Artificial microtubule cytoskeleton construction, manipulation, and modeling via holographic trapping of network nodes

J. Bergman, F. Doval, M. Vershinin

Department of Physics & Astronomy, University of Utah

ABSTRACT

Cytoskeletal networks are 3D arrangements of filaments whose complex spatial structure contributes significantly to their intracellular functions, e.g. biomechanics and cargo motility. Microtubule networks in cells are a particular challenge for *in vitro* modeling because they are sparse and possess overall structure and so cannot be approximated experimentally as a random hydrogel. We have used holographic optical trapping to precisely position and hold multiple microtubule filaments in an *in vitro* assay, where chemical and environmental variables can be carefully controlled. Below we describe the relevant practical details of the approach and demonstrate how our approach can scale to accommodate modeling of molecular motor transport and biomechanics experiments.

KEYWORDS: biophysics, cytoskeleton, 3D, holographic optical trapping, microtubules, transport, biomechanics

INTRODUCTION: Traditional biology, in other words the art of reverse engineering living matter, increasingly relies upon atomic- and molecular-scale forward engineering techniques to enable and accelerate progress. Biological systems provide a seemingly endless stream of examples of practical working nanotechnology (1) but it often takes rebuilding this technology from the ground up to understand how it really works. Despite many recent developments, a quantitative description of biology extending from atomic to organismal level remains elusive, and engineering systems which rival or exceed organismal complexity from the bottom up is not practical.

For decades, nanotechnology has been borderline science fiction. Now that it has arrived, a new challenge has come to the fore: how to scale up. In the field of biology, deciphering how an individual nano-machine (for example a cytoskeletal motor) works is not the end of the road but merely the beginning. But to move forward, we need to deal with the sheer complexity of biological systems without sacrificing the control and precision achievable at the single molecule level. Understanding how living matter functions across multiple length scales, time scales, and complexity scale is a challenge whose scope and importance grows every year (2). For example, the eukaryotic cytoskeleton as a whole is merely a collection of motors, cargos, filaments, and some associated chemicals but how these elements all integrate together to yield functional intracellular logistics and organization remains unclear.

Further progress can and must be accelerated by developing techniques to experimentally and theoretically address biological complexity. The challenge is to set up biologically relevant assays with high complexity and still reproducibly probe how the system changes when just one component is altered or removed. Consider again the cytoskeleton, and even more specifically the cytosolic actin and microtubule filaments (AF and MT respectively) which support active transport. Biology, of course, presents us with a great variety of cytoskeletal arrangements so it is often desirable to restrict consideration to some specific cytoskeleton archetype, e.g. to G_0 state of some non-polar animal cell. One may then plausibly reduce the picture to a textbook scheme(3), whereby MTs form a nearly radial array anchored at the MT organizing center (MTOC) and AFs form a more random hydrogel with some statistical preference for angles between filaments interspersed with actin bundles. In this picture, AFs are far more numerous and dense than MTs and are more prevalent towards the cell periphery. So, we would ideally want an experimental capability to build an *in vitro* model which faithfully reproduces intracellular MT cytoskeleton with a well-defined organizing center. Associated proteins and cargos could then be added under controlled conditions to study the true dynamics of the subsystem. And furthermore, ideally one would then want to be able to move or remove one filament, or one associated protein, or one cargo and rerun the experiments under otherwise identical conditions to see the effect of small perturbations on the complex ensemble. After all, if we desire a quantitative description of biology then by implication we desire to do away with the notion of a phenotype and instead to be able to capture and analyze very small (nearly differential) changes.

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The above vision is still not achievable with today's technology so let's reduce complexity further. Let's neglect the fact that AFs and MTs are typically inter-independent on each other in cells and most of the regulation of cytoskeletal dynamics. Suddenly we are dealing with two independent sub-systems which can be modeled individually. It is quite informative to compare the types of questions we need to answer for these two subsystems and the type of complexity which arises in each case. Below, we will specifically focus on two major roles of the AF and MT cytoskeleton: both are venues for intracellular transport and both serve an important role in cell biomechanics.

