scattered light from untargeted planes, thereby reaching a lateral super-resolution regime that was
128 validated in FFT power spectrum analysis. We also revealed that IRM's theoretical bases for height
129 reconstruction can be valid for SIM-IRM.

130 As a first trial to visualize the fine structure of "actual" biospecimens beyond the diffraction limit (d_{xy}
131 $\sim 200 \text{ nm}$), elementary steps on the surface of protein crystals were monitored, which also can provide
132 deeper insights into the fundamental mechanism of crystal growth¹¹⁻¹³. The crystals of hen egg-white
133 lysozyme (HEWL), widely used to study the growth mechanism of a protein crystal, were placed on a
134 glass substrate using beads (diameter $\sim 100 \text{ nm}$) as spacers (**Figure 2A**, sample preparation: **SI**
135 **method**). 2-D island-like etch pits^{14, 15} of dissolving (110) surface of HEWL crystals was visualized
136 using 2-D/3-D structured illumination, whereas there was less contrast of pits with speckles using
137 unstructured illumination (**Figure 2B**). Visible dark spots under the 2-D/3-D structured illumination are
138 spacer beads (yellow arrowhead). Furthermore, not unstructured but 2-D/3-D structured illumination
139 visualized multiple etch pits (**Figure 2B right**) highlighting the impact of the optical sectioning effect
140 of 2-D/3-D SIM-IRM on crystal surface visualization. Interestingly, there were different interference
141 signals of dissociating crystal surface under 3-D structured illumination (**Figure 2C**); thus we
142 reconstructed the corresponding height image using **Eq. (2)**. Here, I_{\max} was carefully determined not
143 from the crystal-solution interface but from the crystal-substrate interface (**Figure S2A, S2B**) as like
144 our previous studies^{16, 17}. Height profiles across the pits (red arrow) exhibit a height of $h \sim 5 \text{ nm}$, which
145 is in good agreement with that of the elementary step of HEWL crystals^{14, 15}. In addition, height profiles
146 across the other pits showed multiple layers of the elementary step of HEWL crystals^{14, 15} (blue arrow:
147 $h \sim 20 \text{ nm}$ (4 layers), red arrow: $h \sim 40 \text{ nm}$ (8 layers), suggesting the presence of a spatial distribution
148 of impurities and defect on the crystal surface (**Figure 2D**). Furthermore, 3-D SIM-IRM sensitively
149 detected wide varieties of nano-structures during the dissociation of protein crystals (**movie S1.avi**).
150 For instance, 2D-like islands were clearly detected (asterisk, **Figure 2E**) and the radial expansions
151 fused with each other. Topological profiles across both islands indicated the 2 layers of proteins ($t =$
152 0 s , $z \sim 10 \text{ nm}$, **Figure 2F**) leading to the formation of fine pits (circle, $t = 60 \text{ s}$, $xy \sim 100 \text{ nm}$, $z \sim 2 \text{ nm}$).

153 In the other spots after step fusions, multistep-like structures were formed "inside" etch pits (rectangle,
154 **Figure S2C, S2D**, $xy \sim 130$ nm, $z \sim 1$ nm). These results clearly indicate that 3-D structured
155 illumination enhances the contrast in IRM images for crystal growth, leading to visualizing fine
156 structure beyond the diffraction limit ($d_{xy} \sim 200$ nm). In addition, the evaluation of morphological
157 changes inside the etch pits is also crucial in unveiling the mechanism of various crystal dissociation¹⁸.
158 Such different nano-scale dissociation dynamics of proteins also provide physical insights into the
159 spatial distribution of impurities and defects. We succeeded in dynamical imaging (0.5 frames/sec) for
160 dissociating crystals; further high-speed imaging will provide deeper physical insight into the surface
161 dynamics of biospecimens, where the rotary speed of diffraction gratings limits the temporal resolution.
162 Interferometric scattering microscopy (iSCAT) combined with a spinning disk succeeded in visualizing
163 the high-speed dynamics of nanoparticles inside cells¹⁹. Even though the iSCAT did not focus on the
164 surface topological profiles, such a fundamental solution combined with high-speed rotating grating
165 will improve the fundamental frame ratio. Thus, we highlight the advantage of our 3-D SIM-IRM with
166 fine lateral and axial resolution in a label-free manner, while observations with fluorescence labeling
167 and scanning probes potentially disturb the native surface and environmental properties during crystal
168 growth²⁰.

