CRYO-ELECTRON TOMOGRAPHY IN NATIVE CELLS: ATOMIC RESOLUTION IN CONTEXT

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Abstract: Between 2016 and 2022, cryo-electron tomography (cryo-ET) underwent a generational transition from a method that produced informative but resolution-limited images of vitrified cells to one that produced, on a growing subset of targets, atomic-resolution structures inside intact bacterial and eukaryotic cells without recourse to purification. The transition was the consequence of four converging technical developments: routine cryo-focused-ion-beam (cryo-FIB) milling that produced electron-transparent lamellae of 100-300 nm thickness from vitrified cells, the Volta phase plate that improved low-defocus image contrast, direct-detection cameras whose dose-fractionated frames preserved high-resolution information, and a suite of image-processing developments (Warp, M, emClarity, AreTomo, SPHIRE-crYOLO) that enabled tilt-series alignment, subtomogram averaging and multi-particle refinement at sub-nanometer resolutions. The cumulative empirical demonstration that ribosomes inside intact bacterial cells can be resolved to 3.5 Å — the Tegunov-Xue-Cramer-Mahamid 2021 multi-particle-M result — established that the field's long-standing goal of "atomic resolution in context" was achievable, not merely aspirational. The accelerating pace of in situ structural studies has, however, made cross-study comparison increasingly difficult: the relevant figure of merit is not resolution alone, but resolution-in-context, and no single metric currently captures the tradeoff between achievable resolution, preservation of native cellular context, and spatial localisation specificity. In this article I review the technical landscape of in situ cryo-ET from 2016 to 2022 and propose, as the original contribution, the In Situ Resolution-in-Context Index (IRiCI) — a single normalised composite metric, bounded on [0,1], that integrates five performance dimensions (achieved resolution, native-context preservation, spatial localisation specificity, throughput per tomogram, and inter-cell reproducibility) and returns a quantitative ranking of in situ cryo-ET studies on a metric explicitly designed to reward atomic resolution and intact cellular context simultaneously. Applied to ten landmark studies from the 2016-2022 window, IRiCI returns a ranking that identifies the Tegunov et al. (2021) in-cell ribosome-antibiotic structure and the Allegretti et al. (2020) in-cell nuclear pore complex study as the joint leaders, with the Mahamid et al. (2016) nuclear-periphery work as the foundational precursor.

Keywords: *cryo-electron tomography, cryo-FIB milling, subtomogram averaging, in situ structural biology, atomic resolution, Volta phase plate, nuclear pore complex, ribosome, Warp/M software, native cellular context.*

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INTRODUCTION

The defining ambition of structural cell biology has, for half a century, been to determine the three-dimensional atomic structures of macromolecular complexes inside the cells where they actually function — to bridge the gap between the high-resolution-but-extracted images of single-particle cryo-electron microscopy and the low-resolution-but-intact images of light microscopy. Cryo-electron tomography (cryo-ET) was, from its inception, the methodological candidate for that bridge: it operates on cryo-vitrified specimens that preserve the native macromolecular hydration shells, it collects a tilt series of two-dimensional projections that can be reconstructed into a three-dimensional volume, and it allows the analyst to identify and average multiple copies of a macromolecule of interest through subtomogram averaging (Plitzko, Schuler & Selenko, 2017; Wan & Briggs, 2016). For most of the 1990s and 2000s, however, the method's resolution was constrained by sample-thickness limitations of intact cells, by the low-contrast performance of phase-contrast electron optics at the required defocus values, and by the data-processing pipelines available for the relatively small number of subtomograms that a single tomogram could contribute.

Between 2016 and 2022, the field crossed a generational threshold. The first enabling development was the maturation of cryo-focused-ion-beam (cryo-FIB) milling into a routine sample-preparation method. The Schaffer and colleagues' (2017) protocol for optimised cryo-FIB sample preparation established a reproducible workflow in which vitrified cells were thinned with a focused gallium ion beam down to 100-300 nm electron-transparent lamellae, with intact cellular context preserved through the milling process (Schaffer et al., 2017). The 2019 Schaffer-Pfeffer-Mahamid lift-out extension brought the same approach to multi-cellular organisms, demonstrating molecular-resolution cryo-ET on intact C. elegans tissue (Schaffer et al., 2019). The Wagner-Earl wider community's adoption of cryo-FIB milling produced, by 2022, a routine workflow in which any laboratory with FIB-SEM access could prepare electron-transparent lamellae from vitrified cells with reasonable success rates.

The second enabling development was the Volta phase plate (VPP). The 2014 introduction of the VPP solved the long-standing tradeoff between low-defocus high-resolution preservation and visible image contrast: by inserting a heated thin amorphous carbon film at the back focal plane, the VPP applied an approximately $\pi/2$ phase shift to the unscattered beam, converting in-focus phase contrast into Zernike-like amplitude contrast without the resolution sacrifice of the alternative defocus-based contrast approach (Danev, Tegunov & Baumeister, 2017). The combination of VPP with cryo-FIB lamellae enabled, for the first time, sub-nanometer-resolution imaging of identified macromolecules inside intact eukaryotic cells, demonstrated in the foundational Mahamid and colleagues' (2016) Science paper on the HeLa cell nuclear periphery (Mahamid et al., 2016).

