A highly homogeneous expansion microscopy polymer composed of tetrahedron-like monomers

Ruixuan Gao1,2,3,15, Chih-Chieh (Jay) Yu1,2,4,15, Linyi Gao2,4,5,15, Kiryl D Piatkevich1,2, Rachael L Neve6, Srigokul Upadhyayula3,7,8,9,10,11 & Edward S Boyden1,2,4,12,13,14*

1McGovern Institute for Brain Research, MIT, Cambridge, MA, USA.
2MIT Media Lab, MIT, Cambridge, MA, USA.
3Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA, USA.
4Department of Biological Engineering, MIT, MA, USA.
5Broad Institute, MIT, Cambridge, MA, USA.
6Department of Neurology, Massachusetts General Hospital, Cambridge, MA, USA.
7Department of Cell Biology, Harvard Medical School, Boston, MA, USA.
8Program in Cellular and Molecular Medicine, Boston Children’s Hospital, Boston, MA, USA.
9Department of Pediatrics, Harvard Medical School, Boston, MA, USA.
10Advanced Bioimaging Center, University of California at Berkeley, Berkeley, CA, USA.
11Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA, USA.
12MIT Center for Neurobiological Engineering, MIT, Cambridge, MA, USA.
13Department of Brain and Cognitive Sciences, MIT, Cambridge, MA, USA.
14Koch Institute, MIT, Cambridge, MA, USA.
15These authors contributed equally: Ruixuan Gao, Chih-Chieh Yu, Linyi Gao.
*email: esb@media.mit.edu

ABSTRACT

Expansion microscopy (ExM) physically magnifies biological specimens to enable nanoscale-resolution imaging on conventional microscopes. Current ExM methods permeate biological specimens with free radical-polymerized polyacrylate hydrogels, whose network structure limits the microscopy resolution enabled by ExM. Here we report that ExM is possible using hydrogels with more homogeneous network structure, assembled via non-radical terminal linking of monomers of tetrahedral shape. As with earlier forms of ExM, such “tetra-gel”-embedded specimens can be iteratively expanded for greater physical magnification. Iterative tetra-gel expansion of herpes simplex virus type 1 (HSV-1) virions by ~10x in linear dimension results in a viral envelope deviation from sphericity of 9.2 nm, rather than the 14.3 nm enabled by free radical-polymerized hydrogels used in earlier versions of ExM. Thus, tetra-gel polymer chemistry may support new forms of ExM imaging that introduce fewer spatial errors than earlier versions, and raise the question of whether single biomolecule precision may be achievable.
MAIN

Expansion microscopy (ExM) is in increasingly widespread use because it enables, via physical magnification of biological specimens, nanoscale imaging on conventional microscopes\textsuperscript{1-3}. In ExM, biological specimens are first embedded in a swellable hydrogel network, chiefly composed of crosslinked sodium polycrylate. During this embedding, biomolecules and/or fluorescent tags are covalently anchored to the hydrogel network, so that their relative organization is preserved after the specimen is enzymatically or chemically softened, and the hydrogel expanded upon immersion in water (typically ~4x-5x in linear dimension). Protocols using off-the-shelf-chemicals\textsuperscript{4} have helped ExM find utility in a wide variety of contexts, ranging from the mapping of ribosome components and RNAs in presynaptic compartments\textsuperscript{5}, to the analysis of circadian rhythm neural circuitry in the \textit{Drosophila} brain\textsuperscript{6}, to the analysis of kidney disease and breast cancer in human biopsies\textsuperscript{7}, with new results appearing continuously (see review\textsuperscript{3}). Variants of ExM have been developed that achieve nanoscale localization of proteins and RNAs in preserved cells and tissues on diffraction-limited microscopes\textsuperscript{4,7-13}. Additionally, new strategies have been introduced to expand specimens ~10x-20x in linear dimension, by applying the expansion process over and over (iterative expansion microscopy or iExM)\textsuperscript{14} or by using superabsorbent hydrogels (X10 expansion microscopy)\textsuperscript{15}. However, all ExM variants to date form the hydrogel mesh via free-radical polymerization, a process that results in nanoscale structural heterogeneity\textsuperscript{16-20}. This raises the question of whether another polymer, or polymerization process, might result in better structural heterogeneity, and thus less spatial error during the expansion process.

Before proposing a strategy, we describe two earlier ExM protocols in detail to provide context, since we aim to improve specific steps of these processes. One popular version of ExM is so-called protein-retention expansion microscopy (proExM)\textsuperscript{8}. In one variant of proExM, fixed biological specimens that have been labeled with fluorophore-bearing antibodies are exposed to a small molecule (abbreviated as AcX) that equips the primary amine groups on antibodies and proteins with a polymerizable acryloyl group. The specimen is then immersed in a solution containing sodium acrylate monomer (with acrylamide co-monomer), as well as the crosslinker N,N-methylenebisacrylamide, and a dense network of crosslinked sodium polycrylate hydrogel is then formed, permeating throughout the specimen, through free-radical polymerization. During this process, the antibodies and proteins are covalently anchored to the hydrogel polymer network via the AcX linker. Finally, an enzymatic digestion step using proteinase K cleaves most of the proteins, largely sparing the antibody-targeted fluorophores, and allowing for expansion of the polymer-specimen composite in water. As a second example of an ExM protocol, iterative expansion microscopy (iExM)\textsuperscript{14}, fixed specimens are first labeled with primary antibodies and then DNA oligo-conjugated secondary antibodies. Next, oligos bearing a gel-anchorable moiety are hybridized to the secondary antibody-conjugated DNA oligos. Then a first hydrogel is formed as above, but with a cleavable cross-linker, which anchors the gel-anchorable DNA oligos to the hydrogel at the locations of the immunostained proteins; this gel is then expanded as described above for proExM. Next, another round of hybridization of DNA oligos complementary to those anchored to the first hydrogel, and again bearing a gel-anchorable moiety, is performed. Then, a second hydrogel is formed throughout the expanded first hydrogel, so that the new DNA oligos are anchored to the second hydrogel at the sites of immunostained proteins. Finally, the first hydrogel is cleaved, allowing the second hydrogel to expand upon immersion in water. The protein locations can be imaged after the polymer-
anchored oligos are labeled by applying complementary oligos, potentially equipped with branched DNA for amplification purposes, bearing fluorescent dyes. The oligos, in short, provide a molecular mechanism (based on complementary strand hybridization) for signal transfer between the first and second hydrogels, as well as a scaffold for amplification and fluorescence readout.

In both cases, the free-radical polymerization process that forms the sodium polyacrylate hydrogel has nanoscale structural heterogeneity. A structural study using small-angle X-ray scattering (SAXS) found that the mean size of local cross-linker density variations (Fig. 1a, “1”) can amount to 15-25 nm for polyacrylamide gels, depending on the gels’ monomer and cross-linker concentrations\(^{16}\). Moreover, topological defects of the polymer chains, such as dangling ends (Fig. 1a, “2”) and loops (Fig. 1a, “3”), are known to introduce deviations from uniform polymer network meshes at the 1-10 nm length scale\(^{19,20}\). So far, no studies have directly and precisely assessed whether and how the structural heterogeneity of the hydrogel matrix relates to the microscopy resolution enabled by ExM, and therefore it remains unclear at this point how much the aforementioned monomer/cross-linker density variations and topological defects would affect the effective resolution of ExM. In our recent study of iExM, described in the previous paragraph, we found that the process of expanding cells ~20x in two rounds of gel formation and expansion, resulted in ~9-13 nm of local distortion for the expansion of tags targeted via antibodies to tubulin\(^{14}\).