169 Next, we verified the impact of 3-D SIM-IRM imaging on complex biospecimens (*i.e.*, three-
170 dimensional networks of microtubule fibers). The motor proteins (kinesin) functionalized on a glass
171 substrate drive the motion of the deposited microtubule fiber networks in the presence of chemical
172 energy (adenosine triphosphate, ATP) (**Figure 3A**, sample preparation: **SI methods**). This is
173 regarded as an *in vitro* complex model of the cytoskeleton and protein-based actuators²¹. Despite the
174 fluorescence imaging identifying the presence of fibers near the glass substrate, IRM under un-/ 2-D
175 structured illumination could not visualize the adhesion of microtubule fiber networks (**Figure 3B**). In
176 contrast, the 3-D structured illumination facilitates the visualization of fiber-like structures in IRM and
177 fluorescence microscope (FLM) images, even highlighting the impact of 3-D structured illumination to
178 reduce the stray light from complex biospecimens. According to the intensity profiles across the fibers,
179 3-D SIM-IRM enabled the visualization of single fibers (~ 130 nm) beyond the diffraction limit ($d_{xy} \sim$
180 200 nm, **Figure 3C**). To quantitatively confirm the enhancement of contrast of microtubule fibers via
181 the introduction of structured illumination, the less-condensed microtubule fiber networks were
182 imaged (**Figure S3A**). Introducing 3-D structured illumination enhanced the signal-to-noise (SN) ratio
183 five times higher than unstructured illumination (**Figure S3B**). The contrast enhancement highlights
184 the impact of not 2-D but 3-D structured illumination to reduce light scattering from internal fiber
185 networks away from the glass substrate. The interference intensity of fibers corresponds to the fiber-
186 substrate distance according to **Eq. (1)**; thus, we reconstructed height images from a 3-D SIM-IRM
187 image (**Figure 3D left**). 3-D SIM-IRM clearly distinguished whether the fiber is closer (dark line, white
188 arrowheads) or farther (white line, white asterisk) from the substrate, whereas fluorescence imaging
189 under 3-D structured illumination (*i.e.*, 3-D SIM-FLM) identifies the presence of fibers near the
190 substrate (**Figure 3D right**). It should be noted that there was no spatial correlation between
191 reconstructed height and fluorescence intensity, which was different from the case of cells as
192 described below (**Figure 4**). However, these results highlight the impact of simultaneous 3-D SIM-
193 IRM/-FLM imaging leads to a quantitative assessment of the position of microtubule fibers along the
194 optical axis. Next, we applied 3-D SIM-IRM to dynamic microtubule fiber networks with motor protein
195 activated by ATP hydrolysis (**movie S2.avi**). 3-D SIM-FLM monitored the locomotion of a single
196 microtubule fiber to meet the other fibers at $t = 148$ s (**Figure 3E left**). However, the vertical positional
197 relationship between fibers could not be obtained. In contrast, height reconstruction from 3-D SIM-
198 IRM images using **Eq. (2)** indicated that locomoting fibers (red arrowhead, $t = 148$ s) showed 2-4
199 times higher than the ones of tightly contacted to the substrate ($h \leq 40$ nm, blue arrowheads) (**Figure**
200 **3E middle**). The results suggest that the locomoting fiber moved on top of the other fiber, and then
201 showed loose contact during movement (**Figure 3E right**). Although conventional IRM studies
202 evaluated the locomotion speed of isolated microtubule fibers^{22, 23}, highly crosslinked protein fiber
203 networks increased the scattered light from internal fiber networks as seen in **Figure 3A**. Thus, we
204 succeeded in visualizing such dynamical adhesion and its locomotion of the fiber network using fine
205 lateral and axial information which conventional IRM and fluorescence imaging techniques cannot
206 achieve.