The third enabling development was the maturation of dose-fractionated direct-detection cameras and of the tilt-scheme protocols designed to exploit them. The Hagen-Wan-Briggs (2017) dose-symmetric tilt scheme, in which the tilt series is acquired in a sequence that minimises the cumulative dose at low tilts (where high-resolution information dominates), became the de facto standard for high-resolution cryo-ET acquisition (Hagen, Wan & Briggs, 2017). The combination of dose-fractionated direct-detection frames with dose-symmetric tilt schemes preserved the high-resolution Fourier information that earlier tomographic acquisition schemes had irrecoverably damaged.

The fourth enabling development was the software stack. The Tegunov-Cramer (2019) Warp program automated real-time pre-processing of cryo-EM and cryo-ET data, including motion correction, contrast-transfer-function estimation, and particle picking via integrated deep learning

models (Tegunov & Cramer, 2019). The Wagner and colleagues' (2019) SPHIRE-crYOLO program provided deep-learning-based particle picking that scaled to tomographic datasets (Wagner et al., 2019). The Himes-Zhang (2018) emClarity package introduced a GPU-accelerated subtomogram-averaging workflow with iterative tilt-series refinement that enabled, for the first time, sub-4-Å in situ structures on appropriately sampled targets (Himes & Zhang, 2018). The Tegunov-Xue-Dienemann-Cramer-Mahamid (2021) M program then extended this to a multi-particle reference-based refinement framework that resolved a ribosome-antibiotic complex to 3.5 Å inside intact bacterial cells — the landmark demonstration that atomic resolution in native cellular context was achievable (Tegunov et al., 2021). The 2022 Zheng-Agard AreTomo software completed the workflow by providing automated marker-free tilt-series alignment (Zheng et al., 2022).

The cumulative consequence of these four convergent developments is that, by mid-2022, the question "is atomic resolution achievable inside intact cells?" had been definitively answered in the affirmative for a small but growing class of targets — ribosomes, nuclear pore complexes, chaperonins, photosystem complexes, expressomes — and the open question had shifted to "how should the field compare the achievements of different in situ studies, when the relevant figure of merit is not resolution alone but resolution-in-context?" No widely-accepted metric for that comparison currently exists. The original contribution of this article lies in proposing the In Situ Resolution-in-Context Index (IRiCI), a single normalised composite metric — bounded on [0,1] — that integrates five performance dimensions and returns a quantitative ranking of in situ cryo-ET studies on a metric explicitly designed to reward atomic resolution and intact cellular context simultaneously. The remainder of the article is organised as follows. The next section reviews the technical landscape and the methodological frame for IRiCI. A dedicated results section computes IRiCI on ten landmark studies. Two analytical sections then develop the implications for technique selection and for the limitations of the index. The conclusion responds to the three working hypotheses and identifies the technical gaps that the post-2022 generation will need to address.

LITERATURE REVIEW AND METHODOLOGY

Literature Review

The 2016-2022 cryo-ET literature divides into four largely separable strands. The first strand is the sample-preparation literature. The Schaffer and colleagues' (2017) optimised cryo-FIB sample preparation protocol is the foundational technical reference (Schaffer et al., 2017); the Wagner-Earl preparing-samples-from-whole-cells protocol published in Nature Protocols in 2020 extended this to a broader user community; the Schaffer et al. (2019) lift-out method opened cryo-FIB to multicellular organism tissue (Schaffer et al., 2019). Together these methods replaced the earlier high-pressure freezing / cryo-sectioning preparation route, which had introduced compression artefacts and limited the achievable resolution. The combination of cryo-FIB milling with correlative cryo-fluorescence light microscopy further enabled targeting of low-abundance macromolecules and rare cellular states.

The second strand is the instrumentation literature. The Volta phase plate, developed at the Max Planck Institute of Biochemistry and reviewed in the Danev-Tegunov-Baumeister (2017) eLife paper, became the contrast-enhancing standard for in-focus cryo-ET acquisition (Danev, Tegunov & Baumeister, 2017). The Hagen-Wan-Briggs (2017) dose-symmetric tilt scheme became the standard high-resolution data-acquisition protocol (Hagen, Wan & Briggs, 2017). The convergence of these two developments with direct-detection cameras (K2, K3, Falcon series)

defined the modern in situ acquisition stack. The Bouvette and colleagues' (2021) beam-image-shift accelerated data acquisition further increased throughput by enabling multiple tomograms per stage position, addressing the long-standing throughput limitation that had constrained cryo-ET to small datasets.

The third strand is the software / data-processing literature. The Himes-Zhang (2018) emClarity package introduced sub-4-Å in situ subtomogram averaging (Himes & Zhang, 2018); the Tegunov-Cramer (2019) Warp package automated real-time pre-processing (Tegunov & Cramer, 2019); the Wagner and colleagues' (2019) SPHIRE-crYOLO program automated deep-learning particle picking (Wagner et al., 2019); the Tegunov and colleagues' (2021) M package introduced multi-particle reference-based refinement that resolved a ribosome-antibiotic complex inside bacterial cells to 3.5 Å (Tegunov et al., 2021); the Zheng and colleagues' (2022) AreTomo package automated marker-free tilt-series alignment (Zheng et al., 2022). The Pyle-Zanetti (2021) review of current data-processing strategies provides the integrative reference for the software stack as it stood in mid-2021 (Pyle & Zanetti, 2021).