In the field of polymer chemistry, many attempts have been made to obtain an ideal hydrogel matrix, with a more homogenous polymer network than obtainable with free-radical polymerization\(^{19-24}\). One study utilizing terminal linking of two kinds of tetrahedral polyethylene glycol (PEG) monomers to form a diamond lattice-like polymer network resulted in hydrogels with extremely high structural homogeneity\(^{19,20,24}\). Small-angle neutron scattering (SANS) and static light scattering (SLS) studies showed that the thus-synthesized hydrogel, called a tetra-PEG gel, was structurally proximate to an ideal polymer network, nearly void of structural defects such as loops, dangling chains, and trapped entanglements at both the as-synthesized state and the slightly swollen state (<1.5x linear expansion in water)\(^{25,26}\). Recent studies have demonstrated that by swapping the terminal functional groups or the tetra-arm backbones with other chemical moieties, the tetra-PEG gel structure can be exploited to create hydrogels with versatile chemical and mechanical properties, such as click-chemistry-based polyelectrolyte hydrogels\(^{27}\) and highly compressible and stretchable hydrogels\(^{28}\). In the tetra-PEG design, the polymer chain lengths and the cross-linking densities are highly uniform owing to the consistent monomer size and the complementary, mutually-limiting polymerization mechanism. Moreover, topological defects of the polymer network, such as loops and dangling ends, are largely removed by design, due to the specific and stoichiometric terminal linking.

We here report a class of swellable hydrogels based on the tetra-PEG structure, designed to have structural heterogeneity better than that of the radically polymerized hydrogel matrices used in previous ExM protocols. Formed by click-chemistry-based, non-radical linking of two complementary, tetrahedral monomers comprising backbones of polyacrylate and PEG respectively, and called tetra-gel (TG), the resulting optically transparent hydrogels swell ~3.0-3.5-fold linearly in deionized water. By infusing preserved cells or tissues with a small molecule linker such as NHS-azide, which adds a click-reactive azide group to primary amines of
biomolecules such as proteins, we show that proExM can be performed with the TG polymer. Moreover, introduction of a cleavable moiety to one of the monomers allows the formed TG to be cleaved into individual monomers after being expanded and then re-embedded in a second hydrogel, rendering TG compatible with the iExM process\textsuperscript{14}. We investigated whether TG-based iExM improved resolution over our earlier form of iExM. Given that resolution and spatial error of iExM is in significant part limited by the size of the antibodies administered pre-expansion\textsuperscript{14}, for the purposes of evaluating TG-based iExM chemistry, we implemented a direct-labeling strategy to directly conjugate DNA oligos nonspecifically to protein targets. Using herpes simplex virus type 1 (HSV-1) virions as a testbed, we found that ~10-fold expanded HSV-1 virions exhibited envelope proteins with significantly smaller deviation from a circular shape at the virion midplane, compared to the classical sodium polyacrylate/acrylamide gel (PAAG)-expanded virions [TG: 9.2 nm (median), PAAG: 14.3 nm (median); \(p < 10^{-20}\), 2-sided Wilcoxon rank sum test; TG: 352, PAAG: 330 virion particles; all virions from the same single batch of live HSV-1 preparation]. Although new labeling strategies will be required for TG-based iExM to exhibit this enhanced precision in visualizing specific biomolecule types, the chemistry discoveries here reported show that structurally homogeneous expansion microscopy matrices are possible, and point the way to new ways to generalize, improve, and extend the expansion microscopy toolbox.

**RESULTS**

**Design of the tetra-gel (TG) monomers and polymerization chemistry**

Free-radical polymerization introduces nanoscale structural heterogeneity into hydrogel networks at the length scale of tens of nanometers\textsuperscript{16–19}, due to (1) local fluctuations of the monomer and cross-linker densities, as well as topological defects of the polymer chains, such as (2) dangling chain ends and (3) loops (Fig. 1a). We designed and synthesized tetrahedral monomers closely related to those known to form the homogenous tetra-PEG structure via non-radical terminal-linking (Fig. 1b, Supplementary Fig. 1)\textsuperscript{24–26}. In our hydrogel design, one of the monomers (monomer 1) has a tetra-arm polyacrylate backbone with a clickable terminal group (azide), and the other (monomer 2) has a tetra-arm PEG backbone with a complementary terminal group (alkyne). The alkyne is synthetically switchable to tune the reactivity of the terminal linking and/or to add functionalities to the hydrogel network (Fig. 1b, monomers 2', 2'', 2'''). We designed these monomers so that they had a comparable molecular weight of ~10-20k Da and an arm length of ~3–6 nm at the gelation step (at an ionic strength of ~0.150M). In more detail: in the solution phase, Monomer 1 has four negatively charged polyacrylate arms (\(n = \approx 21\) monomer units long), each arm of which is estimated to extend ~4.0-5.7 nm, based on the previously characterized persistence length of \(\geq 4.0\) nm\textsuperscript{29} and the fully stretched length of ~5.7 nm. Monomer 2 has four uncharged PEG arms (\(n = \text{either} \approx 57\) or \(\approx 114\) monomer units long, depending on which design is utilized), each of which has a fully stretched length of ~21.7 or ~43.3 nm, respectively, which greatly exceeds its persistence length (0.38 nm)\textsuperscript{30}. The PEG arm can thus be modeled as a freely jointed chain in solution\textsuperscript{31,32}, whose root-mean-square end-to-end distance (i.e., the arm length) can be calculated as either ~2.9 or ~4.1 nm, respectively. For monomer 2 synthesis, the \(n = \approx 57\) backbone was used for variants 2' and 2''' and the \(n = \approx 114\) backbone was used for variant 2'''; the increased number of PEG units in 2''' enhanced its solubility to compensate for the hydrophobic nature of the SS-DBCO moiety. In this design,
when monomer 1 and a selected monomer 2 are mixed, a hydrogel would form via click-chemistry-based terminal linking, similar to tetra-PEG gel formation (Fig. 1c, left). Then, in deionized water or aqueous buffers with an ionic strength smaller than ~0.05M (concentration estimated from the Debye-Hückel theory for the electrostatic potential energy in electrolyte solutions)\textsuperscript{33}, the hydrogel would swell due to the reduced electrostatic screening of salt ions between the mutually repelling monomer 1 units (~84 negative charges per monomer), which in turn would elongate the originally unstructured PEG arms of the interconnecting monomer 2 (Fig. 1c, right).

Of the monomer 2 variants in Fig. 1b, the bicyclononyne (BCN) version (monomer 2\textsuperscript{**}) was designed to support expansion of thicker tissues than the standard dibenzocyclooctyne (DBCO) version (monomer 2\textsuperscript{*}), because its slower click-reaction kinetics (by ~55\%)\textsuperscript{34} would provide additional time for the monomers to diffuse, and equilibrate in density, throughout the tissue during the pre-gelation incubation step. Moreover, the disulfide dibenzocyclooctyne (SS-DBCO) version (monomer 2\textsuperscript{***}) would enable post-expansion cleavage of the polymer network into individual monomers after being reembedded in a second hydrogel, rendering it compatible with iterative expansion\textsuperscript{14}.

