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Recent advances in single-molecule fluorescence microscopy render structural biology dynamic

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Single-molecule fluorescence microscopy has long been appreciated as a powerful tool to study the structural dynamics that enable biological function of macromolecules. Recent years have witnessed the development of more complex single-molecule fluorescence techniques as well as powerful combinations with structural approaches to obtain mechanistic insights into the workings of various molecular machines and protein complexes. In this review, we highlight these developments that together bring us one step closer to a dynamic understanding of biological processes in atomic details.

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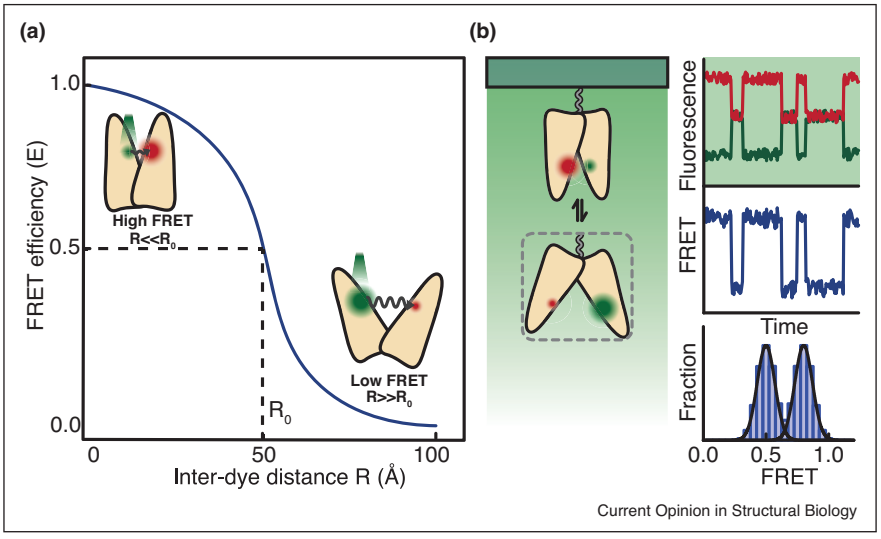
Introduction

Biological processes rely on macromolecules that, to exert their functions, often undergo structural transitions between distinct conformational states. Linking this conformational choreography to the function of a biological macromolecule paves the way for a detailed mechanistic understanding of fundamental processes in biology. The visionary physicist Richard Feynman therefore famously conjectured that, 'everything that living things do can be understood in terms of the jiggling and wiggling of atoms' [1]. An in-depth mechanistic understanding of biological processes would no doubt be facilitated if one could shoot an atomic-resolution movie of the relevant macromolecules that captures all of their distinct conformational states and, at the same time, the dynamics with which these structures interconvert and interact. Such complete insight cannot be gleaned from any single experimental technique currently, despite important advances in the field of structural biology. Cryo-electron microscopy (cryo-EM), for instance, has recently experienced an impressive 'resolution revolution' [2,3] that makes it possible to determine structural, near-atomic resolution snapshots for a small number of distinct conformations of a biological macromolecule. Cryo-EM can therefore provide important information on the structures of a subset of accessible conformations explored by the macromolecule at equilibrium. However, the order and timing of transitions between these conformations cannot typically be inferred from cryo-EM analyses. Moreover, capturing interconversion dynamics for non-equilibrium processes is challenging. To more readily infer molecular mechanisms, biophysical assays are needed to report on the dynamics that connect static structural snapshots.

Single-molecule techniques are ideally poised to satisfy this need as they enable the real-time monitoring of complex molecular processes, a direct observation of transient kinetic intermediate states, and a complete dissection of reaction pathways. The structural interconversion between distinct conformations of biological macromolecules often brings about nanometer-scale distance changes (1−10 nm). At this length scale, Fluorescence Resonance Energy Transfer (FRET) [4,5] allows the observation of distance changes in real time and with high sensitivity. In this spectroscopic technique, a donor and an acceptor fluorophore are introduced at sites of interest, the distance between which is to be monitored. Upon excitation of the donor fluorophore, its energy can be transferred to the acceptor fluorophore in a nonradiative process. The probability for this to occur, termed the efficiency of energy transfer (E), depends sensitively on the distance R between the two fluorophores according to $E = 1/\{1 + (R/R_0)^6\}$, where R_0 is the Förster radius at which $E = 0.5$ (Figure 1a). FRET measurements at the level of single molecules (smFRET) make it possible to record dynamics at a molecular scale that would otherwise be obfuscated by random averaging in ensemble experiments [6–8]. Given that they enable the real-time observation of conformational dynamics of individual molecules, single-molecule techniques have become invaluable for the mechanistic study of a host of important biological systems.