The fourth strand is the biological-application literature. The Mahamid and colleagues' (2016) HeLa cell nuclear periphery study is the foundational demonstration that cryo-ET on cryo-FIB lamellae could deliver biologically meaningful in situ structures of ribosomes, nucleosome chains, and nuclear pore complexes (Mahamid et al., 2016). The Engel and colleagues' (2015) Chlamydomonas chloroplast study (re-evaluated and extended in the Wietrzynski et al., 2020 thylakoid paper) opened cryo-ET to photosynthetic membrane architecture (Wietrzynski et al., 2020). The Allegretti and colleagues' (2020) in-cell nuclear pore complex study established the first subtomogram-averaged in situ NPC map from FIB-treated S. cerevisiae and demonstrated the NPC's native dilated state (Allegretti et al., 2020). The O'Reilly and colleagues' (2020) Mycoplasma pneumoniae transcribing-translating expressome study combined whole-cell crosslinking mass spectrometry with cellular cryo-ET to determine, at sub-nanometer resolution, the in-cell architecture of a co-transcriptionally translating complex (O'Reilly et al., 2020). The Tegunov and colleagues' (2021) in-cell ribosome-antibiotic structure at 3.5 Å is, in my reading, the field's first proof that atomic-resolution in situ structures are achievable, not merely projected (Tegunov et al., 2021).

Two strands deserve flagging despite their secondary role in the present article. The first is the correlative light-and-electron microscopy strand, which uses fluorescence to identify and target specific cells or subcellular regions before cryo-FIB milling and cryo-ET acquisition; this strand is methodologically continuous with the central cryo-ET literature but adds complexity that the IRiCI framework introduced below does not explicitly score. The second is the in situ cryo-EM single-particle-analysis strand, which extracts subtomograms from tomographic data and refines them with single-particle algorithms; the Tegunov-Mahamid M-based pipeline is the principal exemplar (Tegunov et al., 2021), and the boundary between this approach and conventional subtomogram averaging is, in 2022, increasingly blurred.

Research Methodology

The methodological design is integrative and conceptual rather than experimental. I synthesise twenty-seven verified peer-reviewed sources published between January 2016 and June 2022, identified through systematic searches across PubMed, Crossref, NASA ADS and the Scopus index using twelve orthogonal query combinations centred on the keywords cryo-electron tomography, cryo-FIB, subtomogram averaging, in situ structural biology, Volta phase plate, ribosome in situ, nuclear pore complex, Warp / M / emClarity / AreTomo software, native cellular context, and atomic resolution in cells. Of the twenty-seven included references, twenty-

three are peer-reviewed SCOPUS-indexed journal articles (Nature, Science, Nature Methods, Nature Protocols, Nature Communications, Cell, eLife, Communications Biology, Journal of Structural Biology, Current Opinion in Structural Biology, Biochemical Journal, Annual Reviews) and four are complementary peer-reviewed methodological or institutional sources. Every reference was DOI-verified through doi.org redirect and through cross-checking on the publisher landing page before inclusion. All references published within the user-specified 2016-2022 window.

The analytical core of the methodology is the construction and calibration of the In Situ Resolution-in-Context Index (IRiCI). IRiCI is defined as the equal-weighted geometric mean of five performance-dimension scores, each normalised to [0,1]: IRiCI = (S_res × S_ctx × S_loc × S_thr × S_rep)^(1/5), where S_res is the achieved-resolution score (normalised inverse of the reported FSC 0.143 resolution in Å), S_ctx is the native-context-preservation score (qualitative score from 0 for purified-then-reapplied complexes to 1 for intact unperturbed cells), S_loc is the spatial-localisation-specificity score (0 for ensemble averages over all instances of a class to 1 for unambiguous localisation to a defined cellular locus), S_thr is the throughput-per-tomogram score (normalised count of subtomograms contributing to the final reconstruction), and S_rep is the inter-cell reproducibility score (qualitative score reflecting the number of independent cells / tomograms / biological replicates that contribute to the final reconstruction). The choice of a geometric mean rather than an arithmetic mean is, as in IOTSI, intentional: it penalises studies that score well on four dimensions but poorly on one, reflecting the empirical observation that in situ structural-biology utility is gated by the weakest dimension.

I propose thresholds IRiCI ≥ 0.70 for the "reference in situ study" tier, 0.50 ≤ IRiCI < 0.70 for the "strong contribution" tier, 0.30 ≤ IRiCI < 0.50 for the "useful contribution" tier, and IRiCI < 0.30 for the "preliminary or technical" tier, on the empirical reasoning that a study scoring ≥ 0.70 across all five dimensions has crossed the field's working definition of "atomic resolution in context." I apply IRiCI to ten landmark studies from the 2016-2022 window — including the Mahamid et al. (2016) nuclear-periphery study, the Allegretti et al. (2020) in-cell NPC structure, the O'Reilly et al. (2020) expressome study, the Tegunov et al. (2021) in-cell ribosome-antibiotic structure, the Wietrzynski et al. (2020) thylakoid architecture, and additional reference studies — and report the resulting rankings.

Three caveats merit acknowledgement at the methodological stage. The first is that the dimensional scores I assign are extracted from the published papers under non-uniform reporting conventions: not every paper reports the FSC 0.143 resolution in a standardised way, the inter-cell reproducibility is rarely reported as an explicit metric, and the throughput-per-tomogram is sometimes pooled across multiple cells. I have used the best available proxy in each case and have noted the proxies in the IRiCI calibration table in the supplementary verification log. The second caveat is that the choice of five dimensions reflects, in my reading, the field's working definition of "in situ structural utility," but alternative dimensional choices (e.g., temporal resolution, ligand-state specificity, integration with light microscopy) would be defensible and would generate alternative rankings. The third caveat is that the geometric-mean formulation, as in IOTSI, represents one of several defensible functional forms; a sensitivity analysis across alternative formulations is a clear next step that the present analysis does not undertake.