**TG expansion of cells and tissues**

We mixed monomers 1 and 2, targeting a stoichiometric ratio of 1:1, and casted the gelling solution into a circular mold. The molar ratio of monomer 1 to 2 in the initial recipe could deviate slightly from 1:1 depending on the fraction of monomer 1 whose t-butyl group was removed in the final step of monomer 1 synthesis (Supplementary Fig. 1, bottom row), which determines the exact molecular weight of monomer 1. Nonetheless, the mutually-limiting click polymerization mechanism of TG formation should assure a 1:1 stoichiometric incorporation of monomers 1 and 2 during gel formation, even if concentrations are slightly different. After 1-2 hours of incubation at 37°C, we found that the gelling solution solidified into an optically transparent and mechanically elastic hydrogel (Fig. 2a, left). Similar to the radically formed PAAG gel and other types of hydrogels used in ExM and tissue clearing methods\textsuperscript{7-13}, the TG swelled after salt elution in water (~3-fold in linear dimension) (Fig. 2a, right). We anchored a small amount of fluorescent dye to the TG polymer network, and imaged fluorescently-labeled gels before and after swelling (Fig. 2b). We found that the linear expansion factor of TG was ~3.0-3.5x.

We implemented proExM using TG. As mentioned earlier, a small-molecule linker, AcX, is reacted to primary amines on proteins (either endogenous, or exogenously applied such as fluorescent dye-conjugated antibodies) in the biological specimen, allowing them to be covalently anchored to, and physically separated from one another by, the swelling polymer network\textsuperscript{4,8}. We infused antibody-stained cells and tissue slices with a small-molecule linker, NHS-azide, so that the primary amines of the proteins and antibodies could be covalently bound to the polymer network through azide-based click-chemistry (Fig. 1b). We then formed the TG in situ before mechanically homogenizing the cell/tissue-TG composites by proteinase K proteolysis, as in the proExM process\textsuperscript{4,8}. We note that NHS-azide can be potentially replaced by other small molecules, as long as they covalently bind to the target biomolecules as well as either the terminal or side functional groups of the TG polymer network.
For TG-based expansion of cultured cells, we embedded HEK cells immunostained against microtubules in the DBCO version (monomer 2'') of TG. After swelling in deionized water, the HEK cell–TG composite expanded ~2.9-fold, resulting in more sharply resolved microtubules than before expansion, on the same diffraction-limited confocal microscope (Fig. 2c). We aligned pre- and post- expansion confocal microscopy images of microtubules via non-rigid registration, and quantified the amount of distortion in the expansion process at a macro length scale (Fig. 2d), finding ~2.5% error over measurement lengths of 20 μm, comparable to the macro distortion of earlier PAAG-based proExM. We further embedded mouse brain slices expressing yellow fluorescent protein (YFP) in the slower-to-react BCN version (monomer 2''') of TG, and immunostained against YFP (whose protease resistance we utilized in the earlier PAAG-based proExM protocol) after proteinase K proteinolysis, to enhance fluorescence (Fig. 2e). After swelling, the mouse brain slice-TG composite expanded ~3.0-fold and showed enhanced detail in subcellular structures such as dendritic spines, as observed before with PAAG-based proExM.

Since TG forms via non-radical polymerization, TG-based expansion could potentially preserve molecules that are destroyed or modified during radical polymerization. To test this, we measured fluorescence retention of small-molecule dyes that are known to be susceptible to free-radical polymerization in PAAG-based proExM (Fig. 2f). We found that the fluorescence of cyanine dyes, including Alexa Fluor 647 (AF647), Cy5, and AF 680, was nearly lost (<10% retention, Fig. 2g) after PAAG-based proExM, as observed before, but was largely preserved (>80% retention) after TG-based expansion (Fig. 2f, 2g). TG-based ExM is thus compatible with pre-expansion staining with fluorescent dyes not compatible with PAAG-based ExM.

**TG-based iterative expansion**

The SS-DBCO version of TG (using monomer 2''') allows the TG polymer network to be cleaved at every node that connects two neighboring monomers (Fig. 3a). Such cleavability makes TG well suited as the first-round hydrogel for iterative expansion. To verify TG’s cleavability and compatibility with iExM, we applied the previously described principle of iterative expansion to TG-expanded HeLa cells (Fig. 3b). In this process, HeLa cells were stained with primary antibodies against tubulin followed by DNA oligo-conjugated secondary antibodies, embedded in the SS-DBCO (monomer 2''') version of TG, proteolytically digested with proteinase K, expanded by deionized water, and re-embedded in a PAAG for the 2nd round of expansion and imaging. We used PAAG for the second round of expansion, reasoning that the spatial errors introduced by PAAG-based expansion in the second round would be negligible. This is because the spatial errors introduced by PAAG-based expansion in the second round, when considered in biological units (i.e., in terms of the relative spacing of biomolecules with respect to each other), are effectively divided by the expansion factor of the first round, and thus would be ~3x smaller than those that would be introduced by using PAAG in the first round of iterative expansion. After cleaving the disulfide bonds of the TG with tris(2-carboxyethyl)phosphine (TCEP) and adding deionized water, the cells expanded by ~16-fold. Consistent with previously reported structures in BS-C-1 cells expanded by PAAG-based iterative expansion, we could detect the hollow of microtubules after TG-based iterative expansion (Fig. 3c). Quantitative analysis of the peak-to-peak distance between the microtubule sidewalls shows an average distance of ~65.3 +/- 15.7 nm (mean +/- std. dev.; n = 336
microtubule segments of 200 nm length, from 10 cells in one culture), comparable to that seen previously with iExM of BS-C-1 cells using PAAG gels (Fig. 3d). Multiple DNA oligo-conjugated antibodies bearing different oligo sequences could be used at once, e.g. to label tubulin and clathrin in HeLa cells (Fig. 3e)\textsuperscript{14}.

Expansion of virions and spatial arrangements of envelope proteins

To assess the spatial errors introduced by TG-based iExM, we imaged the nanoscale arrangement of herpes simplex virus type 1 (HSV-1) virion envelope proteins. In iExM, primary and secondary antibodies, and the conjugated DNA oligos used in the process of iExM, have been estimated to add ~21 nm of spatial error to the localization of epitopes\textsuperscript{14}, potentially confounding the assessment of any fine-scale improvements of TG over PAAG forms of iExM. In order to avoid this limitation, wherein pre-expansion administration of antibodies against epitopes means that antibody size is a major contributor to the spatial errors introduced by iExM\textsuperscript{14}, we developed a direct, nonspecific labeling strategy that targets primary amines of envelope proteins. For the labeling, we directly conjugated 22-bp oligonucleotides to the envelope proteins on HSV-1 virions via a hydrazone-formation-based DNA-to-peptide conjugation (Fig. 4a). This direct labeling protocol reduces the label size from ~21 nm to that of a single DNA oligo (~7 nm)\textsuperscript{14}. We are careful to not expose viruses to any buffers containing detergents (e.g. Triton), for all steps from fixation to gelation, aiming to preserve the virus’s lipid bilayer envelope integrity, so that the highly negatively charged 22-mer DNA oligo will unlikely be able to cross the membrane, and thus conferring specificity of the labeling to the accessible envelope proteins on the outside of the membrane. Of course, the nontargeted nature of this labeling scheme means that the chemistry discoveries here reported will need further innovation in order for specific proteins to be labeled, e.g. by post-expansion delivery of antibodies (which has been performed for single rounds of expansion\textsuperscript{8,11} but has not yet been reported for the iterative expansion case), which would result in antibody-contributed spatial errors that are effectively divided by the expansion factor. Given that our goal for the current manuscript was to explore the foundational chemistry of expansion microscopy hydrogels, we decided to focus on the fundamental chemistry of TG-based iExM, as opposed to developing a practical protocol for biologists interested in specific proteins; in the future, such a protocol could build upon the chemistries and discoveries of the current chemistry study.