FRET efficiencies can be recorded for freely diffusing molecules when they pass through the observation volume of a confocal microscope. Such diffusion-based smFRET has been applied with great success to

Figure 1



Cartoon schematic of smFRET detection with surface-immobilized molecules. **(a)** When donor (green) and acceptor (red) fluorophores are close (<100 Å), excitation of the donor can result in acceptor fluorescence due to FRET. The efficiency of FRET sensitively depends on the distance between the two fluorophores. **(b)** Time series of FRET efficiency can be recorded from individual surface-immobilized FRET-labeled molecules, reporting on conformational dynamics at the single-molecule level on timescales ranging from ~100 ms to tens of seconds. When a histogram of individual FRET values from multiple molecules is plotted, distinct conformational states become apparent, which is not the case for bulk FRET. Surface immobilization allows for a continuous smFRET readout during buffer exchange, making smFRET detection with surface-immobilized molecules particularly useful for the study of non-equilibrium processes.

investigate various fundamental biological processes, including protein folding [9]. A key advantage of the diffusion-based smFRET implementation is its ability to access short-lived processes within the 10 μs–100 ms time regime. However, non-equilibrium dynamics are not accessible by diffusion-based smFRET.

SmFRET can alternatively be measured with surface-anchored molecules, which enables the observation of conformational dynamics on a slower time scale of 100 ms–10 s, where longer observation times are required. Here, the temporal evolution of donor and acceptor fluorescence emissions from individual surface-immobilized molecules is directly recorded. The time trajectory of FRET efficiency for an individual molecule and consequently the dwell times it spends in the distinct FRET states can thus be readily obtained and non-equilibrium processes can be observed (Figure 1b). Using this approach, rigorous control experiments are required to first establish that the immobilization of the biological macromolecule does not significantly impact on its function.

SmFRET has been successfully applied to a cornucopia of biological systems including, among many others, membrane transporters [10,11], receptors [12,13], channels [14,15], helicases [16–18], and CRISPR-associated endonucleases [19,20]. Here, we will focus on recent examples of how smFRET obtained from surface-immobilized molecules has been utilized to study protein-nucleic acid interactions. Given the extreme versatility of this approach with a vast number of applications [21,22••,23–25], we focus here on a small subset of recent examples that serve to highlight particularly important recent developments.

Multi-coordinate single-molecule FRET experiments

Conventional two-color smFRET can only report on distance changes along a single coordinate given by the distance between the donor and acceptor fluorophores. This limitation can impede the observation of more complex interactions and conformational changes and has prompted the development of more complex approaches that seek to combine smFRET with other single-molecule techniques.