For either filament network we are in general faced with the need to model 3D geometric complexity (or if we are thinking of the filaments as forming a network then one might also consider the topological complexity of this network). However the nature of this complexity is different in each case. For example, AFs in lamellipodia are typically shorter (length distribution on the order of a few microns), quite dense and cross-linked by factors which cannot be neglected such as the Arp2/3 complex(4). Because of the sheer filament number and density, we may be justified in many cases to consider this system in an averaged sense. Indeed, the role of an individual AF is likely to be small because as far as transport is concerned it forms only a short segment within a mesh and that single filament will not contribute much to the overall mechanical response of the system, since the flexural rigidity of actin is quite low (of order 1e-25 Nm^2 (5)). Hence, it is common and entirely reasonable (in some unsettlingly gross approximation) to model actin subsystem as a hydrogel and control its properties via overall filament density and via its cross-linkers (6–9). By contrast, MTs are not a hydrogel in a great number of cells no matter how much one coarse-grains the description. MTs in many cells are sparse. MT-MT distances on the order of 100 nm are common (10, 11). Reptation of MTs with a MT network is usually not biologically relevant but by contrast reptation of MT filaments in actin gels has received attention (12). The number of MTs in cells is fairly small, to the point where modern imaging and computing potentially allows for all filaments to potentially be tracked. For example fairly large mammalian BSC-1 cells were reported to contain only ~700 MTs (13). Crucially, rigidity of a MT filament (5) is several orders of magnitude higher than that of an AF (of order 1e-23 Nm^2) and MT lengths can be sufficient to span the cell or even extend from MTOC to cell periphery and bend backwards (14). It is not our intent to trivialize the enormous variety of MT networks (e.g. (15)) even under our simplifying assumptions, but the net result of the above considerations is that for a great number of cells the contribution of even a single MT filament to intracellular transport and biomechanics can potentially be accounted for and should not be treated in an averaged sense. Hence, it is often entirely unreasonable to generally model the MT subsystem in cells as a hydrogel (although this may be applicable in some specific cases) and one cannot simply control its properties via filament density or cross-linkers alone. Particularly for MT cytoskeleton, capturing the complexity of the network requires the ability to position multiple filaments individually and precisely in 3D. The task is complicated by the fact that MTs are polar filaments, so that one end is chemically different from the other. Therefore 3D MT assemblies must recapitulate not only the desired filament geometry but also the desired polarity of each filament. Once this minimal set of requirements is achieved, one of course further requires the potential to add associated proteins, cargos and other complexity in a controlled manner.

The benefits associated with precisely modeling transport and mechanics at the level of a complex cytoskeletal network (rather than at the scale of an individual filament) are numerous. When it comes to biomechanics, several key questions become accessible to experimental probing. We know at the level of a single filament that MT rigidity and persistence length depend on the filament contour length (16). This is a simple and intuitive consequence of the fact that longitudinal bonds within each protofilament are mechanically different from lateral tubulin-tubulin bonds. But it is unclear how this picture scales for larger MT assemblies. If individual MTs have a characteristic length scale over which mechanical properties significantly change, then does this length scale also govern the mechanics and spatial dynamics of MT filament networks? It is also unclear how MT-MT cross-links (especially multi-motor cargos) affect network properties. First, rather obviously, cross-links themselves can have variable mechanical properties. Less obvious is the fact that cross-linking of MTs by e.g. motors on cargos occurs via binding to individual tubulin binding sites. Therefore cross-links can potentially have differing effects on network properties depending on how they mechanically couple to the intrinsic anisotropy of each filament. The ability to build 3D MT structures would allow experimental access to all of the above issues. In addition, it would help clarify things on a conceptual level. There is a long standing debate (partially reviewed in 17) as to how much MT cytoskeleton can contribute to cell mechanical properties. The ability to construct increasingly complex MT networks and to study them as a function of their degree of crosslinking and spatial scale would finally establish experimental limits for this broad class of problems.

The ability to precisely model 3D MT networks would also be highly beneficial to studies of molecular motor driven transport. While much experimental modeling to date has been done *in vitro* in bead assays or with *ex vivo* cargo transport on single filaments, these assays only capture a subset of actual cargo navigation in cells. MTs are certainly sparse in many types of eukaryotic cells so there are undoubtedly stretches of cytosolic space where a single filament is all that is accessible to a given cargo. However, much of the recent super-resolution work (10, 11, 18) demonstrates that in many locations MTs are spaced close enough to each other that a single cargo's motors could geometrically reach two or more filaments (a locus which is natural to term MT intersection despite the fact that the terminology is then cargo-specific). So the question of how cargos are routed on realistic MT arrangements (or in physics terms, the problem of experimentally measuring the two point correlation function for active transport on a complex network) cannot be answered without the ability to build these arrangements and to also introduce known and well-characterized cargos into these networks. Ideally, one would start with experimentally modeling traffic rules in the simplest case: a single MT-MT crossing. The resulting statistical picture could then be applied to two or more MT-MT crossings. In principle, cargo behavior at each crossing should be governed by the same overall traffic rules and the motility of a cargo along the clear filament stretches which connect the intersections is already amenable to theoretical modeling. Therefore it should be possible to reduce the routing on a complex network to a Markov chain of navigation traversal events. However, the extent to which rules at each intersections scale for a chain of intersections needs to be experimentally constrained. In other words, if we desire to understand cargo transport on complex cytoskeletal networks it is necessary but not sufficient to be able to experimentally model just one intersection in 3D. The ability to scale up network geometry and topology cleanly and reproducibly is essential for the full benefit to accrue.