207 To highlight the impact of simultaneous 3-D SIM-IRM/-FLM imaging for further complex
208 biospecimens, we visualized the adhesion of a single myoblast cell (expressing fluorescent F-actin).
209 In contrast to unstructured IRM/FLM only detected larger dark patch-like structures with blurred
210 fluorescent fibers, 3-D SIM-IRM/-FLM visualized dark and mesh-like structures of the cell membrane
211 which was supported by a fine network of fluorescent actin fibers (**Figure 4A**). Magnification of 3-D
212 SIM-IRM in **Figure 4A** clearly detected fiber-like structures with dark contrast (**Figure 4B**, arrowhead)
213 and the size (~ 130 nm) was beyond the diffraction limit ($d_{xy} \sim 200$ nm) (**Figure 4C**). These results
214 highlight the impact of optical sectioning via 3-D structured illumination, that efficiently eliminates stray
215 light outside a focal plane (e.g., organelles, cytoskeleton, etc.) that obscured fluorescence signals⁹.
216 Next, we obtained a spatial correlation between the cell membrane and cytoskeleton structures in cell
217 adhesion. Height reconstruction from 3-D SIM-IRM images using **Eq. (2)** enables us to classify cell
218 adhesion area into tight contact area (T) and loose contact area (or unadhered area) (L) at the
219 threshold of cell-substrate distance, $h \sim 40$ nm (**Figure 4D left**), which is the comparable size to the
220 extracellular domain of the cell adhesion molecules¹⁷. The structure of cell adhesion can be further
221 classified according to the fluorescent level of F-actin (**Figure 4D right**), then adding text as a
222 subscript to T and L . As schematically illustrated in **Figure 4E (blue)**, T_{High} region (tight contact with
223 higher fluorescence (subscript)) suggests the formation of macromolecular assemblies (i.e., focal
224 complex) around the cell periphery via biological key-lock interaction, whereas T_{Low} region (tight
225 contact with lower fluorescence (subscript)) is attributed to direct contact via generic physical
226 interaction²⁴ (e.g., coulomb force and van der Waals forces, etc.). Also, as schematically illustrated
227 in **Figure 4E (red)**, the L_{High} region (loose contact with higher fluorescence) indicates repulsive
228 interaction²⁴ with supporting cell morphology²⁵, whereas L_{Low} (loose contact with lower fluorescence)
229 defines repulsive free membrane possibly via undulation interactions²⁴. Together, these results
230 clearly highlight the advantages of simultaneous 3-D SIM-IRM/-FLM imaging- with fine lateral and
231 axial resolutions- for characterizing the local distribution of adhesion structures in single cells.

232 Finally, we applied simultaneous 3-D SIM-IRM/-FLM imaging to characterize the adhesion of
233 multicellular myoblasts during myogenic differentiation. Cultivation of myoblasts at high cell densities
234 activates cellular fusion (culturing procedures: **SI method**), resulting in differentiated myotubes with
235 various biological characteristics (e.g., multiple nuclei, expression of myogenin in **Figure S4A and**
236 **S4B**). However, no previous report exists to answer whether classifying the local distribution of
237 adhesion structures enables us to characterize the differentiation states from myoblasts to myotubes.
238 Reconstructed height images from 3-D SIM-IRM images using **Eq. (2)** represent a monotonic
239 decrease in height, indicating the adhesion transition from loose to tight contact according to the
240 myogenic differentiation (**Figure 4F left**). 3-D SIM-FLM visualized a decrease in fluorescence
241 intensity (Day 3), and an increase in the orientation of actin fibers (Day 6) (**Figure 4F right**). To visibly
242 evaluate the local distribution of adhesion structures, we simultaneously plotted the height and
243 fluorescence intensity of each pixel from 3-D SIM-IRM/-FLM images as density plots (**Figure 4G**).
244 Adhesion of myoblasts (Day 0) showed loose contact with a broad expression of actin (L_{High} and L_{Low})
245 via repulsive biophysical interaction. After induction of differentiation by serum starvation, the width of
246 the distribution of dots became narrow due to a simultaneous decrease in height and fluorescence
247 intensity (Day 3, myocytes). The peak changed to broad with shifting to the tight contact with higher
248 fluorescence intensity (T_{High}) for myotubes (Day 6). These results indicate that simultaneous super-
249 resolution imaging of cell membranes and cytoskeletal structures clearly detected the adhesion
250 transition from loose to tight contact during myogenic differentiation (**Figure 4H, left and right**).
251 Furthermore, we sensitively detected the intermediate differentiating stage with a narrow distribution
252 of loose contact with low expression of actin (**Figure 4H, middle**). Based on the obtained results,
253 simultaneous imaging of 3-D SIM-IRM and 3-D SIM-FLM may provide new, physical and biological
254 fingerprints for myogenic differentiation. It should be noted that biological immunostaining only
255 detected myotubes at a "fixed" time point (**Figure S4B**) demonstrating the potential applicability of
256 our 3-D SIM-IRM/-FLM to unveil "dynamical" local complex mechanisms of the differentiation state
257 triggered by cell-matrix adhesion.