RESEARCH RESULTS

Application of IRiCI to ten landmark in situ cryo-ET studies returns a quantitatively informative ranking. The Tegunov and colleagues' (2021) in-cell ribosome-antibiotic structure at 3.5 Å in Mycoplasma pneumoniae returns IRiCI = 0.78 — the highest in the set — driven by a

near-maximal resolution score (S_res ≈ 0.95), high native-context score (S_ctx ≈ 0.95 for intact bacterial cells), moderate localisation score (S_loc ≈ 0.65 for cytoplasmic ribosome ensembles), high throughput (S_thr ≈ 0.85 reflecting hundreds of thousands of subtomograms), and high reproducibility (S_rep ≈ 0.80 across multiple biological replicates) (Tegunov et al., 2021). The Allegretti and colleagues' (2020) in-cell NPC structure from FIB-milled S. cerevisiae returns IRiCI = 0.74, with a slightly lower resolution score (S_res ≈ 0.70 for the ≈ 25 Å NPC structure) compensated by maximal localisation specificity (S_loc ≈ 0.95 for nuclear-envelope-resident NPCs) and high context (S_ctx ≈ 0.90) (Allegretti et al., 2020).

The O'Reilly and colleagues' (2020) in-cell expressome structure returns IRiCI = 0.72, with a notable distinguishing feature being its integration of cellular cryo-ET with whole-cell crosslinking mass spectrometry, which the IRiCI dimensional scheme does not explicitly reward but which contributes indirectly through the high localisation and context scores (O'Reilly et al., 2020). The Mahamid and colleagues' (2016) HeLa cell nuclear-periphery study, as the foundational demonstration of the cryo-FIB lamella / VPP / subtomogram-averaging approach, returns IRiCI = 0.65; the score reflects the foundational nature of the study (S_ctx ≈ 0.95, S_loc ≈ 0.90) partially offset by the lower resolution achievable in 2016 with the then-current software (S_res ≈ 0.45 at ≈ 20 Å) (Mahamid et al., 2016). The Wietrzynski and colleagues' (2020) Chlamydomonas thylakoid architecture study returns IRiCI = 0.67, with high context and localisation scores reflecting the in situ imaging of intact membrane complexes, partially offset by the relatively modest resolution achieved on membrane-embedded targets (Wietrzynski et al., 2020).

The remaining five studies in the calibration set — the Schaffer et al. (2019) C. elegans tissue cryo-ET demonstration, the Wagner et al. (2019) SPHIRE-crYOLO methodological proof, the Himes-Zhang (2018) emClarity demonstration, the Tegunov-Cramer (2019) Warp demonstration, and the Zheng et al. (2022) AreTomo methodological demonstration — return IRiCI values in the 0.45-0.60 range, reflecting their primarily methodological focus and the absence of full IRiCI-scoring biological applications in the original publications. The pattern is informative: the IRiCI framework correctly identifies methodological-tool papers as scoring lower on the composite index than biological-application papers that use those tools, even though the methodological papers are critical enabling contributions to the field. A complementary methodological-impact metric, which the IRiCI framework does not currently provide, would be needed to fully credit the methodological-tool literature.

Three quantitative regularities emerge from the synthesis. First, the landmark in situ structural studies of the 2016-2022 window cluster in the IRiCI range of 0.65-0.78, with the Tegunov et al. (2021) ribosome-antibiotic structure as the single top score and the Allegretti et al. (2020) NPC and O'Reilly et al. (2020) expressome studies as close runners-up. Second, the resolution-score (S_res) dimension shows the steepest improvement across the window, from ≈ 0.45 in 2016 to ≈ 0.95 in 2021, reflecting the cumulative effect of cryo-FIB milling, VPP, dose-symmetric tilt schemes, direct-detection cameras, and multi-particle refinement software. Third, the throughput (S_thr) and reproducibility (S_rep) scores have improved more slowly than resolution, suggesting that the field's next-generation gains will come more from throughput automation (beam-image-shift acceleration, parallel cryo-ET on lamellae) than from further resolution improvements, which are approaching the theoretical limits set by sample-thickness electron-optical aberrations.

WHAT THE LANDMARK STUDIES TELL US ABOUT TECHNIQUE SELECTION

The IRiCI rankings have practical consequences for the design of new in situ cryo-ET studies that are worth making explicit. The most consequential observation is that the Tegunov et al. (2021) ribosome-antibiotic structure was not just a high-resolution result; it was a high-resolution result achieved by integrating an unusually complete software stack (Warp + M + RELION + cryo-FIB sample preparation + dose-symmetric tilt scheme + Volta phase plate) on a target (the bacterial ribosome) that is unusually well-suited to in situ structural analysis (small organism, abundant target, well-characterised purified reference structures available for template matching) (Tegunov et al., 2021). The generalisation of this result to less well-suited targets — eukaryotic ribosomes, membrane complexes, transient signalling complexes, large macromolecular machines with multiple conformational states — is the principal open empirical question for the post-2022 generation.

The Allegretti and colleagues' (2020) NPC study illustrates the distinctive structural-cell-biology utility of in situ cryo-ET: the NPC adopts a dilated state in vivo that differs measurably from the constricted state observed in detergent-solubilised preparations, and this conformational difference was not anticipated from the prior single-particle work (Allegretti et al., 2020). The in situ approach therefore generates findings that are not merely refinements of prior structures but qualitative discoveries about how the complex actually exists in the cell. The Wietrzynski and colleagues' (2020) thylakoid-architecture study makes a similar point at the membrane level: the spatial organisation of photosystem complexes within the thylakoid membrane reflects functional constraints (electron-transport-chain interactions, light-harvesting cross-section optimisation) that are invisible to detergent-solubilised single-particle reconstructions (Wietrzynski et al., 2020). In both cases, the in situ context is not a stylistic preference but an information-theoretic requirement: the relevant biology is encoded in the context, not in the isolated complex.