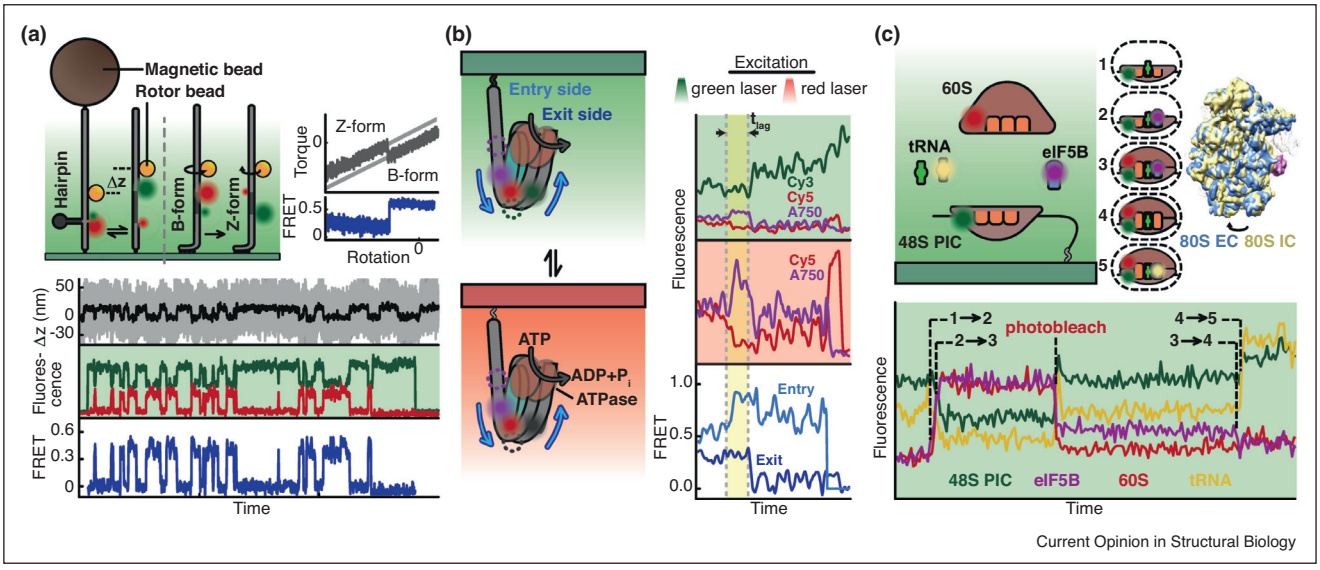
Of particular note are combinations of single-molecule fluorescence microscopy with force spectroscopy methods, including magnetic tweezers [26,27] and optical trapping [28]. Such combined approaches have proven highly efficient in addressing a vast array of biological topics, such as the mechanical properties of various DNA structures [29–31] and the mechanisms of action of diverse nucleic acid-interacting proteins [32–34,35•]. Ivanov *et al.* have recently demonstrated a powerful combination of smFRET and rotor bead tracking, an advanced

magnetic tweezers technique [36] that allows the rotational and translational movements of a DNA segment to be tracked in real time [37••] (Figure 2a). In this FluoRBT (Fluorescence Rotor Bead Tracking) approach, a DNA molecule is tethered between the surface and a magnetic bead. The magnetic bead can be used to apply force and twist to the DNA, while a second, non-magnetic (rotor) bead is attached to the side of the DNA molecule to report on the rotation of the DNA as well as the distance between the surface and the bead attachment point. The power of this new technique is demonstrated by simultaneously monitoring changes in DNA torque, measured through the amplitude of the rotor bead rotational motion, and structural rearrangements, reported by FRET, during a transition between right-handed B-form and left-handed Z-form in response to twist [37••]. More recently, the Bryant and Doudna groups have leveraged the potential of FluoRBT to investigate the mechanism of DNA interrogation by Cas9 [38]. In the future, a number of advanced optical trapping techniques, including angular trapping [39] and high-throughput nanophotonic trapping [40] could also form compelling combinations with smFRET.

Another way to overcome limitations of conventional two-color smFRET is to use additional fluorescence channels to achieve three-color [41,42] and even four-color single-molecule fluorescence microscopy [43]. Although technically challenging, these multi-color smFRET approaches hold great potential for the dissection of the coordinated motions that underlie the function of complex molecular machines [44,45•,46–48,49••,50].

For example, Sabantsev *et al.* have recently developed a three-color smFRET approach to monitor the coordinated movements of DNA during nucleosome sliding by chromatin remodelers [45•] (Figure 2b). By placing two fluorophores on the DNA at opposite sides of the nucleosome and a third fluorophore on the histone octamer, the authors were able to independently monitor the real-time movements of DNA relative to the histone octamer on both sides of the nucleosomes. This enabled the observation of a discontinuous DNA movement during remodeling, where additional DNA first moves onto the nucleosome from the entry side, while the exit side DNA is pushed out of the nucleosome only after an ATP-dependent delay. Together with cross-linking data, these