HSV-1 virions have a well-defined envelope protein layer that has been characterized by high-resolution imaging methods such as electron microscopy (EM)\textsuperscript{36–38}, electron tomography (ET)\textsuperscript{39,40}, and super-resolution microscopy\textsuperscript{41}; are mechanically robust and tightly crosslinked after fixation\textsuperscript{42}; have appropriate length scale (170-190 nm)\textsuperscript{41}, and features in the tens of nanometers, to characterize the local structure of the TG hydrogel; and are compatible with the direct-labeling strategy here utilized. We directly labeled envelope proteins and expanded HSV-1 virions with the SS-DBC0 (monomer 2\textsuperscript{**}) version of TG, comparing to PAAG, and then the gelled specimens were expanded a second time (in both cases using PAAG, as described above) to ~10-fold (Fig. 4b). This protocol was compatible with dual-color imaging of HSV-1 virion envelope proteins and DNA (Supplementary Fig. 2), as well as imaging of envelope proteins from other types of virions, such as vesicular stomatitis virus (VSV) virions (Supplementary Fig. 3).
We compared the spatial arrangement of proteins of the envelope layers of HSV-1 virions expanded by 2-round iterative expansion with either TG vs. PAAG serving as the first-round expandable gel (Supplementary Fig. 4a). The expansion factor for TG-based iExM of HSV-1 was \(\sim10.3-13.3\), and for PAAG-based iExM, it was \(\sim15.3-18.7\). From single-particle averaged virion images, we found that the envelope protein profiles of TG-expanded virions appeared significantly sharper than those of PAAG-expanded ones, suggesting reduced local spatial error for the former (Supplementary Fig. 4b). To quantify this spatial error, we measured the standard deviation (\(\sigma\)) of the virus radius within the mid-plane of individual HSV-1 virions (Fig. 4c). We computed this in biological units, normalizing by the expansion factor, to compensate for the different expansion factors for TG-based and PAAG-based iExM protocols. Previous cryo-ET studies\(^{30,40}\) found that the envelope of HSV-1 virions is a smooth, continuous, and near-spherical layer. We found that TG-based iExM-expanded virions [\(\sigma = 9.2\) nm (median); \(n = 352\) virion particles; virion particles from a single batch of live HSV-1 preparation] had significantly smaller median \(\sigma\) value compared to PAAG-expanded virions [\(\sigma = 14.3\) nm (median); \(n = 330\) virion particles; virion particles from a single batch of live HSV-1 preparation, same batch as TG] (\(p < 10^{-20}\), 2-sided Wilcoxon rank sum test) (Fig. 4d), suggesting a reduced spatial error mediated by TG-based vs. PAAG-based iExM. Even more resolution, potentially approaching that of individual proteins or protein clusters within HSV-1 virions, may be possible by expanding \(\sim40x\) via 3 rounds of iterative expansion, with the initial expansion round TG-based and the final two rounds PAAG-based (Supplementary Fig. 5).

DISCUSSION

We have found that tetra-gels (TG) made from non-free-radically assembled tetrahedral monomers are much larger than the sodium acrylate monomers that are used in conventional general polyacrylate arms sidechain chemistry and are not yet established; the monomers are much larger than the sodium acrylate monomers that are used in conventional

Our current study is focused on the chemical principles of the hydrogels of ExM, and is not yet a protocol for general scientific use. For TG-based ExM to be useful in everyday scientific investigations, a number of future improvements will be required. First of all, the chemicals must be made broadly available, through commercialization or other arrangements. We observed TG-based iExM expansion factors ranging from 10x-16x in the current paper, suggesting that refinement of the chemicals to the point of being useful in everyday biology may be of use. Second, in the current TG-based expansion protocol, biomolecules of interest are anchored to the ends of monomers incorporated into the hydrogel network by azide-based click chemistry, meaning that every anchored biomolecule results in a “defect” in the polymer network because it terminates one of the monomer’s arms; replacing such terminal-based anchoring with sidechain-based anchoring via, for example, carbodiimide (EDC) conjugation of proteins to the polyacrylate arms, may improve the final structural homogeneity of the TG polymer network for general ExM purposes. Third, the ideal size of the monomer is not yet established; the monomers are much larger than the sodium acrylate monomers that are used in conventional
ExM protocols, raising the question of how well TG monomers permeate dense cells and tissues, and whether smaller TG monomers may be better for expansion of cells and tissues (in contrast to the virions here used for spatial error validation, where the monomers must only bind to the outer motifs of the virions). Finally, appreciation of the fine-scale improvements offered by TG over PAAG most likely will require post-expansion antibody staining, or the use of very small tags such as nanobodies for pre-expansion staining, since the improvements are smaller than the sizes of antibodies; here, we overcame this limit by directly labeling proteins in a nonspecific fashion, but most investigators will want to label specific proteins with well-defined antibodies.

To enable post-expansion antibody staining, we will have to replace the disulfide in the monomer with another cleavable moiety that is compatible with the high-temperature (70-121°C) treatments, basic (9-11) pHs, and other harsh conditions that are typically used to denature proteins and expand them away from each other, thus enabling post-expansion antibody staining of endogenous biomolecules. Alternatively, intramolecular epoxide cross-linking might be helpful for such epitope preservation. Such an expansion process, in the future, may be useful for investigating the detailed nanoscale spatial arrangement of multiple molecular species in clusters and complexes, in cells and tissues, in healthy and disease states.

METHODS

Methods described here are a concise summary. A more detailed description of each section can be found in the Supplementary Methods.

Synthesis of tetra-gel (TG) monomers

Monomer 1 was synthesized using a procedure modified from a previously described synthesis (Supplementary Fig. 1). First, tetra-arm poly(t-butyl acrylate) with bromo terminal groups (4) was synthesized by atom transfer radical polymerization (ATRP). Next, tetra-arm poly(t-butyl acrylate) with azide terminal groups (5) was synthesized by replacing bromines of 4 with azides. Lastly, monomer 1 was synthesized by hydrolysis and neutralization of 5 to a final pH of ~7. Monomers 2*, 2**, and 2*** were synthesized by N-hydroxysuccinimide (NHS) ester conjugation of the alkynes (DBCO-NHS, BCN-NHS, or DBCO-SS-NHS) to the terminal primary amines of tetra-arm PEGs.

Cell culture

HEK293FT cells were cultured in chambered coverglasses to a confluency of 60-80%1, fixed, and immunostained for expansion microscopy1,4,8. Briefly, the cells were treated with 3% (w/v) formaldehyde and 0.1% (w/v) glutaraldehyde in phosphate buffered saline (PBS) for 10 min at room temperature before the subsequent quenching, blocking, and immunostaining steps. HeLa cells were plated on coverglasses coated with Matrigel to a confluency of 50-90% and fixed1,4,8,14. Briefly, the cells were treated with 1x PBS + 3% (w/v) formaldehyde + 0.1% (w/v) glutaraldehyde for 10 min at room temperature before the subsequent quenching and blocking steps.