Finally, we must not overlook the fact that MTs are a fascinating materials system for nanoscale and micron-scale engineering (19). As Howard lab pointed out, the Young's modulus of MTs is "similar to Plexiglas and rigid plastics" (5). However the aspect ratios of the tubular shapes are dramatic (of order 2000:1). These structures are thus suitable for building *in vitro* scaffolds and (if the approach were sufficiently scalable) MT-based metamaterials. In this respect, one complication is that MTs can be unstable at higher temperatures and can depolymerize or change conformation due to the presence of additional chemical agents. Therefore, for engineering purposes, it is important to be able to stabilize MT structures *in situ*.

RESULTS: We have recently demonstrated an experimental approach to build 3D MT structures *in vitro* (20). Briefly, our system (21) is built around an inverted Nikon Ti-U microscope and has two optical trapping lines admitted from the objective side via a dichroic beam combiner. One optical trapping line is driven by a 1W laser at 1064 nm and is spatially modulated by a holographic mirror to allow the creation of multiple holographic optical traps (HOTs) in the field of view in 3D (i.e. not just in the focal plane of the objective). The other trapping line is driven by a high power diode laser at 980 nm and can be quickly moved in the field of view via either a piezo driven mirror (high trapping power and deflection range) or an acousto-optic deflector (maximum speed). The fast-moving ordinary trap thus compensates for the one weakness of holography – slow spatial reconfiguration.

Given our microscopy capabilities, the fundamental idea is to attach refractive microspheres to MTs and to then use these optically trappable nodes as bead handles (BHs). We assure bead affinity for MTs by decorating their surface with enzymatically dead full-length kinesin-1. Precise MT manipulation requires at least two BHs attached to it, preferably far away from each other (we usually refer to such an assembly as a dumbbell). Once such an assembly is built, it in principle allows a MT to be positioned in 3D via HOT of the two BHs. Crucially, we have shown that dumbbells can be built with high precision and high workflow throughput. In addition, we have demonstrated that model cargos and regulatory chemical agents can be introduced into the system to gradually expand experimental complexity. Our approach to constructing 3D cytoskeletal structures is optimized for MT filaments although other high aspect ratio reasonably rigid building blocks may be used by extending our basic idea.

Previous relevant publications from our lab were aimed at describing the underlying technology and at demonstrating that the approach is feasible and practical. Here, we will discuss how our approach works in practice and how the workflow can be optimized and scaled up.

Storage Depot: If one desires to build a simple 3D MT-MT crossing arrangement (described before 20), then it is often convenient to construct a dumbbell, release it from trapping so that it is allowed to settle to the coverslip "floor" of the chamber. The glass BHs are sufficiently denser than the water-based buffer that they readily sink and do not appreciably diffuse one on the coverslip, especially if both BHs are 0.5 μm in diameter or larger. The location of the dumbbell can then be remembered via some local surface imperfection and it is then usually possible for us to