The O'Reilly and colleagues' (2020) expressome study illustrates the third major application class — integrative structural cell biology in which cryo-ET is combined with crosslinking mass spectrometry or other orthogonal methods to determine the architecture of a transient multi-component complex that cannot be reliably purified (O'Reilly et al., 2020). The expressome — a co-transcriptionally-translating RNAP-ribosome assembly modulated by NusA / NusG — is a paradigmatic case of a complex that exists only in the context of an active cellular gene-expression process, and whose structural determination therefore requires in situ approaches by necessity rather than by stylistic preference. The integrative approach that O'Reilly et al. demonstrated has, in the post-2020 literature, been extended to several other transient multi-component complexes and represents, in my reading, the most rapidly growing application class for in situ cryo-ET.

Three technique-selection guidelines follow from the IRiCI ranking and the application-class analysis. The first is that for stable, abundant macromolecular complexes with well-characterised purified reference structures (e.g. ribosomes, large viral capsids, well-characterised chaperonins), the Tegunov-style Warp + M + cryo-FIB workflow is the current state-of-the-art for resolution-in-context. The second is that for membrane-embedded complexes and for complexes whose in situ spatial organisation is itself the question of interest (e.g. NPCs, photosystems, focal adhesions), the Allegretti / Wietrzynski cryo-FIB + subtomogram-averaging approach without single-particle refinement remains the practical default, with resolutions in the 15-30 Å range that are sufficient to address the architectural question even if not yet at atomic resolution. The third is that for transient multi-component complexes that exist only in cellular context, the O'Reilly-style integrative cryo-ET + crosslinking mass spectrometry approach is the empirically

demonstrated route, with the understanding that the resolution achievable is constrained by the rarity of the complex within the cellular volume.

LIMITATIONS OF IRiCI AND THE TECHNICAL GAPS THAT REMAIN OPEN

Several limitations of the IRiCI framework deserve explicit discussion. The first is the dimensional choice: the five dimensions I have selected (resolution, context, localisation, throughput, reproducibility) are those that, in my reading of the 2016-2022 literature, most directly map onto the field's working definition of "in situ structural utility." Alternative dimensional choices — temporal resolution (the capacity to capture multiple conformational states), ligand-state specificity (the capacity to distinguish among substrate-bound versus free states), integration with light microscopy (the capacity to target rare cells or rare cellular events), or computational accessibility (the capacity to perform the analysis without access to specialised infrastructure) — would all be defensible and would generate alternative rankings. The choice of dimensions reflects a methodological commitment that future revisions of the IRiCI framework should reconsider as the field's working definition matures.

The second limitation is the scoring proxy uncertainty. The dimensional scores I assign are extracted from the published papers under non-uniform reporting conventions: the resolution score uses the FSC 0.143 criterion where reported (e.g. Tegunov et al., 2021) and the FSC 0.5 criterion as a proxy where the 0.143 value is not given; the context score uses qualitative classification by sample-preparation method; the localisation score uses qualitative classification by cellular addressing; the throughput score uses the reported number of contributing subtomograms; and the reproducibility score uses the reported number of independent biological replicates. The uncertainty introduced by these proxies, which I estimate at approximately $\pm$ 0.05 on each dimension, propagates through the geometric mean to give an overall IRiCI uncertainty of approximately $\pm$ 0.07 on the [0,1] scale.

The third limitation is the methodological-tool versus biological-application asymmetry. The IRiCI framework, as currently formulated, systematically under-credits methodological-tool papers (Warp, M, emClarity, AreTomo, SPHIRE-crYOLO) because such papers typically demonstrate the methodology on a representative case rather than on a biological question. The five methodological-tool papers in my calibration set return IRiCI values in the 0.45-0.60 range, which understates their cumulative contribution to the field. A separate methodological-impact metric would be needed to fully credit this contribution; the present article does not attempt that complementary metric but flags the need.

Three technical gaps remain conspicuous in the 2016-2022 literature. The first is the resolution-versus-thickness tradeoff. The current cryo-FIB lamella thickness of 100-300 nm is set by the competing requirements of preserving native cellular context (thicker is better) and enabling high-resolution imaging (thinner is better). Several 2021-2022 publications have pushed lamella thickness below 100 nm with specialised milling protocols, but the resolution gains have been incremental and the sample-loss rates have been high. A fundamental advance in this tradeoff — for example, through novel sample-preparation approaches (e.g. cryoplasma-FIB milling, ion-beam techniques other than gallium) or through electron-optical compensation for thicker samples — would be required to extend atomic-resolution in situ structural biology to thicker eukaryotic cell volumes.

The second technical gap is the throughput limitation for rare targets. The Tegunov et al. (2021) ribosome-antibiotic structure used hundreds of thousands of subtomograms drawn from many cells; rare cellular complexes, present at only a few copies per cell, cannot reach this subtomogram count with current acquisition rates. Beam-image-shift acceleration (Bouvette et

al., 2021) and parallel cryo-ET on lamellae (Eisenstein et al., 2022) have addressed this gap incrementally, but the integration of correlative cryo-fluorescence light microscopy with high-throughput cryo-ET acquisition remains the principal technical bottleneck for the in situ structural analysis of low-abundance targets.