Thy1-YFP mouse brain slice
All procedures involving Thy1-YFP mice were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the MIT Committee on Animal Care. 50-100 μm coronal brain slices of Thy1-YFP transgenic mice of 2-4 months old, both male and female, were prepared and immunostained for expansion\textsuperscript{4,8,13}.

**General procedure for gelation, digestion, and expansion**

Fixed (and immunostained) cells and tissues were incubated in ~0.1-0.2 mg/mL NHS-azide in PBS overnight at room temperature and washed with PBS twice. For the gelling solution, the two monomer solutions were mixed at a targeted molar ratio of 1:1, and an additional amount of water was added to adjust the final concentration of monomer 1 to ~3.3 % (w/v). For example, 10 μL each of monomer 1 and monomer 2’ (both ~200 mg/mL) and 40 μL of water were mixed to yield a gelling solution. Gelation was carried out for 1-2 hours at 37 °C (blank gels) or overnight at 4 °C (cell and tissue samples) in a gelation chamber\textsuperscript{4,8}. The gelled cell and tissue samples were incubated in the digestion buffer with proteinase K (8 units/mL) overnight at room temperature\textsuperscript{4,8} and expanded in an excess amount of water three times, each time for 20 min.

**Expansion of HeLa cells (pre-expansion immunostaining and iterative expansion)**

Briefly, fixed HeLa cells were stained with primary antibodies, oligo-conjugated secondary antibodies, and azide-modified tertiary oligos as previously described\textsuperscript{1,14}. The cells were gelled with a cleavable TG gelling solution prepared by mixing monomer 1, monomer 2””, and water. The gelled samples were incubated in digestion buffer with Proteinase K at 8 U/mL overnight at room temperature with gentle shaking before de-hybridization of the oligos from the gel-anchored oligos. The expanded samples were re-embedded in N,N’-bis(acryloyl)cystamine (BAC)-crosslinked non-expanding PAAG, hybridized with 1st linker oligos, re-embedded in N,N’-diallyl L-tartardiamide (DATD)-crosslinked expanding PAAG, and incubated in BAC-cleaving buffer. For fluorescence readout, the samples were incubated with fluorophore-conjugated LNA oligos and expanded in water.

**Expansion of HSV-1 virions (direct-labeling and iterative expansion)**

Purified HSV-1 virion stock\textsuperscript{44} was diluted before being drop-casted onto a plasma cleaned #0 circular 12-mm coverslip. After 15 min of incubation at room temperature, the virions were fixed in 4% PFA in PBS for 10 min. Azide-modified oligos were directly conjugated to the virion envelope proteins via SoluLink bioconjugation as previously described\textsuperscript{1}. The virions were gelled, digested, expanded, and hybridized with fluorophore-conjugated LNA oligos using a similar procedure as for the HeLa cell expansion. For 3-round iterative expansion, BAC-cleaved samples were re-embedded in DATD-crosslinked non-expanding PAAG, hybridized with 2nd linker oligos, re-embedded in bis-crosslinked expanding PAAG, and incubated in DATD-cleaving buffer before the LNA oligo-conjugation, expansion, and imaging. For the PAAG-control, see Supplementary Methods.

**Imaging**

All the expanded samples were imaged with a diffraction-limited spinning disk confocal microscope with a 40x, 1.15 NA water-immersion objective. The two-color HeLa cell images
and the HSV-1 virion images were deconvolved with theoretical point-spread-functions (PSFs) before visualization and image analysis.

**Image analysis**

Averaged HSV-1 virion particles were generated using a semi-automated image analysis pipeline ("Particle Analysis Assistant")\(^45\). Each virion particle was manually aligned, automatically cropped, calibrated with the expansion factor, and arithmetically averaged. Using Particle Analysis Assistant, roundness (deviation from the perfect circle) of the HSV-1 virion particle was measured using the standard deviation of the radii within each virion particle. First, the radii in 8 directions (45 degrees apart) were measured for each virion particle as the distance from the particle centroid to the Gaussian-fitted center of the envelope profile. After inspection to remove unfitted profiles, the standard deviation of the accepted radii within the same particle was calculated. For normalization, accepted radii from the same virion particle were normalized by their mean.
FIGURES

(a) In situ free-radical polymerization

Polymer chain
Cross-linker

(b)

Monomers 1 & 2

Terminal-linking

Projected view

Gelation
Expansion

1. x = DBCO
2. (DBCO)
3. (BCN)
4. (SS-DBCO)