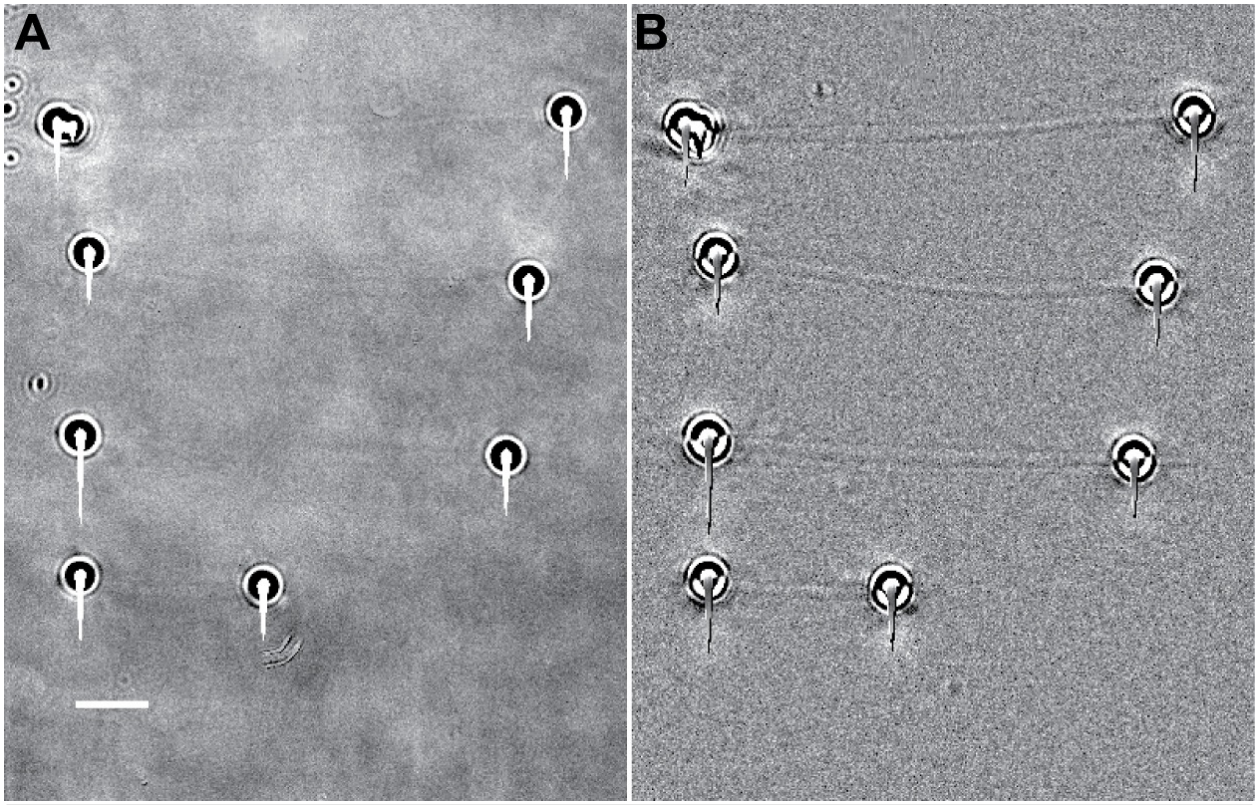

Fig. 1. Four MT dumbbells are resting on the glass coverslip in a designated area. (A) Raw image shows random impurity spots on the coverslip surface. Each bead is imaged with a streaking artefact due to camera oversaturation under our lighting conditions. (B) Filtered frame image showing MT locations more clearly. Here two nearby frames were high-pass filtered and then subtracted from each other to eliminate static background. Scale bar: 5 μm.

build another dumbbell close enough to the first location that finding the first dumbbell is easy. However this approach fails when the number of dumbbells grows. In such cases, we can build dumbbells one by one and drop them off to the coverslip in a selected location (Fig. 1).

In this way, an entire depot of building blocks can be prefabricated and made available for future use. Finding a depot routinely can be aided by adding micrometer scales to the sample stage. The scales need not be extremely high resolution; 20 micron ticks are sufficient assuming that the depot storage covers most of the field of view. Stages with built-in digital readout of global absolute position could offer even more convenience.

Although dumbbells do not diffuse much over experimental time scales when lying on the surface, they can be inadvertently "bumped" by a trap manipulating another object within the depot. That could then lead to undesirable BH-MT cross-linking. To avoid this, some minimal spacing between objects, typically on the scale of a few microns, is practical.

Bead-MT arrangements: The strength of BH-MT affinity can be easily modulated by changing the density of enzymatically dead kinesin-1 on bead surface. We have observed a continuum of assay conditions, from no BH to MT binding to very robust attachment. In the intermediate case, attachment may form slowly or not at all in each case. Testing for whether attachment occurs can be easily done by moving the BH relative to MT and observing whether a MT deforms. We usually aim for our assays to have robust BH-MT attachment. Under those conditions we can form dumbbells most efficiently as previously described (20) by finding a bead with one MT already attached randomly ("mace" arrangement). Another BH can then be placed on the MT at a desired displacement from the first bead.

The use of dumbbells is the most reliable way to assemble 3D shapes but in some cases maces can be the more flexible building blocks. To give a simple example, we can use them to build V-shaped assemblies as shown in

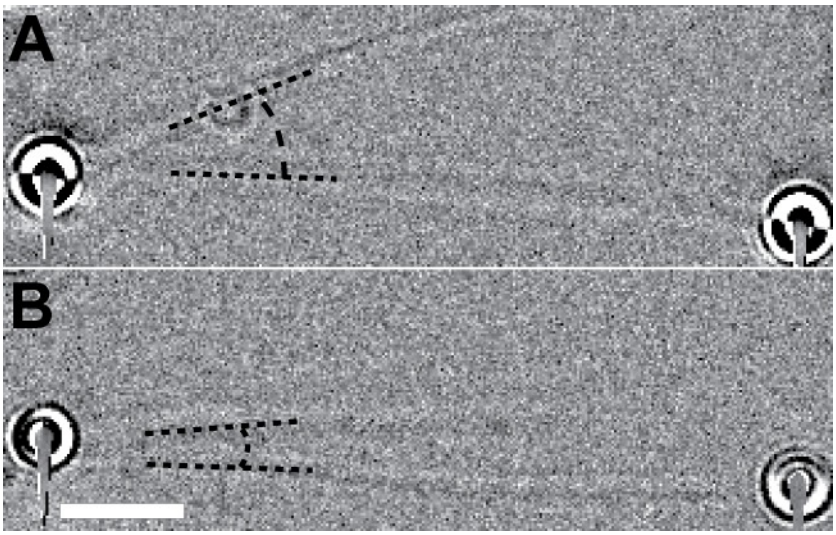

Fig. 2. A dumbbell with a side MT can be a useful construction block. In addition they inform us about the BH-MT attachment - no nodal point pivoting between a reference MT (fixed by the dumbbell) and the MT with one free end is seen. Here pivoting in the range of ~10° to ~25° is seen but larger angles are not attained even when the system is perturbed by the dumbbell moving through solution or the stage moving. Scale bar: 5 μm. Filtering as in Fig. 1B.