Figure 2



Expanding the smFRET methodology to include additional readouts. **(a)** FluoRBT combines rotor bead tracking and smFRET. Top left: schematic illustrating an experiment with a DNA hairpin (left) and a B-form to Z-form transition (right). Top right: correlated torque and FRET changes upon B to Z transition in response to twist. Bottom: example DNA hairpin data showing correlated FRET changes and rotor bead movements upon hairpin opening and closing. Adapted with permission from Ref. [37••]. **(b)** Three-color smFRET sheds light on the coordination of DNA movements during chromatin remodeling. Left: a schematic of the experiment showing the nucleosome labeling scheme and alternating laser excitation used to simultaneously resolve entry-side and exit-side FRET. Dotted circles represent the initial positions of DNA labels, while the arrows indicate the direction of DNA movements during remodeling. Note that the green dye is not excited by the red laser. Right: an example of a remodeling three-color FRET trace that shows the coordination between entry-side and exit-side DNA movements during remodeling. Entry side movement starts first, while exit side moves after an ATP-dependent delay designated t_{lag}. Adapted with permission from Ref. [45•]. **(c)** Multi-color single-molecule fluorescence microscopy is used to study the transition from initiation to elongation during translation. Top left: cartoon schematic of the ribosome labeling scheme. Top center: ribosome states observed in the experiment. Top right: overlay of the initiation and elongation complexes resolved by cryo-EM. Bottom: example four-color single-molecule fluorescence time trace capturing the transition from the pre-initiation to the elongation complex. Note that 60S fluorophore undergoes photobleaching during the course of the recording. Adapted with permission from Ref. [49••].

findings demonstrated that during remodeling, one or a few additional basepairs of DNA are transiently buffered on the nucleosome.

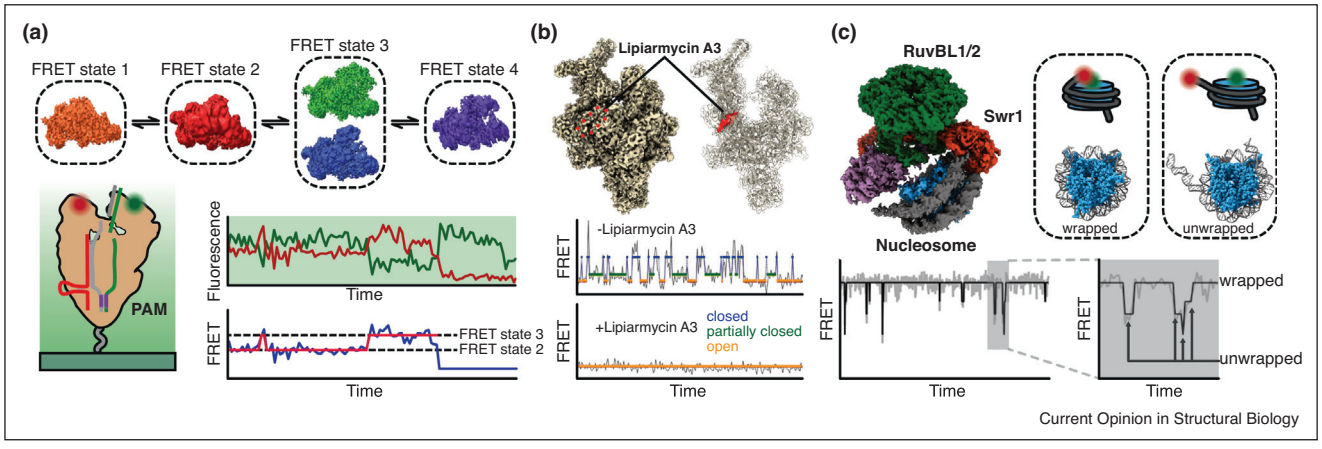
Multi-color single-molecule fluorescence microscopy has also facilitated the study of the translation machinery [47,48,49••]. A particularly elegant example is given by recent work from the Puglisi group [49••]. Here, the authors used smFRET with four-channel detection to dissect the sequence of events during the initiation of translation in yeast (Figure 2c). For multi-color detection, both ribosome subunits, eukaryotic initiation factor 5B (eIF5B), and the first elongator aminoacyl-tRNA were labeled with different fluorophores. Zero-mode waveguides, photonic nanostructures that guide the excitation light into highly confined optical observation volumes, were then used to lower the background from fluorescent molecules in solution despite their required relatively high concentrations. This strategy provided a window into the transition from pre-initiation complex to elongation in as-of-yet unprecedented detail. The study demonstrated that eIF5B GTP hydrolysis and subsequent dissociation serves as the checkpoint on the way to elongation. Insights into the timescale of this process from the single-molecule fluorescence microscopy data also facilitated capturing a cryo-EM structure of the on-pathway initiation complex. More specifically, the single-molecule data informed on the ideal time point at which to freeze ribosomes undergoing initiation, clearly illustrating the merits of an intimate connection between single-molecule fluorescence microscopy and structural methods, in particular cryo-EM.