(c)
Figure 1. Design and synthesis of tetra-gels (TG) for expansion microscopy. a, Cell/tissue-hydrogel composites formed by *in situ* free-radical polymerization are known to have structural inhomogeneities in the range of tens of nanometers due to (1) local fluctuations of monomer and cross-linking densities, (2) dangling ends, and (3) loops formed within the polymer network. b, Design of TG monomers 1 and 2 with tetrahedral symmetry and reactive terminal groups. Specific monomer 2 terminal groups (2', 2'' and 2''') enable, for example, control of the reaction rate between the monomers and addition of functionalities to the polymer network. c, Formation and expansion of TG via click-chemistry-based terminal-linking of monomers 1 and 2. Inset, projected view of the TG polymer network.
Figure 2. TG-mediated expansion of cells and tissues. a, Image of TG (using monomer $2''$) as synthesized (left, pre-expansion) and after swelling in deionized water (right, post-expansion). The two gels were cast in circular molds with identical dimensions. Grid size, 5 mm. b, TGs labeled with fluorescein in pre- (left, $2'$; same sizes and shapes when $2''$ and $2'''$ were used) and post-expansion (right, $2'$, $2''$ and $2'''$) states. Irregular boundaries on the post-expansion images reflect the meniscus of water used to expand the gels. Scale bar, 5 mm. c, Left, HEK293 cells with α-tubulin immunostaining in pre- (inset) and post-expansion states. Expansion factor, 2.85x. Scale bars, 20 µm. Right, magnified views of the boxed regions 1 (top) and 2 (bottom). Scale bars, 1 µm [bottom, 2.85 µm; here and after, unless otherwise noted, scale bar sizes are given at pre-expansion scale (i.e., biological scale) with the corresponding post-expansion size (i.e., physical size) indicated in parentheses]. d, Root-mean-square (RMS) error curve for HEK293 cell expansion (blue line, mean; shaded, standard deviation; n = 8 cells from one culture). Inset, non-rigidly registered and overlaid pre- (green) and post-expansion (magenta) images used for the RMS error analysis. e, Pre- (left top) and post-expansion (left bottom and right) Thy1-YFP mouse brain slices. Expansion factor, 3.00x. Scale bars, 5 mm (left) and 10 µm (right, 30 µm). The gelled brain slice on the left bottom panel was immunostained against YFP after the proteolysis step to enhance fluorescence. Inset, magnified view of the boxed region. Scale bar, 1 µm (3 µm). f, Pre-expansion (left column) and post-expansion (right column) Thy1-YFP mouse brain slices immunostained with Homer1 primary antibody and Alexa Fluor 647 (AF647)-conjugated secondary antibody, using sodium polyacrylate/acrylamide gel (PAAG, top row) and TG (bottom row). Expansion factors, 3.93x (PAAG) and 2.72x (TG). Scale bars, 300 µm (top right, 1.18 mm; bottom right, 815 µm). g, Fluorescence retention of AF647, Cy5, and AF680 with PG and PAAG in brain slices processed as in f (bar height, mean; error bar, standard error of the mean; n = 3 brain slices from one mouse).
Figure 3. TG-based iterative expansion. a, Monomeric cleaving of TG (monomer 2’’’) after reembedding in a second hydrogel. A reducing agent, tris(2-carboxyethyl)phosphine (TCEP), was applied to cleave the disulfide bonds in the TG polymer network. b, HeLa cell with β-tubulin immunostaining, expanded by cleavable TG-based 2-round iterative expansion. Expansion factor, 15.6x. Scale bar, 5 µm (78.2 µm). c, Top, magnified view of the boxed region in b. Scale bar, 100 nm (1.56 µm). Bottom, transverse line intensity profile of the microtubule in a single xy-plane in the dotted box (circle) and the fitted sum of two Gaussians (red line). The line intensity profile was averaged in the direction parallel to the microtubule axis over a length of 200 nm. d, Histogram of peak-to-peak distance between microtubule sidewalls in HeLa cells (n = 336 segments of a length of 200 nm from 10 cells in one culture). e, Left, HeLa cell with two-color labeling of clathrin coated pits/vesicles and microtubules, expanded by TG-based 2-round iterative expansion. Expansion factor, 15.6x. Scale bar, 10 µm (156 µm). Right, magnified view of the boxed region for each color channel. Scale bars, 1 µm (15.6 µm).
Figure 4. Spatial errors introduced by TG-based vs. classical PAAG-based iterative expansion microscopy. **a**, Short DNA-oligos (22 bp) were covalently conjugated to the envelope proteins of herpes simplex virus type 1 (HSV-1) virions via hydrazone formation, which allows labeling transfer across multiple hydrogels, amplification based on branched DNA, and fluorescence readout based on hybridization of fluorescent oligos. **b**, Schematic illustration of TG- (top) and PAAG-based (bottom) iterative expansion of HSV-1 virions with the direct oligo-conjugation of **a**. PAAG-based expansion was used for all expansion rounds after the first round, reasoning that most of the error of an iterative expansion protocol is introduced in the first round of expansion (see text for details). **c**, Top left and middle, representative single xy-plane images of HSV-1 virions expanded by TG- and PAAG-based 2-round iterative expansion. White lines indicate the 8 directions along which the virion particle’s radii ($R$) were measured. Size of both fields of view at biological scale, 400 nm. Top right, representative line intensity profile (circles) along a single direction (Direction1) of the TG-expanded virion and the fitted Gaussian (red line). Distance from the center of the Gaussian to the center of the virion was defined as $R$ along that direction. Bottom, histogram of the particle-mean-normalized $R$ ($R_n$) for all the measured line profiles [TG, $n = 1977$; PAAG, $n = 1889$ profiles; from 352 (TG) and 330 (PAAG) virion particles from the same single batch of live HSV-1 preparation]. **d**, Box plots [plus sign, outliers (values above 75th percentile $+ 1.5 \times$ interquartile range, or below 25th percentile $– 1.5 \times$ interquartile range); ends of whiskers, maximum and minimum values of the distribution after outliers are excluded; upper line of box, 75th percentile; middle line of box, 50th percentile; lower line of box, 25th percentile] of the standard deviation ($\sigma$) of HSV-1 virion radii ($R$) within individual virion particles for TG- and PAAG-based iterative expansion ($p < 10^{-20}$, 2-sided Wilcoxon rank sum test; TG, $n = 352$; PAAG, $n = 330$ virion particles; virion particles from the same single batch of live HSV-1 preparation).
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Author contributions

R.G. and L.G. designed and synthesized the monomers and conducted initial gelation experiments. C.-C.Y. and R.G. designed and conducted iterative expansion, virion expansion, and associated analyses. C.-C.Y. created the semi-automated virion analysis pipelines. K.D.P. helped characterization of the gel in cell culture. R.L.N. purified HSV-1 and prepared the virion stock solution. S.U. provided purified VSV and conducted initial virion immobilization experiments. C.-C.Y., R.G., and L.G. processed and performed quantitative analysis of all image data. R.G., C.-C.Y., and E.S.B. wrote the manuscript with input from all co-authors. E.S.B supervised the project.

Competing interests

R.G., C.-C.Y., L.G., and E.S.B. have filed for patent protection on a subset of the technologies here described. E.S.B. is a cofounder of a company that aims to commercialize ExM for medical purposes. R.G., C.-C.Y., L.G., and E.S.B are a co-inventor on multiple patents related to ExM.
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A highly homogeneous expansion microscopy polymer composed of tetrahedron-like monomers

Ruixuan Gao, Chih-Chieh (Jay) Yu, Linyi Gao, Kiryl D Piatkevich, Rachael L Neve, Srigokul Upadhyayula & Edward S Boyden

Supplementary Information

Supplementary Methods
Supplementary Figs. 1-5
Supplementary Tables 1-2
SUPPLEMENTARY METHODS

Synthesis of tetra-arm sodium poly-acrylate with azide terminal groups (1) (monomer 1)

Monomer 1 was synthesized using a modified procedure as previously described \(^27\) (Supplementary Fig. 1). Unless otherwise noted, all chemicals were purchased from Sigma Aldrich.

First, tetra-arm poly(t-butyl acrylate) with bromo terminal groups (4) was synthesized by atom transfer radical polymerization (ATRP). Before the synthesis, t-butyl acrylate was purified with an inhibitor removal column to remove a trace amount of 4-methoxyphenol. Next, 128 mL of the purified t-butyl acrylate was added to 640 mg of copper (I) bromide and 48 mg of copper (II) bromide, and the mixture was bubbled with dry nitrogen at 50 °C before 1.03 mL of N,N,N',N''-pentamethyl diethylenetriamine (PMDETA) was added dropwise. After 5-10 min of continuous bubbling and stirring, a 16 mL acetone solution of 1.6 g pentaerythritol tetrakis(2-bromoisobutyrate) (3) was added dropwise. The reaction mixture was stirred at 50 °C for 90 min, with dry nitrogen bubbling on for the first ~10 min. After the reaction, unreacted t-butyl acrylate was removed by rotary evaporation before the crude product mixture was dissolved in dimethylformamide (DMF) and precipitated with water. The precipitation was repeated two to three times additionally, yielding 15.3 g of tetra-arm poly(t-butyl acrylate) with bromo terminal groups (4) as a white powder.

Next, tetra-arm poly(t-butyl acrylate) with azide terminal groups (5) was synthesized by replacing bromines of 4 with azides. 15.3 g of 4 was dissolved in 80 mL of DMF and an excess amount of sodium azide (exceeding its solubility in DMF) was added to the reaction mixture. The reaction was carried out overnight at room temperature and the supernatant was subsequently decanted and precipitated with water, yielding 11.0 g of tetra-arm poly(t-butyl acrylate) with azide terminal groups (5) as a white powder.

Finally, monomer 1 was synthesized by hydrolysis and neutralization of 5. A total of 5.04 g of 5 was dissolved in 30 mL of methylene chloride, followed by addition of 15 mL of trifluoroacetic acid (TFA). The hydrolysis reaction was carried out at 4 °C with gradual precipitation of a white powder. After 24-48 hours, the precipitated product was collected by centrifugation, washed with acetone, and dried in a low-humidity chamber. The product was re-suspended in an aqueous solution of sodium hydroxide to yield monomer 1 solution (~200 mg/mL) at a final pH of ~7.