Figure 2. These assemblies are useful if one wants to test whether BH-MT bond is via a single dead kinesin homodimer. In particular, we expect to see nodal point pivoting (22, 23) for single kinesin-MT bonds because we use full-length kinesin molecules with unaltered stalk. However, for strong bead-MT attachment assays, we never see such pivoting, suggesting that the bonds are mediated by multiple motors which collectively constrain the angle by which a MT can pivot. One can also build V shapes where all nodes are fixed via BHs but it is not clear at the moment whether actively forcing the MTs to pivot would have the resolution to probe the exact number of BH-MT attachment cross-bridges.

Building complexity: HOT-based construction has to work well in the face of multiple challenges. Among those is the occasional difficulty of MT visualization and the fact that MT diffusion, if left unconstrained, can make it challenging to space the BHs on MTs as desired. For example, we have found placing two beads simultaneously on a completely unconstrained MT with any degree of precision difficult. It is far better to place one bead reliably and then to place the second bead at a desired offset. Therefore, we do not advise building dumbbells and then manipulating them in parallel to try to link them up via a free MT with no attached BHs. Attempting to make multiple BH-MT bonds simultaneously almost never works out well in our hands. Building MT structure one building block at a time is far more prudent.

Populating the depot area with appropriate building blocks is therefore a good start for building 3D filament shapes. Care must then be taken to assemble individual pieces into the final assembly to avoid inadvertent crosslinks and to assure that cross-links are formed at desired locations. For example, consider the pentagonal shape in Figure 3. An interesting challenge here is that this network, as shown, cannot be assembled from dumbbells alone. Instead, maces are used to build a flexible skeleton and the shape is then linked up into the final form using two dumbbells.

Manipulation of maces to allow their proper positioning can be more challenging than for dumbbells because a MT is only controlled via one node. This of course is not sufficient for deterministic 3D manipulation. Nevertheless mace-based construction, as in Fig. 3, is not too onerous. Several factors make assembly process more reliable. First, a single mace can be oriented as desired by moving the bead in space fast enough to flow-align its MT. Second, assembling "starter" shapes from maces can be performed near the surface which provides an effective steric constraint for MT rotation. Finally, one usually does not need to manipulate a single mace beyond building the key "starter" shapes. Once even just two maces are linked up, we have a dumbbell and a MT hanging off of it (a