258 In summary, we introduce the optical sectioning concept of structured illumination to the classical
259 IRM, to obtain the surface visualization of complex biospecimens beyond the diffraction limit ($d_{xy} \sim$
260 200 nm) (**Figure S5**). Lateral super-resolution was confirmed by the visualizing isolated fluorescent
261 nanobeads (**Figure S6a-b**) and distinguishing adjacent fluorescent nanobeads (**Figure S6c-e**). Not

262 2-D but 3-D structure illumination introduced to IRM, successfully reduced stray light and visualized
263 dynamical structures of protein crystal growth and microtubule fiber motion. Simultaneous imaging of
264 3-D SIM-IRM/-FLM enabled us to classify the adhesion of cells and provide new physical fingerprints
265 for myogenic differentiation. Microscopic interference artifacts in the background (~ 2 μm peak to peak
266 like **Figure 2B** and **Figure S3A**) do not interrupt the lateral super-resolution imaging. Still, future
267 mathematical efforts should develop reconstruction algorithms that are specialized for our structured
268 IRM. We foresee that advanced interferometry with 3-D structured illumination is expected to facilitate
269 the discovery of critical fine structures of complex biospecimens from biomolecule assemblies to cells
270 that regulate biological phenomena (e.g., embryonic development and diseases).

271

272 **Associated contents**

273 ○ **Supporting Information**

274 Supporting methods, figures (**Figure S1-S6**), and movies (**Movie S1-S2**) are available free of charge
275 via the Internet at <http://pubs.acs.org>.

276

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286

287 **Author contribution**

288 TM and HYY conceived the study, designed/performed the experiments, collected/analyzed the data,
289 and authored the manuscript. RK, YY, HT, MF, ST performed the experiments under TM and HYY's
290 technical guidance and expertise. RK, AY, FH, SIT, MS, SN. SS, JKG authored and reviewed the
291 manuscript.

292

293 **Declaration of interests**

294 This study was conducted while Shivani Sharma was employed at the University of California, Los
295 Angeles. The opinions expressed in this article are the author's own and do not reflect the view of
296 the National Institutes of Health and Human Services or the United States government. The authors
297 declare no competing financial interests.