Towards a close intermarriage of single-molecule and structural approaches

Such close intermarriage between structural and single-molecule approaches is extremely powerful. For one, knowledge of the structures of biological macromolecules facilitates the successful design of single-molecule experiments. Single-molecule experiments in turn offer the dynamic information required to correctly join static structural snapshots together into a quantitative picture of the underlying mechanisms. Recent work from several groups has capitalized on this mutually beneficial combination of approaches.

For example, Stella *et al.* were able to dissect the activation pathway of the CRISPR-Cas12a endonuclease by combining structural information from cryo-EM with insight into conformational dynamics from smFRET data [51••] (Figure 3a). Five distinct conformations of Cas12a complexed with crRNA and target DNA could be resolved that exhibited varying degrees of hybridization between the crRNA and the target DNA strand. These Cas12a structures highlighted regions that are involved in target recognition and in triggering nuclease activity, and demonstrated large-scale conformational flexibility of the complex. smFRET revealed the thermodynamics and kinetics of these conformational changes at different stages of the reaction. Based on the smFRET data, in

Figure 3



Examples of synergy between single-molecule fluorescence microscopy and structural methods. **(a)** Cryo-EM and smFRET were used to establish a conformational pathway leading to the activation of Cas12a upon target binding. Top: Cas12a conformations observed by cryo-EM grouped according to their corresponding FRET states. Bottom: a schematic of the FRET labeling scheme (left) and an example FRET trace demonstrating transitions between different Cas12a conformations. Adapted with permission from Ref. [51••]. **(b)** The combination of smFRET and structural information was used to uncover the mechanism of transcription inhibition by the antibiotic Lipiarmycin A3. Top: cryo-EM structure of RNA polymerase in complex with Lipiarmycin A3 (red). Bottom: FRET traces demonstrating how RNA polymerase conformational distribution changes upon drug binding. Adapted with permission from Ref. [52••]. **(c)** Combination of structural and dynamic information was used to clarify the mechanism of histone exchange by SWR1 chromatin remodeler. Top: cryo-EM structure of the SWR1-nucleosome complex demonstrating DNA unwrapping from the nucleosome and the FRET labeling scheme used to probe unwrapping in solution. Bottom: a FRET trace demonstrating nucleosome unwrapping dynamics in the presence of SWR1 and ATP-γ-S. Adapted with permission from Ref. [57••].

the apo, crRNA-bound, and target-bound states, Cas12a samples a number of different states with distinct FRET efficiencies that could be assigned to specific conformations visualized by cryo-EM. An analysis of the dwell times and transition rates for the different conformations using Hidden Markov Modeling shed light on the pathway leading to the enzyme activation upon target binding.

Lin *et al.* used a combination of cryo-EM and smFRET to determine the mechanism by which Lipiarmycin A3 inhibits transcription [52••]. The authors obtained a structure of the drug-bound bacterial RNA polymerase (RNAP), which adopts a catalytically incompetent open-clamp conformation. Data from smFRET experiments demonstrated that the addition of Lipiarmycin A3 freezes the RNAP in the open conformation, in stark contrast to the multiple conformations that the clamp was observed to sample in the absence of the inhibitor. Here, smFRET data were able to directly correlate static structural information with function and thus helped to uncover the mechanism of action for an important antibacterial drug (Figure 3b).