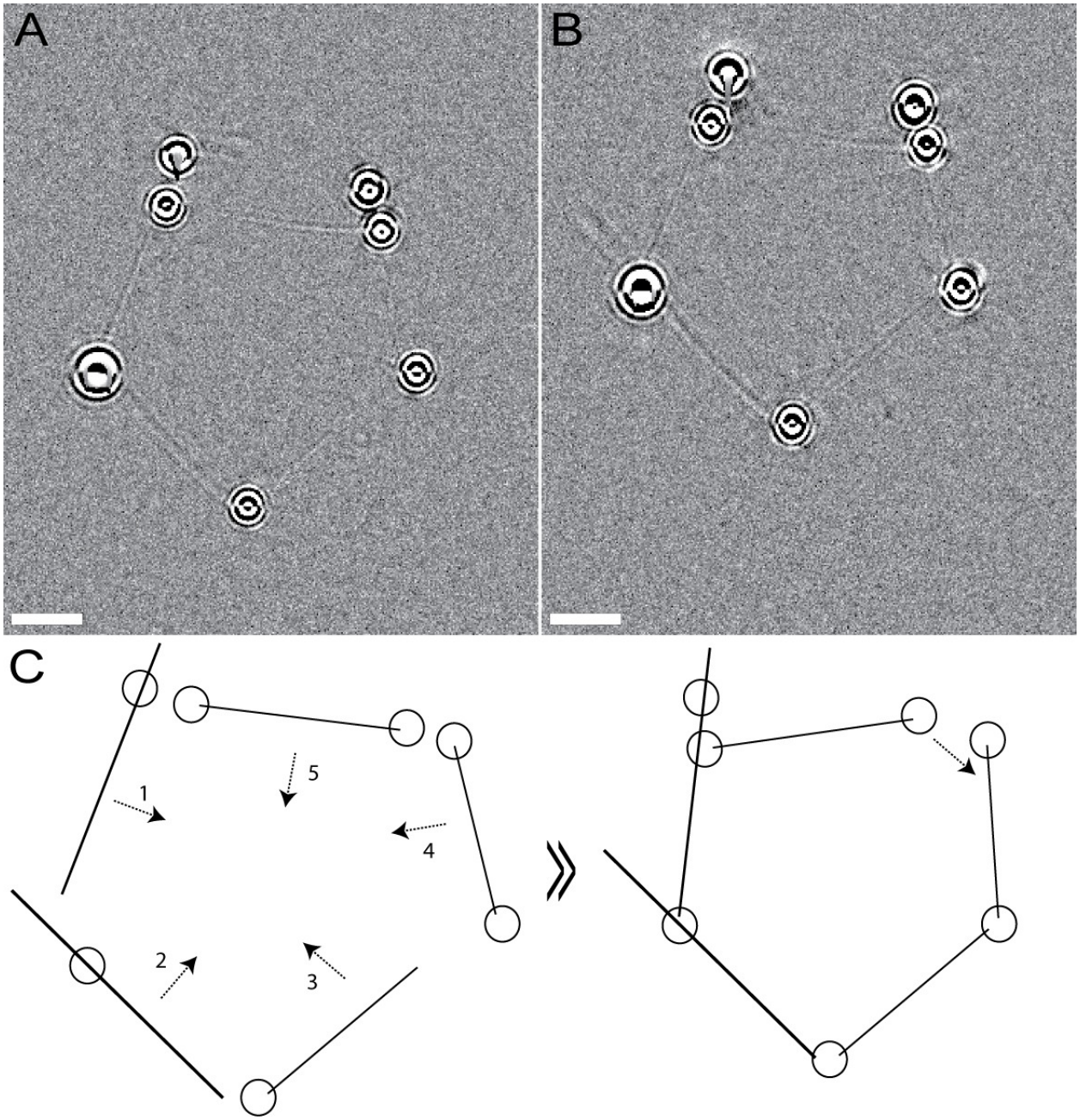

Fig. 3. Pentagonal shape is assembled out of five MTs and seven BHs. Once built (A), the shape can be moved as a whole (B). The assembly process (shown as an Ikea diagram) helps avoid inadvertent cross-links. Scale bar: 5 μm. Filtering as in Fig. 1B.

situation similar to Fig. 2). As discussed above, the MT with an unconstrained end will not diffuse around much thus minimizing the risks of inadvertent crosslinks.

Let us now consider construction into the third dimension. For our current implementation, the requirement for good visualization of MTs in 3D is somewhat limiting the complexity of what can be built with efficient workflow, hence our preference for initially building key components in plane. Construction within one 2D layer can often be facilitated by bringing building blocks in first raising above or below the plane of construction and then

lower or raising them into place. For 3D construction, building blocks can often still be built in a plane and then rotated appropriately before the final assembly.

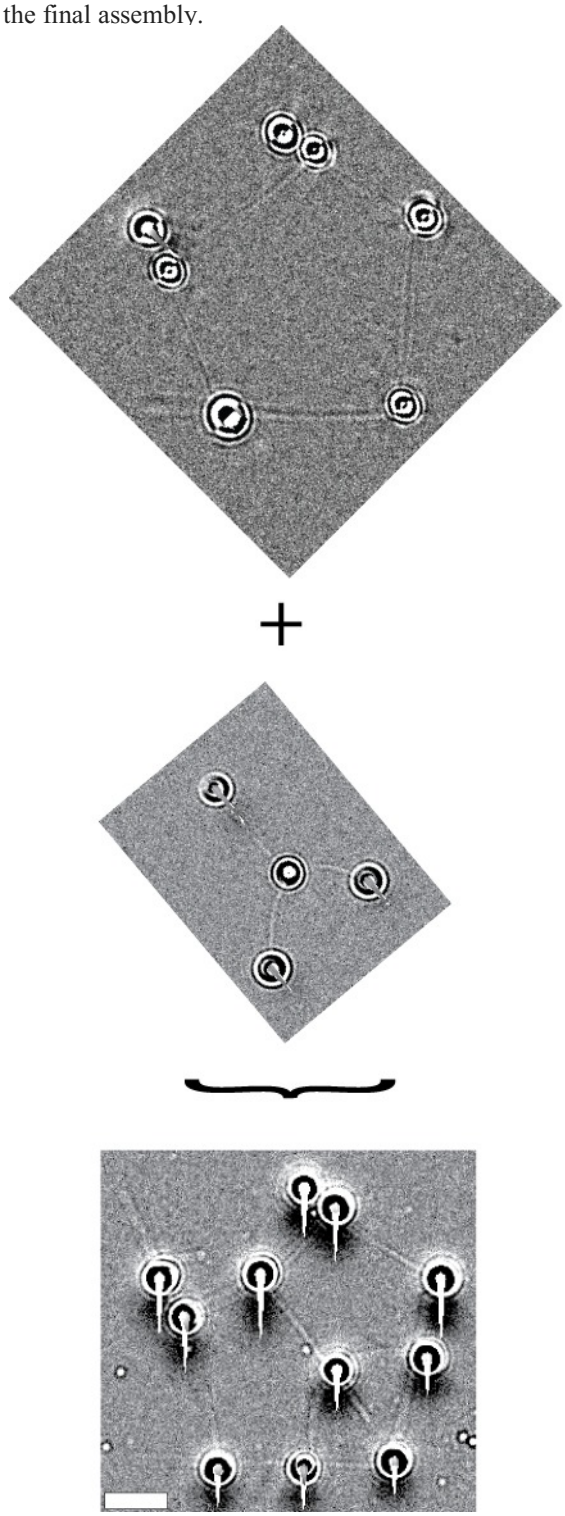

Fig. 4. Pentagonal and Y building blocks are combined to form a more complex arrangement which would be challenging to achieve efficiently by other means of nano-assembly. Assembly time (from design to completion) was ~2 hours. Scale bar: 5 μm. Filtering as in Fig. 1B.