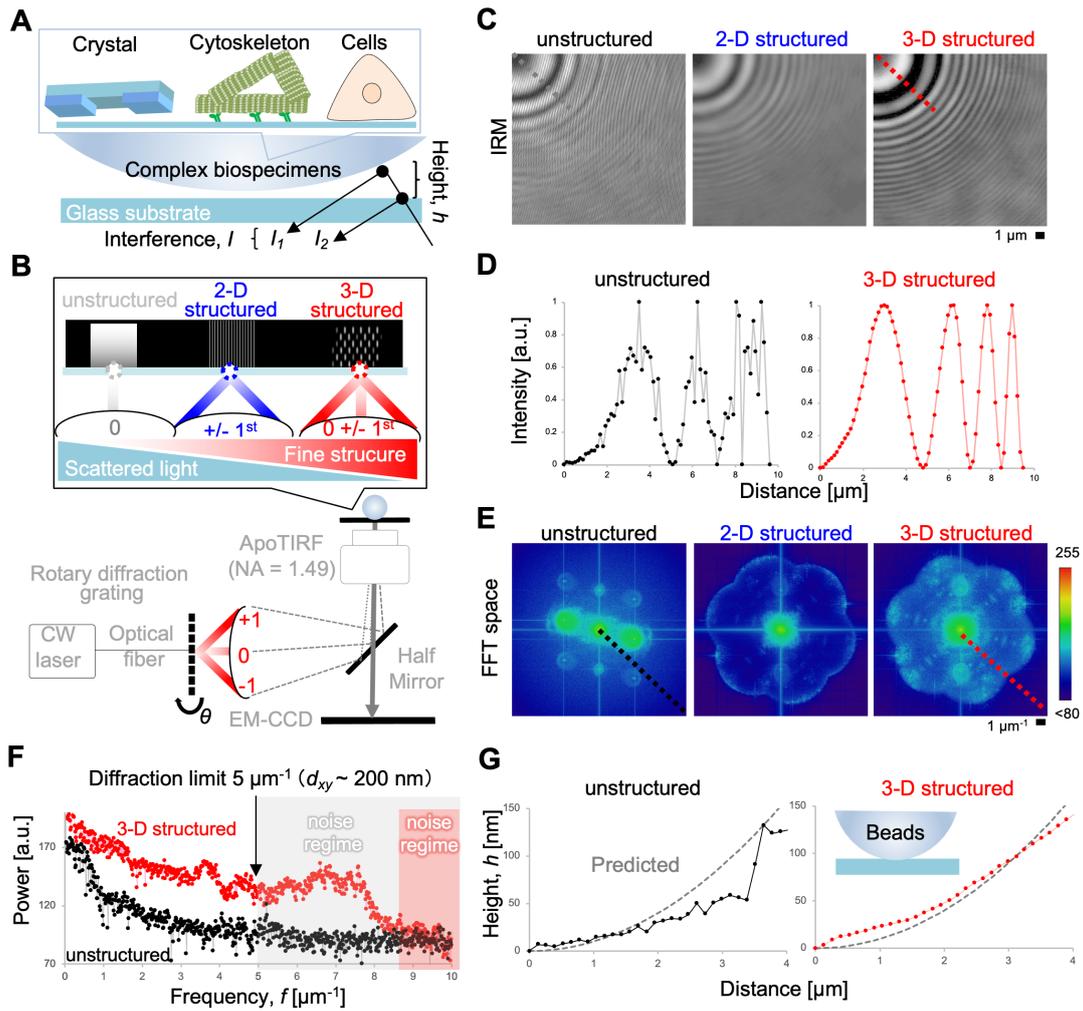
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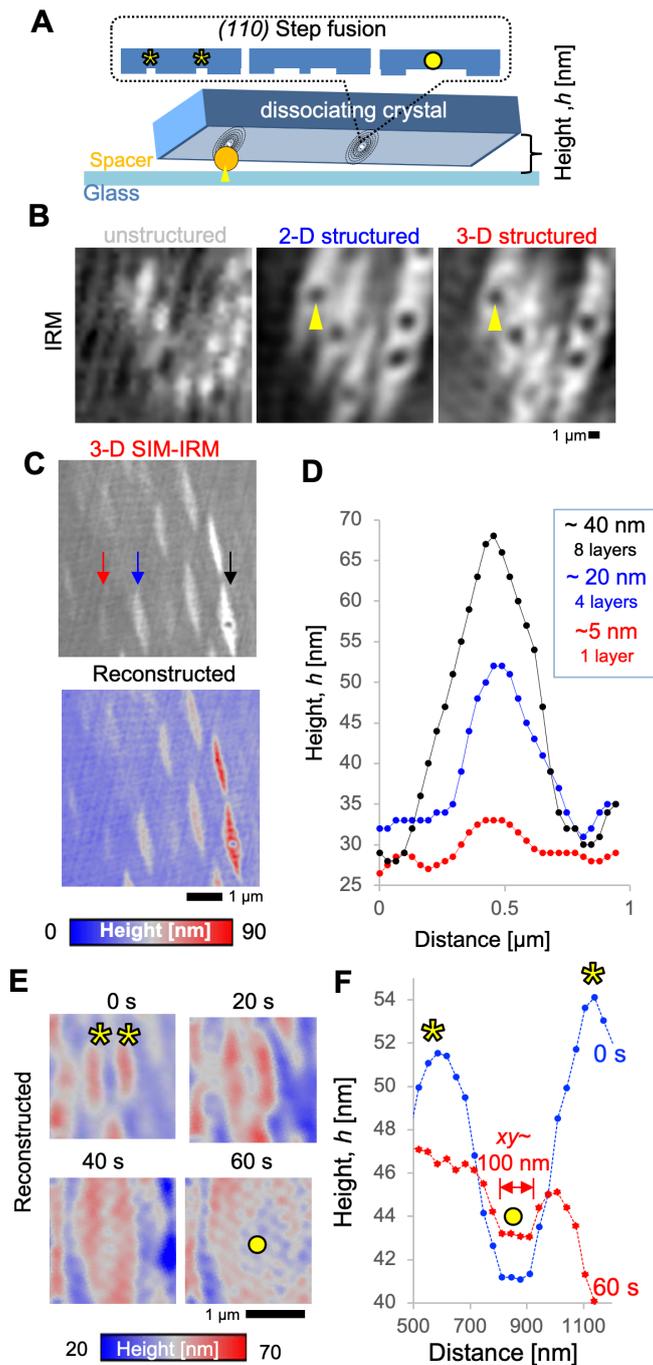
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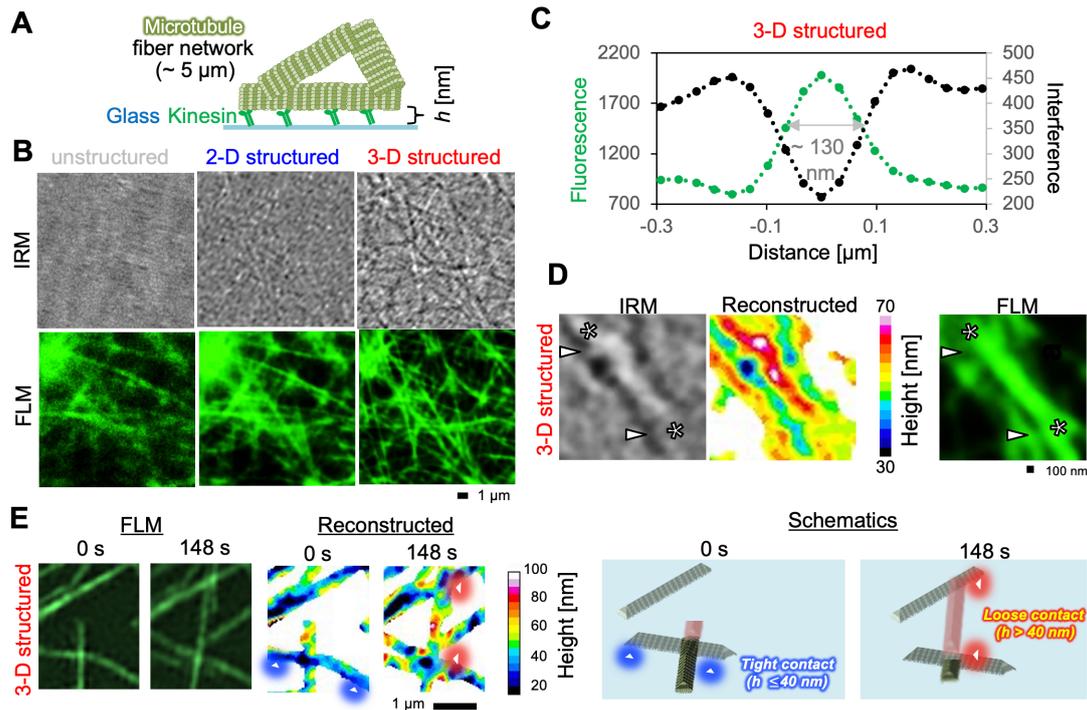
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Figure 1: 3-D structured illumination enhances the image contrast, and IRM's theoretical basis for height reconstruction remains. (A) Concept of 3-D SIM-IRM for visualizing the surface fine structure of complex biospecimens. Principles of visualizing biospecimens-substrate height (h) via interference between I_1 and I_2 . (B) Optical setup, and schematics of prepared structured illumination. A CW laser combined with rotary diffraction grating provides laterally (2-D) and axially (3-D) aligned Moiré patterns, reducing multiple-scattered reflections from biospecimens. (C) IRM images of a polystyrene bead (diameter, $\phi = 100 \mu\text{m}$) on a glass substrate under un/2-D/3-D structured illuminations ($\lambda = 488 \text{ nm}$) and corresponding (D) intensity profiles according to the dotted line in C. (E) Calculated FFT images from C and (F) power spectrum according to the dotted line in E. (G) Reconstructed height profiles from D using Eq 2. The predicted height profile of a spherical bead is displayed as a gray line.