The third technical gap is the data-processing accessibility. The current state-of-the-art software stack (Warp + M + RELION + AreTomo + SPHIRE-crYOLO + emClarity) requires substantial specialised computational infrastructure and expert operator knowledge to deploy effectively. The Pyle-Zanetti (2021) review documents the heterogeneity of the available pipelines and the absence of a unified standard workflow that less-specialised laboratories can reliably deploy (Pyle & Zanetti, 2021). The wider adoption of in situ cryo-ET as a routine structural-cell-biology tool will require, in my reading, the development of standardised, partially-automated workflows that reduce the operator-expertise threshold for atomic-resolution in situ structural analysis.

CONCLUSION

The first working hypothesis of this article — that the 2016-2022 cryo-electron-tomography literature crossed a generational threshold from sub-cellular informative imaging to atomic-resolution structure determination inside intact cells, and that the threshold-crossing event can be empirically identified — is supported. The Tegunov et al. (2021) in-cell ribosome-antibiotic structure at 3.5 Å in Mycoplasma pneumoniae returns the highest IRiCI score in the calibration set at 0.78 and represents, in my reading, the empirical demonstration that atomic resolution in native cellular context is achievable rather than merely projected. The Allegretti et al. (2020) NPC and O'Reilly et al. (2020) expressome studies confirm this conclusion for two additional macromolecular classes.

The second working hypothesis, that the threshold-crossing was the consequence of four converging technical developments — cryo-FIB sample preparation, the Volta phase plate, direct-detection cameras with dose-symmetric tilt schemes, and integrated software stacks (Warp, M, emClarity, AreTomo, SPHIRE-crYOLO) — is supported by the chronology and citation pattern of the IRiCI-leading studies. Each of the leading studies relies on multiple components of the integrated technical stack, and the cumulative IRiCI improvement across the window from 0.45-0.55 in 2016-2017 to 0.65-0.78 in 2020-2022 closely tracks the cumulative deployment of these components. The third working hypothesis, that the field's next-generation gains will come more from throughput automation and accessibility improvements than from further resolution improvements, is consistent with the observation that the resolution dimension (S_res) has approached its theoretical ceiling for the leading targets while the throughput and reproducibility dimensions (S_thr, S_rep) continue to show measurable headroom.

The principal original contribution of this article is the formulation and calibration of the In Situ Resolution-in-Context Index (IRiCI). IRiCI is a single normalised composite metric — bounded on [0,1] — that integrates five performance dimensions of in situ cryo-ET studies (achieved resolution, native-context preservation, spatial localisation specificity, throughput per tomogram, and inter-cell reproducibility) and returns a quantitative ranking on a metric explicitly designed to reward atomic resolution and intact cellular context simultaneously. The metric is not novel in its constituent parts: each of the five dimensions has been independently discussed in the published 2016-2022 literature, and informal qualitative comparisons across in situ studies are routine in the field's review sections. The original contribution is the formalisation of the multi-dimensional comparison as a single computable index, the calibration of that index on ten landmark studies, and the use of the index to identify the Tegunov et al. (2021) ribosome-

antibiotic structure as the empirical threshold-crossing event for atomic resolution in cellular context. I do not claim that IRiCI is the only viable composite metric; I do claim that the field's reliance on qualitative cross-study comparison has become impractical as the in situ structural-biology literature has expanded, and that an explicit computable index even an imperfect first-pass one improves on the implicit alternative.

Four limitations of the present study merit explicit acknowledgement. The first is the dimensional-choice commitment, which omits temporal resolution, ligand-state specificity, light-microscopy integration, and computational accessibility; future revisions of the index should reconsider these candidate dimensions. The second is the scoring-proxy uncertainty, which I estimate at ± 0.07 on the [0,1] scale and which reflects the non-uniform reporting conventions of the source papers. The third is the methodological-tool versus biological-application asymmetry, which systematically under-credits methodological-tool papers and which a complementary methodological-impact metric would need to address. The fourth is the geometric-mean functional form, which represents one of several defensible choices and which would benefit from a sensitivity analysis across alternative formulations. The future research priorities that follow are five: a community-agreed reporting standard for the five IRiCI dimensions, the development of standardised partially-automated software workflows that reduce the operator-expertise threshold for in situ cryo-ET, the integration of correlative cryo-fluorescence light microscopy with high-throughput cryo-ET acquisition for rare-target structural biology, the extension of atomic-resolution in situ structural analysis to thicker eukaryotic cell volumes, and the application of the IRiCI framework to the rapidly growing post-2022 in situ literature as a community-wide consistency check on the field's claims of cumulative progress.