Increasing complexity: Building blocks are not necessarily limited to just maces and dumbbells. As mentioned above, more complex building blocks can be manipulated as a whole and dropped into other shapes to combine into the desired final product (Fig. 4). In the case shown, the Y building block was first assembled from two dumbbells (not shown). The building block was then lifted above the pentagon and finally lowered into place. The Y shape was slightly angled as it was lowered to make sure that one BH at a time made contact with the pentagon MTs. Building blocks such as the ones shown here can in principle also be stored in designated depot areas.

Another barrier to increasing complexity that warrants discussion is available space. The exact dimensions of the volume accessible to HOT vary with the choice of the objective: our typical choices are Nikon 60X water immersion and 100X oil high NA objectives and of course the 60X allows for higher accessible volume. With 100X objective, our volume is nearly cubic (~50 micron on the side) though working at high z depth requires additional care. Despite this space limitation, the building capacity of the system is far greater. For example, we manipulated the pentagon shape (Fig.3 and 4) for some duration of time via only some nodes while other nodes were moved off-screen. The final assembly occurred fully within the field of view however it is now clear that stable shapes can be moved off screen so long as they are structurally connected to the structure being trapped within the field of view. Therefore MT structures can potentially be scaled from sub-micron scale to sub-millimeter scale or even larger.

Additional cytoskeletal structures: The structures considered so far have been chosen to illustrate building techniques and practical assembly issues which we have dealt with to assure reliable, scalable, high throughput construction. It is also worth highlighting how the current system can be used to build other types of biologically relevant topologies. For example, a very exciting development in the field of MT networks has been the discovery that in some cases MTs can nucleate along other MTs giving rise to internally branched MT asters (aka extended star topology). These types of topologies are experimentally accessible via HOT construction (Fig. 5).

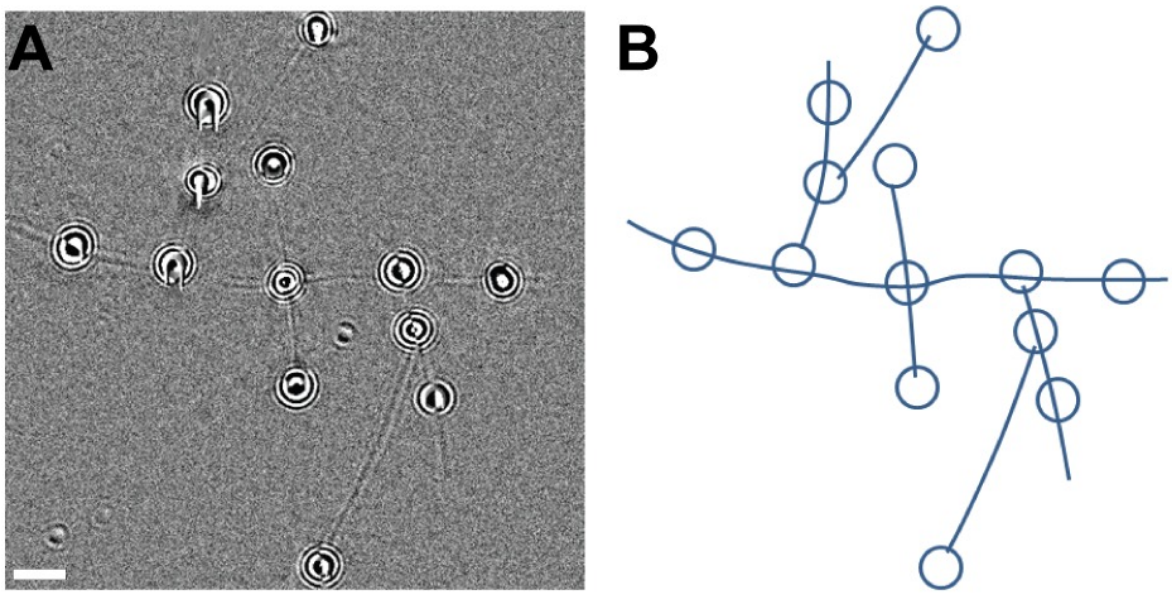

Fig. 5. An example of an extended star MT network (A) and a schematic of its topology (B) constructed *in vitro*. The network shown here is resting on the coverslip floor and its nodes are thus free to undergo limited diffusion. Thus the shape changes over time but MT bending and rotation are not sufficient to bring BHs next to nearby MTs and cross-link the structure. Scale bar: 5 μm. Filtering as in Fig. 1B.