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Figure 2: Elementary step-scale visualization of the dissolution dynamics of lysozyme crystal on (110) surface. Experimental setup for observing lysozyme-substrate height. Silica beads ($\phi = 100$ nm) were used as spacers (yellow arrowhead). (B) IRM images during crystal dissolution under un/2-D/3-D structured illumination ($\lambda = 488$ nm). (C) A 3-D SIM-IRM image of dissolving crystals and its reconstructed height images. (D) Height profiles along the etch pit in C: red (single layer of protein molecule), blue (4 layers), and black (8 layers). (E) Timelapse of height change in dissolving crystal and its (F) topological profiles across etch pits. Adjacent pits ($h \sim 10$ nm, $t = 0$ s asterisk) fuse to form a single etch pit ($t = 60$ s, circle) with a fine structure ($xy \sim 100$ nm, $z \sim 2$ nm).



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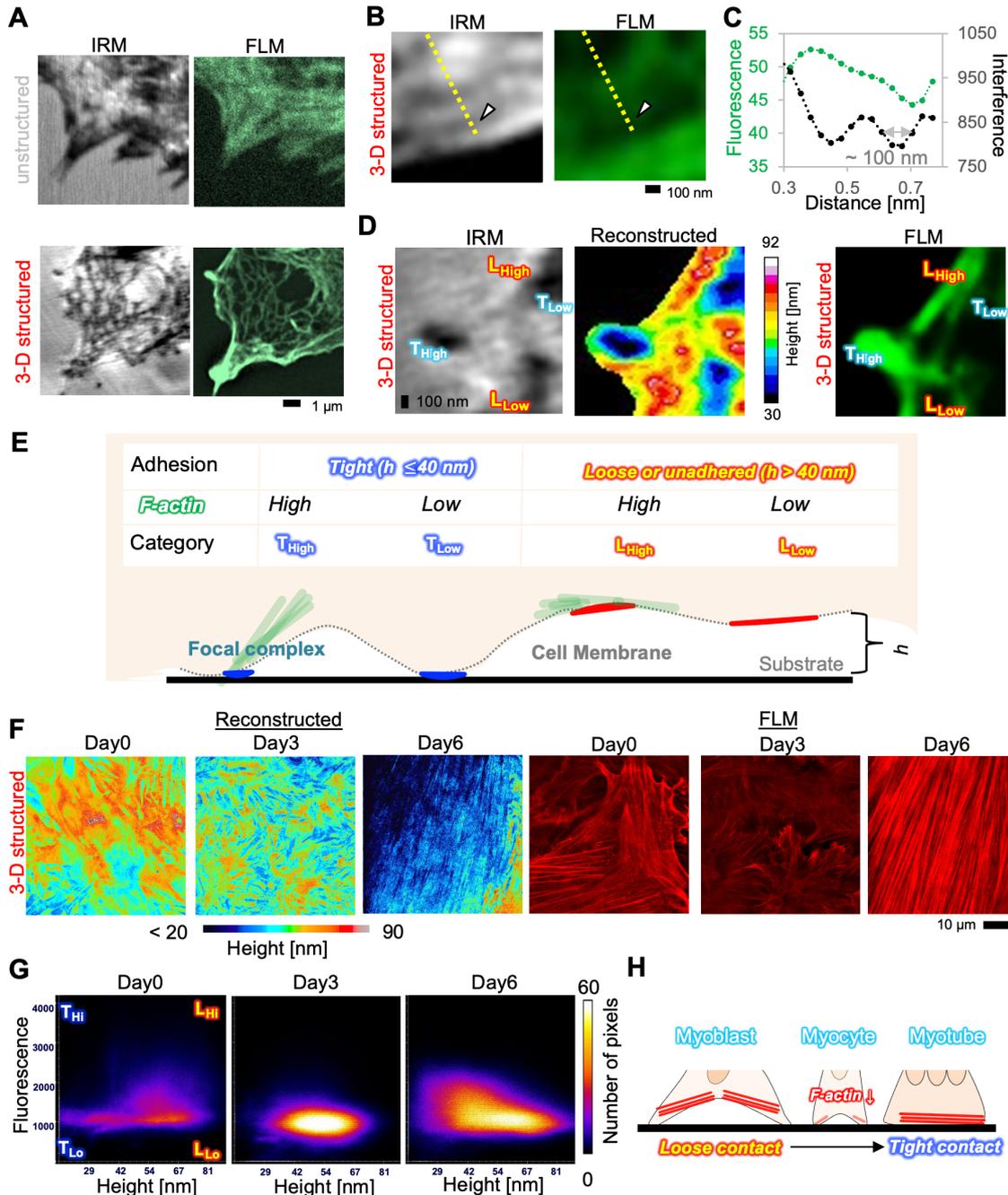
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Figure 3: Not 2-D but 3-D SIM-IRM visualized complex microtubule fiber networks unveiling adhesion dynamics along the optical axis. (A) Experimental setup for preparing a microtubule network on the glass substrate. Motor protein (kinesin) drives the locomotion of the network by adding ATP. (B) IRM and FLM (fluorescence microscope) images of the networks under un/2-D-/3-D-structured illumination ($\lambda = 488$ nm (IRM), $\lambda = 561$ nm (FLM)). (C) Representative intensity profiles across the microtubule fiber in the network. The full width at half maximum (FWHM) is approximately 130 nm, which is beyond the diffraction limit of conventional IRM. (D) Images of 3-D SIM-IRM, its reconstructed height, and 3-D SIM-FLM of the microtubule fiber networks. Different interference intensities originate from height, h from the underlying substrate (close: arrowhead, farther: asterisk) (E) Timelapse of 3-D SIM-FLM images, reconstructed height images from 3-D SIM-IRM images, and its schematics of the locomoting networks in the presence of ATP. An increase in height (red arrowhead) is beyond the diameter of a microtubule fiber²⁶; thus the fibers locomote onto the fibers.