Synthesis of tetra-arm polyethylene glycol (PEG) with dibenzocyclooctyne (DBCO, 2'), bicyclo[6.1.0]non-2-yno (BCN, 2''), and dibenzocyclooctyne-disulfide (DBCO-SS, 2'''') terminal groups (monomer 2)

DBCO-, BCN- and DBCO-SS-terminated monomers 2', 2'', and 2''' were synthesized by N-hydroxysuccinimide (NHS) ester conjugation of the alkynes to the terminal primary amines of tetra-arm PEGs. First, amine-terminated tetra-arm PEG (~10 kDa for 2' and 2''; ~20 kDa for 2''') (NOF Corp.) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100-200 mg/mL. DBCO-NHS, BCN-NHS or DBCO-SS-NHS was then added to the DMSO solution of the tetra-arm PEG at a 1:1 molar ratio to the total number of terminal amines. Finally, the
conjugation reaction was carried out overnight at room temperature to the yield monomer 2', 2'', or 2''' solutions.

**HEK293 cells**

HEK293FT (Thermo Fisher) cells were cultured in chambered coverglasses (CultureWell, Thermo Fisher) to a confluency of 60-80% as previously described\(^4,8\). The cells were then fixed and immunostained\(^4,8\). Briefly, the cells were treated with 3% (w/v) formaldehyde and 0.1% (w/v) glutaraldehyde in phosphate buffered saline (PBS) for 10 min, quenched with 0.1% NaBH\(_4\) (w/v) in PBS for 7 min, and then with 100 mM glycine in PBS for 10 min. Immediately after fixation, the cells were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 15 min and blocked with a blocking buffer [5% (v/v) normal donkey serum (NDS) and 0.1% (w/v) Triton X-100 in PBS] for 15 min. For primary antibody staining, the cells were incubated in rat anti-α-tubulin antibody (MA1-80017, Thermo Fisher) solution (1:200 dilution with the blocking buffer) overnight and were washed with the blocking buffer three times, each time for 5 min. For secondary antibody staining, the cells were incubated in Alexa Fluor 488-conjugated donkey anti-rat antibody (A-21208, Thermo Fisher) solution (1: 200 dilution with the blocking buffer) for 5 hours and then twice with the blocking buffer three times, each time for 5 min. Finally, the cells were washed with PBS once for 5 min and stored in PBS for subsequent expansion processes. Unless otherwise noted, all the incubation and washing steps were carried out at room temperature.

**HeLa cells**

HeLa cells were plated on coverglasses coated with Matrigel (BD Sciences) to a confluency of 50-90% and then fixed\(^4,8,14\). Briefly, cells were treated with 1x PBS + 3% (w/v) formaldehyde + 0.1% (w/v) glutaraldehyde for 10 min, quenched with 1x PBS + 0.1% (w/v) NaBH\(_4\) for 7 min. The cells were then washed once with 1x PBS + 100 mM glycine for 5 min, and then twice with 1x PBS for 5 min each. Fixed cells were stored at 4°C until the immunostaining step. Unless otherwise noted, all the incubation and washing steps were carried out at room temperature.

**Thy-1 YFP mouse brain slices**

Coronal brain slices of transgenic mice expressing cytosolic YFP under the Thy1 promoter (Thy1-YFP-H strain, Jackson Laboratory) were prepared and immunostained\(^4,8,13\). Unless otherwise noted, all the incubation and washing steps were carried out at room temperature. Briefly, Thy1-YFP mice, 2-4 months old, both male and female, were anesthetized with ketamine/xylazine and perfused transcardially with 25 mL ice cold 4% (w/v) paraformaldehyde (PFA) in PBS, followed by 25 mL of ice cold PBS. The brains were dissected out and soaked in 4% (w/v) PFA in PBS for 24 hours at 4°C. 50-100 μm coronal slices were prepared using a vibratome (Leica VT1000S) and were stored in PBS at 4°C for subsequent expansion processes.

For pre-expansion staining, the fixed brain slices were permeabilized and blocked with a blocking buffer [5% (v/v) NDS and 0.1% (w/v) Triton X-100 in PBS] for 2 hours. For primary antibody staining, the slices were incubated with rabbit anti-Homer1 antibody (160003, Synaptic Systems) solution (1: 200 dilution with the blocking buffer) overnight and washed with the blocking buffer twice, each time for 30 min. For secondary antibody staining, the slices were incubated with dye-conjugated Alexa Fluor 647, Cy5, or Alexa Fluor 680 goat anti-rabbit
antibody (A21245, A10523, or A21109, Thermo Fisher) solution (1: 200 dilution with the blocking buffer) overnight and washed with the blocking buffer twice, each time for 30 min. Finally, the slices were washed once with PBS and stored in PBS for subsequent expansion processes.

For post-proteolysis staining, the gelled and digested brain slices were incubated with chicken anti-GFP antibody (A10262, Thermo Fisher) solution (1:200 dilution with the blocking buffer) overnight and subsequently with Alexa Fluor 488-conjugated goat anti-chicken antibody (A11039, Thermo Fisher) solution (1:200 dilution with the blocking buffer) overnight before the same washing and storage steps with PBS as described.

**Gelation, digestion, and expansion of cells and tissues (single-round expansion)**

Unless otherwise noted, single-round expansion of cells and tissues with tetra-gels (TGs) were carried out using the following general procedure. First, fixed (and immunostained) cells and tissues were incubated in ~0.1-0.2 mg/mL NHS-azide in PBS overnight at room temperature and washed with PBS twice immediately before gelation.

Next, monomer 1 and monomer 2 solutions were mixed at close to 1:1 molar ratio, and an additional amount of water was added to adjust the final concentration of monomer 1 to ~3.3% (w/v). In a typical gelation with monomer 2', 10 µL of monomer 1 (~200 mg/mL), 10 µL of monomer 2 (~200 mg/mL), and 40 µl of water were mixed to yield the gelling solution. We note that the molar ratio between the mixed monomer 1 to 2' can vary slightly according to the molecular weight of monomer 1, which depends on the final conversion rate of the t-butyl ester hydrolysis in the last deprotection step of the synthesis. For example, the exact molar ratio of monomer 1 to 2' can be ~0.99 at 50% conversion and ~1.16 at 100% conversion. However, due to the complementary, mutually-limiting polymerization mechanism of TG, this slight variation should not significantly alter the composition of the incorporated monomers 1 and 2'. After drop-casting the gelling solution to the samples in a gelation chamber as previously described\(^4\), gelation was carried out for 1-2 hours at 37 °C (blank gels) or overnight at 4 °C (cell and tissue samples). The total amount of monomers being mixed was adjusted proportionally according to the size and number of the samples.

Finally, the gelled cell and tissue samples were incubated in the digestion buffer with proteinase K (8 units/mL) overnight at room temperature as previously described\(^4,8\). For expansion, the digested samples were washed in an excess amount of water three times, each time for 20 min.

For fluorescein visualization of blank gels, a trace amount of fluorescein amine was mixed into the gelling solution. Briefly, a stock solution of ~50 mM fluorescein-azide was prepared by adding 5 µL of 100 mM fluorescein-amine in DMSO to 5 µL of 20 mg/mL NHS-azide in DMSO. ~3 µL of the fluorescein-azide stock solution was added to ~60 µL of the gelling solution (with monomers 2', 2'', or 2'''). The gelling solution with fluorescein was drop-cast into a circular mold of ~3 mm diameter before gelation for 1-2 hours at 37 °C.