The combination of cryo-EM and smFRET has been particularly effective in studies of nucleosome remodeling [53–55,56•,57••,58,59], for the following reasons. First, the nucleosome structure can be affected by vitrification [60,61]. Cryo-EM structures of vitrified nucleosome complexes (reviewed in Ref. [62]) that display deviations from the canonical nucleosome structure [63,64] should therefore be validated using solution methods, and smFRET has proven highly efficient for this purpose [55,57••]. Second, chromatin remodeling as an essentially non-equilibrium process typically relies on multiple ATP hydrolysis cycles for completion [45•,65]. Cryo-EM studies have captured structures of remodeler-nucleosome complexes in several different nucleotide-bound states and provided invaluable insights into the structural rearrangements associated with a single ATP hydrolysis cycle of the remodeler ATPase motor [56•]. However, a complete understanding of the remodeling mechanism additionally requires knowledge of the remodeling intermediates that are formed during consecutive cycles of ATP hydrolysis, and smFRET can provide a window into these intermediates [45•,65–68]. The power of combining both cryo-EM and smFRET for studying chromatin remodeling was recently highlighted in an elegant study on the yeast SWR1 remodeler that facilitates the exchange of canonical H2A/H2B dimers for Htz1/H2B in an ATP-dependent manner [57••]. The authors reported a cryo-EM structure of the nucleosome-SWR1 remodeling complex with the transition state analog ADP-BeF_3 bound in the active center (Figure 3c). The structure showed that binding of ADP-BeF_3-complexed SWR1 to the nucleosome causes substantial DNA unwrapping and a single-base pair DNA translocation from the entry side towards the site where the ATPase motor subunit engages the nucleosome, at superhelical location 2 (SHL2). This mode of engagement suggested unwrapping-induced exposure of the H2A/H2B dimer interface as an integral part of the histone exchange mechanism. Indeed, such unwrapping of nucleosomal DNA was directly observed in solution using smFRET experiments. Upon SWR1 binding to the nucleosome in the presence of ATP or ATP-γ-S, conformational dynamics of the nucleosomal DNA were markedly increased. Notably, binding of the remodeler in the absence of nucleotides did not yield any FRET change, whereas under both ATP and ATP-γ-S conditions, nucleosomal DNA reversibly visited up to four distinct unwrapped states. The smFRET data revealed that ATP binding was both necessary and sufficient for unwrapping, even though ATP hydrolysis was required for histone exchange. Together, cryo-EM and smFRET data provide an unprecedented window into the SWR1 remodeling mechanism.

Future directions

With the advent of commercial equipment, smFRET is becoming more and more widely accessible, emphasizing the importance of common standards for smFRET experiments. The reproducibility of smFRET measurements has been recently assessed in an important multi-laboratory benchmark study [69] that suggested unified protocols and analysis methods for accurate and reproducible measurements of FRET efficiency. In principle, FRET can also be used to measure the actual distance between fluorophore attachment points, yielding direct structural information [70] (see Ref. [22••] for additional discussion).

The essentially single-molecule nature of cryo-EM makes it suitable for the analysis of heterogeneous populations of macromolecules, allowing in some cases for a number of distinct conformations within a single sample to be distinguished. However, the distinction between different conformations crucially relies on them exhibiting sufficient structural differences that can be detected at the level of individual particle images. As such, this fascinating capability of single-particle analysis cryo-EM is currently limited to the study of especially favorable contrast-rich specimens. We therefore find it intriguing to speculate on a possible future correlative smFRET-cryo-EM approach. Here, smFRET could report specifically and more sensitively on macromolecular conformation at the single-molecule level and therefore greatly enhance particle classification in cryo-EM samples. Improved particle classification might enable, for instance, the specific enrichment of a rare short-lived conformational state that is otherwise indistinguishable from the rest, but may play a critical mechanistic role.

Clearly, combining smFRET with structural methods such as cryo-EM represents a powerful means for dissecting the molecular mechanism of biological

macromolecules and complexes. Nonetheless, how they structurally transition between the most prominent conformations, that is, what short-lived structural intermediates exist, typically remains inaccessible. Molecular dynamics (MD) simulations hold great potential for filling in this remaining gap on the way to a dynamic and atomic-level understanding of macromolecular mechanisms [71–74]. Although successful examples are scarce as of yet, we anticipate that in the future, MD simulations will play a more important role in complementing static structural information and dynamics revealed by smFRET. Increasing computational power and the development of specialized MD clusters continuously improve both length and time scales of MD simulations. We eagerly await direct comparisons between all-atom MD simulations of slow conformational changes on the millisecond time scale with smFRET data.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Luka Bacic: Visualization, Writing - review & editing. **Anton Sabantsev:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision. **Sebastian Deindl:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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- •• of outstanding interest

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