BIBLIOGRAPHY

Allegretti, M., Zimmerli, C. E., Rantos, V., Wilfling, F., Ronchi, P., Fung, H. K. H., Lee, C.-W., Hagen, W., Turonova, B., Karius, K., Bormer, X., Glavy, J. S., Buthe, H., Pulupa, J., Hummer, G., Locker, J. K., Beck, M., & Kosinski, J. (2020). In-cell architecture of the nuclear pore complex and snapshots of its turnover. *Nature*, *586*(7831), 796–800. https://doi.org/10.1038/s41586-020-2670-5

Bouvette, J., Liu, H.-F., Du, X., Zhou, Y., Sikkema, A. P., da Fonseca Rezende e Mello, J., Klemm, B. P., Huang, R., Schaaper, R. M., Borgnia, M. J., & Bartesaghi, A. (2021). Beam image-shift accelerated data acquisition for near-atomic resolution single-particle cryo-electron tomography. *Nature Communications*, *12*, 1957. https://doi.org/10.1038/s41467-021-22251-8

Danev, R., Tegunov, D., & Baumeister, W. (2017). Using the Volta phase plate with defocus for cryo-EM single particle analysis. *eLife*, *6*, e23006. https://doi.org/10.7554/eLife.23006

Hagen, W. J. H., Wan, W., & Briggs, J. A. G. (2017). Implementation of a cryo-electron tomography tilt-scheme optimized for high resolution subtomogram averaging. *Journal of Structural Biology*, *197*(2), 191–198. https://doi.org/10.1016/j.jsb.2016.06.007

Himes, B. A., & Zhang, P. (2018). emClarity: Software for high-resolution cryo-electron tomography and subtomogram averaging. *Nature Methods*, *15*(11), 955–961. https://doi.org/10.1038/s41592-018-0167-z

Mahamid, J., Pfeffer, S., Schaffer, M., Villa, E., Danev, R., Cuellar, L. K., Förster, F., Hyman, A. A., Plitzko, J. M., & Baumeister, W. (2016). Visualizing the molecular sociology at the HeLa cell nuclear periphery. *Science*, *351*(6276), 969–972. https://doi.org/10.1126/science.aad8857

O'Reilly, F. J., Xue, L., Graziadei, A., Sinn, L., Lenz, S., Tegunov, D., Blötz, C., Singh, N., Hagen, W. J. H., Cramer, P., Stülke, J., Mahamid, J., & Rappsilber, J. (2020). In-cell architecture of an actively transcribing-translating expressome. *Science*, *369*(6503), 554–557. https://doi.org/10.1126/science.abb3758

Plitzko, J. M., Schuler, B., & Selenko, P. (2017). Structural biology outside the box—inside the cell. *Current Opinion in Structural Biology*, *46*, 110–121. https://doi.org/10.1016/j.sbi.2017.06.007

Pyle, E., & Zanetti, G. (2021). Current data processing strategies for cryo-electron tomography and subtomogram averaging. *Biochemical Journal*, *478*(10), 1827–1845. https://doi.org/10.1042/BCJ20200715

Schaffer, M., Mahamid, J., Engel, B. D., Laugks, T., Baumeister, W., & Plitzko, J. M. (2017). Optimized cryo-focused ion beam sample preparation aimed at in situ structural studies of membrane proteins. *Journal of Structural Biology*, *197*(2), 73–82. https://doi.org/10.1016/j.jsb.2016.07.010

Schaffer, M., Pfeffer, S., Mahamid, J., Kleindiek, S., Laugks, T., Albert, S., Engel, B. D., Rummel, A., Smith, A. J., Baumeister, W., & Plitzko, J. M. (2019). A cryo-FIB lift-out technique enables molecular-resolution cryo-ET within native Caenorhabditis elegans tissue. *Nature Methods*, *16*(8), 757–762. https://doi.org/10.1038/s41592-019-0497-5

Tegunov, D., & Cramer, P. (2019). Real-time cryo-electron microscopy data preprocessing with Warp. *Nature Methods*, *16*(11), 1146–1152. https://doi.org/10.1038/s41592-019-0580-y

Tegunov, D., Xue, L., Dienemann, C., Cramer, P., & Mahamid, J. (2021). Multi-particle cryo-EM refinement with M visualizes ribosome-antibiotic complex at 3.5 Å in cells. *Nature Methods*, *18*(2), 186–193. https://doi.org/10.1038/s41592-020-01054-7

Wagner, T., Merino, F., Stabrin, M., Moriya, T., Antoni, C., Apelbaum, A., Hagel, P., Sitsel, O., Raisch, T., Prumbaum, D., Quentin, D., Roderer, D., Tacke, S., Siebolds, B., Schubert, E., Shaikh, T. R., Lill, P., Gatsogiannis, C., & Raunser, S. (2019). SPHIRE-crYOLO is a fast and accurate fully automated particle picker for cryo-EM. *Communications Biology*, *2*, 218. https://doi.org/10.1038/s42003-019-0437-z

Wan, W., & Briggs, J. A. G. (2016). Cryo-electron tomography and subtomogram averaging. *Methods in Enzymology*, *579*, 329–367. https://doi.org/10.1016/bs.mie.2016.04.014

Wietrzynski, W., Schaffer, M., Tegunov, D., Albert, S., Kanazawa, A., Plitzko, J. M., Baumeister, W., & Engel, B. D. (2020). Charting the native architecture of Chlamydomonas thylakoid membranes with single-molecule precision. *eLife*, *9*, e53740. https://doi.org/10.7554/eLife.53740

Zheng, S., Wolff, G., Greenan, G., Chen, Z., Faas, F. G. A., Bárcena, M., Koster, A. J., Cheng, Y., & Agard, D. A. (2022). AreTomo: An integrated software package for automated marker-free, motion-corrected cryo-electron tomographic alignment and reconstruction. *Journal of Structural Biology: X*, *6*, 100068. https://doi.org/10.1016/j.yjsbx.2022.100068

Turoňová, B., Schur, F. K. M., Wan, W., & Briggs, J. A. G. (2017). Efficient 3D-CTF correction for cryo-electron tomography using NovaCTF improves subtomogram averaging resolution to 3.4 Å. *Journal of Structural Biology*, *199*(3), 187–195. https://doi.org/10.1016/j.jsb.2017.07.007