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Figure 4: Simultaneous interference/fluorescence imaging of membrane/cytoskeleton structures classified the local distribution of fine adhesion structures, and detected adhesion transition during myogenic differentiation. (A) IRM and FLM images of different myoblasts expressing fluorescent *F-actin* under un/3-D- structured illumination ($\lambda = 488$ nm (IRM), $\lambda = 561$ nm (FLM)). (B) Magnification of 3-D SIM-IRM/-FLM images and (C) corresponding intensity profiles according to the dotted lines in B. FWHM of dark fibrous structure (arrowhead) in an IRM image showed approximately 100 nm, which is beyond the diffraction limit ($d_{xy} \sim 200$ nm). (D) 3-D SIM-IRM, reconstructed height, and 3-D SIM-FLM images of myoblast. Here, the adhesion's structure is first classified into tight contact (T_{High} and T_{Low}) and loose contact (or unadhered, L_{High} and L_{Low}) at the threshold $h \sim 40$ nm, which is the comparable size of the cell adhesion molecules¹⁷. Then, the structure of cell adhesion can be further classified according to the higher fluorescent (T_{High} and L_{High}) and lower fluorescent (T_{Low} and L_{Low}) of *F-actin*. (E) Schematic illustration of spatial and axial structures at cell membrane of D. (F) Timelapse of reconstructed height and 3-D SIM-FLM images of myoblasts during myogenic differentiation from myoblast (Day 0), myocyte (Day 3) to myotubes (Day

419 6). Low serum medium activates cellular fusion from Day 4 (**Figure S4A**). (G) Colocalization plots
420 between fluorescence intensity and height of each pixel from F. The spatial size of each dot
421 corresponds to the nano-scale adhesion structure ($\sim 30 \times 30$ nm) with the classification according to
422 the categories in E. (H) Schematics of the change in cell adhesion structure during differentiation. The
423 narrowing of plot distribution (Day 3) and peak shift toward the region of T_{High} (Day 6) in G supports
424 the new physical marker for myogenic differentiation.
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