Likewise, we have previously shown that MT-MT crosses can be built *in vitro* and used to study transport at filament intersections (20). However this demonstration focused on a single intersection. Yet, as discussed above, it will soon be necessary to show that the rules which will be experimentally established for a single intersection can scale to more complex case of multiple intersections. In this example, it is important that the MTs are held in place by optical traps. The geometry can then be adjusted on the fly as desired (Fig. 6). Of course the other benefit is that forces exerted by motors during tug-of-war events can be quantified (20).

Remaining challenges and conclusions: Several improvements to the presented technique are clearly worth pursuing in the future.

1. It is increasingly clear that the efficiency of the assembly process depends heavily on our ability to analyze the structure to be built and partition it into optimal building blocks. What constitutes optimal is a balance of several factors. The blocks themselves need to be easy to build, and space-efficient to store in a depot. Of course they should also allow for a straightforward assembly workflow which would minimize the risk of inadvertent MT-BH crosslinking.

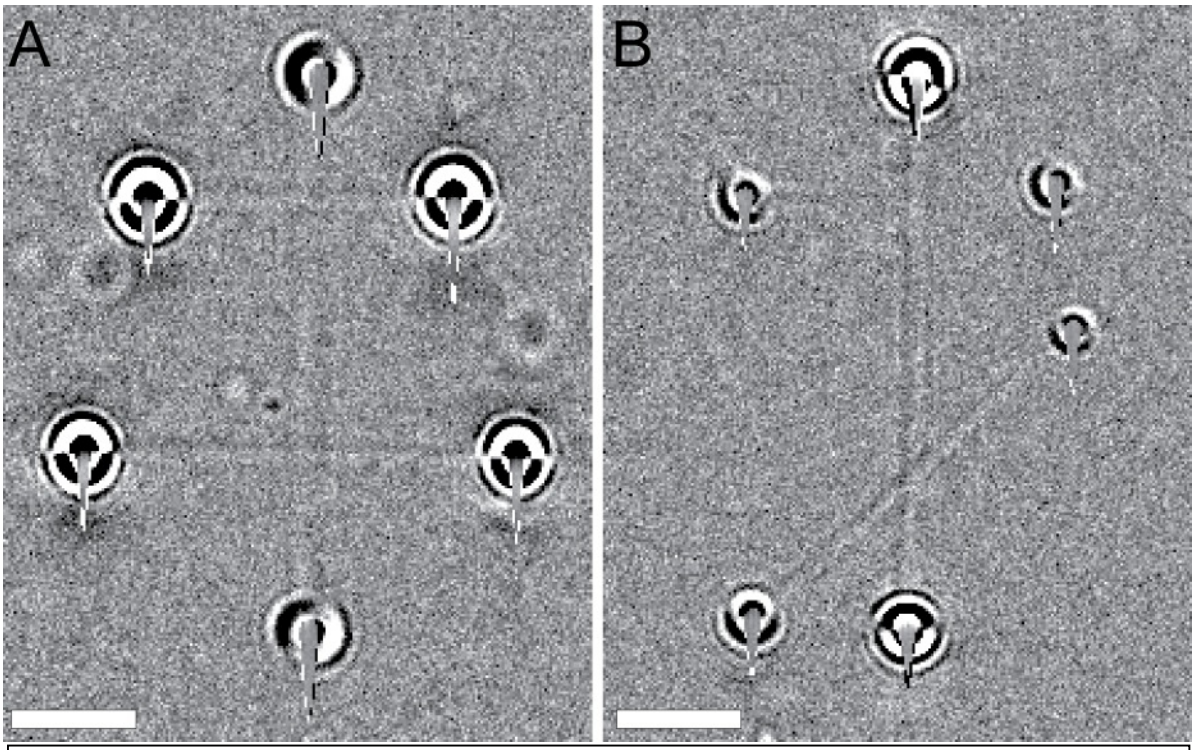

Fig. 6. Two MT-MT crosses are formed by three dumbbells. All dumbbells are held in independent traps, so that the structures are not crosslinked and intersections angle can be adjusted from e.g. normal (A) to 45° (B) for one of the intersections. Scale bar: 5 μm. Filtering as in Fig. 1B.

2. It is important to improve MT imaging. So far, label-free imaging has proven adequate but it is clear that an alternative is desirable, especially for maximizing the 3D potential of the technique. The problem is that structural assembly and subsequent imaging can take hours of work with illumination conditions dictated by the needs of the experiment. Therefore any signal type that can be readily bleached out via excessive illumination is likely not optimal. Techniques like iScat may be preferable in this case but have not yet been explored.

3. As design and build rules become more set, it will clearly be desirable to automate the construction of MT-based structures. This development would allow potentially much faster construction with higher complexity.

The MT cytoskeleton modeling technique we have developed certainly can be improved upon but as is it already holds much promise for biophysical studies, e.g. in biomechanics, MT-based transport and nanoengineering. We demonstrated here that the approach is highly scalable and extensible. Incorporating more MTs into the structures and building larger structures is possible and in fact straightforward for many biologically relevant designs.

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