Eisenstein, F., Yanagisawa, H., Kashihara, H., Kikkawa, M., Tsukita, S., & Danev, R. (2022). Parallel cryo electron tomography on in situ lamellae. *Nature Methods*, *19*(11), 1465–1473. https://doi.org/10.1038/s41592-022-01690-1

Castaño-Díez, D., & Zanetti, G. (2019). In situ structure determination by subtomogram averaging. *Current Opinion in Structural Biology*, *58*, 68–75. https://doi.org/10.1016/j.sbi.2019.05.011

Bharat, T. A. M., & Scheres, S. H. W. (2016). Resolving macromolecular structures from electron cryo-tomography data using subtomogram averaging in RELION. *Nature Protocols*, *11*(11), 2054–2065. https://doi.org/10.1038/nprot.2016.124

Liu, H.-F., Zhou, Y., Huang, Q., Piland, J., Jin, W., Mandel, J., Du, X., Martin, J., & Bartesaghi, A. (2022). nextPYP: A comprehensive and scalable platform for characterizing protein variability in situ using single-particle cryo-electron tomography. *Nature Methods*, *19*(2), 224–231. https://doi.org/10.1038/s41592-021-01382-2

Wagner, F. R., Watanabe, R., Schampers, R., Singh, D., Persoon, H., Schaffer, M., Fruhstorfer, P., Plitzko, J., & Villa, E. (2020). Preparing samples from whole cells using focused-ion-beam milling for cryo-electron tomography. *Nature Protocols*, *15*(6), 2041–2070. https://doi.org/10.1038/s41596-020-0320-x

EMPIAR — Electron Microscopy Public Image Archive. (2022). *Repository entries for in situ cryo-electron tomography datasets including EMPIAR-10499 (Tegunov et al., Mycoplasma), EMPIAR-10546 (Allegretti et al., S. cerevisiae NPC)*. EMBL-EBI. https://www.ebi.ac.uk/empiar/

RCSB Protein Data Bank. (2022). *PDB entries for in situ cryo-ET reconstructions including 6Z6N (Allegretti et al. NPC), 6ZTJ (O'Reilly et al. expressome)*. RCSB PDB. https://www.rcsb.org/

Mahamid Laboratory. (2022). *Publications database — EMBL Heidelberg*. European Molecular Biology Laboratory. https://www.embl.org/groups/mahamid/

Beck Group. (2022). *Publications database — EMBL Heidelberg and Max Planck Institute of Biophysics*. https://www.beck.embl.de/publications/

KRIO-ELEKTRONSKA TOMOGRAFIJA U NATIVNIM ĆELIJAMA: ATOMSKA REZOLUCIJA U KONTEKSTU

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Sažetak: Između 2016. i 2022. godine, krio-elektronska tomografija (cryo-ET) prošla je generacijsku tranziciju iz metode koja je proizvodila informativne ali rezolucijom ograničene slike vitrificiranih ćelija u onu koja je proizvodila, na rastućem podskupu meta, atomske rezolucije strukture unutar netaknutih bakterijskih i eukariotskih ćelija bez pribjegavanja prečišćavanju. Tranzicija je bila posljedica četiri konvergentna tehnička razvoja: rutinskog krio-fokusiranog jonskog-snopa (cryo-FIB) glodanja koje je proizvodilo elektronski prozirne lamele debljine 100-300 nm iz vitrificiranih ćelija, Volta faznih ploča koje su poboljšale kontrast slike pri niskom defokusu, kamera s direktnom detekcijom čiji su dozno-frakcionisani frame-ovi sačuvali informacije visoke rezolucije, te paketa razvojnih softvera za obradu slike (Warp, M, emClarity, AreTomo, SPHIRE-crYOLO) koji su omogućili poravnanje tilt-serija, prosjek subtomograma i refinement više čestica pri subnanometarskim rezolucijama. Kumulativna empirijska demonstracija da se ribosomi unutar netaknutih bakterijskih ćelija mogu razriješiti do 3,5 Å — Tegunov-Xue-Cramer-Mahamid 2021. rezultat sa multi-particle M-om — utvrdila je da je dugogodišnji cilj polja "atomske rezolucije u kontekstu" ostvariv, ne samo aspiracionalan. U ovom članku pregledavam tehnički krajolik in situ cryo-ET-a od 2016. do 2022. i predlažem, kao originalni doprinos, In Situ Resolution-in-Context Index (IRiCI) — jednu normalizovanu kompozitnu metriku, ograničenu na [0,1], koja integriše pet performansnih dimenzija (postignuta rezolucija, očuvanje nativnog konteksta, specifičnost prostorne lokalizacije, propusnost po tomogramu, te međućelijska reproducibilnost) i vraća kvantitativno rangiranje in situ cryo-ET studija. Primijenjen na deset značajnih studija iz prozora 2016-2022, IRiCI vraća rangiranje koje identifikuje Tegunov et al. (2021) strukturu ribosom-antibiotika u ćeliji i Allegretti et al. (2020) studiju nuklearnog kompleksa pora u ćeliji kao zajedničke vodeće, sa Mahamid et al. (2016) radom o nuklearnoj periferiji kao temeljnim prethodnikom.

Ključne riječi: *krio-elektronska tomografija, cryo-FIB glodanje, prosjek subtomograma, in situ strukturna biologija, atomska rezolucija, Volta fazna ploča, nuklearni kompleks pora, ribosom, Warp/M softver, nativni ćelijski kontekst.*