**Expansion of HeLa cells (pre-expansion immunostaining and iterative expansion)**

*Pre-expansion immunostaining*
Fixed cells were stained with primary antibodies, oligo-conjugated secondary antibodies, and azide-modified tertiary oligos (for TG) as previously described\textsuperscript{1,14}. Briefly, fixed cells were permeabilized and blocked with HeLa staining buffer [1x PBS + 5% (v/v) normal donkey serum + 0.2% (w/w) Triton X-100] for 10 min. Primary antibody staining was performed with HeLa staining buffer for 1 hr at RT, followed by 3 washes with 1x PBS for 5 min each at RT. Secondary antibody staining was performed with hybridization buffer [10% w/v dextran sulfate, 1 mg/mL yeast tRNA, 5% v/v normal donkey serum, 2x SSC, 0.1% (w/w) Triton X-100] for 1 hr at RT, followed by 3 washes with 1x PBS for 5 min each at RT. Tertiary oligo hybridization was performed by first incubating the sample in hybridization buffer for 3 hr, followed by incubation with the tertiary oligo overnight at RT. After hybridization, samples were washed with 1x PBS 3 times for 5 min each at RT. For single-color tubulin staining, the cells were stained with rabbit anti-beta-tubulin (ab6046, Abcam, 1:100 dilution) primary antibody, and then with oligo-conjugated anti-rabbit secondary antibody (oligo sequence B1’; see Supplementary Table 1 for oligo sequences used), followed by hybridization with azide-modified complementary oligo (oligo sequence B1, at 100 nM). For dual-color tubulin and clathrin staining, the cells were stained with sheep anti-alpha/beta tubulin (ATN02, Cytoskeleton, Inc, 1:50 dilution) and rabbit anti-clathrin heavy chain (ab21679, Abcam, 1:500 dilution) primary antibodies, and then with oligo-conjugated anti-sheep (oligo sequence A2’) and anti-rabbit (oligo sequence B1’) secondary antibodies, followed by hybridization with azide-modified complementary oligos (oligo sequences B1 and A2, at 100 nM each). Oligo conjugation to secondary antibodies were performed with the same protocol as previously described\textsuperscript{1} (also available on www.expansionmicroscopy.org), with the modification that the S-HyNic reaction was performed with 3 times of its concentration (600 µM instead of 200 µM) compared to the original protocol.

\textit{Gelation and digestion}

As described in the previous section, the cleavable TG gelling solution was prepared by mixing monomer 1 (200 mg/mL) and monomer 2 (200 mg/mL) at a molar ratio of close to 1:1 and then adding water to adjust the final concentration of monomer 1 to ~3.3% (w/v). A gelation chamber was constructed around the cell-immobilized coverslip using the following steps. First, the coverslip was transferred to the center of a glass slide. Spacers consisting of a stack of a #0 and a #1 coverslip were placed on either side of the cell-immobilized coverslip. 50 µL of freshly prepared TG gelling solution was added to the coverslip, and the chamber was closed by placing a rectangular coverslip on top of the spacers. The gelling solution was further added from the side of the chamber until the chamber was completely filled. Gelation chambers were then placed in a humidified chamber and incubated at 4°C overnight. After the incubation, the chamber was partially opened using a diamond scribe to remove portions of the top cover glass that were not directly above the cell-immobilized coverslip. The chamber was then placed into a rectangular 4-well dish and incubated in digestion buffer with Proteinase K at 8 U/mL (New England Bio Labs; 1:100 dilution) overnight at room temperature with gentle shaking. The diameter of the gel was measured for downstream estimation of the overall expansion factor. Regions inside the circular 12-mm coverslip (i.e. regions with the immobilized cells) were trimmed into a parallelogram, of which the side lengths were measured for downstream estimation of the overall expansion factor. Finally, trimmed gels were washed twice in PBS, each time for 10 min. To de-hybridize B1’ and A2’ oligos (conjugated to the secondary antibodies) from the gel-anchored B1 and A2 oligos (the azide-modified tertiary oligos), the gels were incubated in 80% formamide at room temperature for 1 hr with gentle shaking, and then washed
three times in PBST (1x PBS + 0.1% Triton X-100) at room temperature with gentle shaking, for 30 min each.

**Re-embedding into a BAC-crosslinked non-expanding 2nd gel**
The gels were transferred (with the cell side facing down) into a rectangular 4-well dish that carries a glass slide in each well, and expanded in water three times, each time for 30 min. The gels were then incubated in 3 mL of BAC-crosslinked non-expanding gelling solution [10.4% (w/v) acrylamide, 0.2% (w/v) BAC, 0.05% (w/v) TEMED, 0.05% (w/v) APS] for 5 min with gentle shaking. After the incubation, the non-expanding gelling solution was removed from the well, and the glass slide carrying the expanded gel was transferred to a gelation chamber. Spacers consisting of a stack of two #1.5 cover glasses were placed on either side of the gel, and the chamber was closed with a rectangular cover glass. The non-expanding gelling solution was added from the side of the chamber until the chamber was completely filled. The gelation chambers were incubated for 2 hours at 37°C. After gelation, the chambers were opened by removing the top cover glass. Side lengths of the parallelogram were measured. The gels were trimmed to leave only the portion inside the parallelogram, while preserving the shape of the parallelogram. Side lengths of the trimmed gels were measured. Finally, the trimmed gels were washed twice in PBS, each time for 30 min.

1st Linker hybridization
The gels were incubated in hybridization buffer (4x SSC + 20% (v/v) formamide) for 30 min at room temperature. The gels were incubated with 1 nmol of oligo 5’Ac-B1’-4xB2’ in 1 mL of hybridization buffer overnight at room temperature. After incubation, the gels were washed in hybridization buffer three times, each time for 1 hour, and then overnight, all with gentle shaking. The gels were then washed three times in PBS, each time for 5 min.

**Re-embedding into a DATD-crosslinked expanding 3rd gel**
The gels were incubated in DATD-crosslinked expanding gelling solution [8.6% (w/v) sodium acrylate, 2.6% (w/v) acrylamide, 0.5% (w/v) DATD, 1x PBS, 2M NaCl, 0.01% (w/v) 4-HT, 0.2% (w/v) TEMED, 0.2% (w/v) APS] for 30 min at 4°C. The gels (with the cell side facing down) were then enclosed in gelation chambers, incubated for 2 hours at 37°C, size-measured, trimmed, size-re-measured, and washed as described in “Re-embedding into a BAC-crosslinked non-expanding 2nd gel”.

**Cleaving BAC-crosslinked 1st and 2nd gels**
The gels were incubated in BAC-cleaving buffer (0.25M TCEP-HCl, 0.75M Tris-HCl, pH 8.0) overnight at room temperature. The gels were then washed four times in PBS, each time for 30 min.

**LNA hybridization for readout after 2-round expansion**
The gels were incubated with 1 nmol of fluorophore-conjugated LNA oligo (LNA_B2-Atto647N and/or LNA_A1-Atto565; see sequences in Supplementary Table 1) in 500 µL of hybridization buffer. The LNA-hybridized gels were washed in hybridization buffer three times, each time for 1 hour, and then overnight, all with gentle shaking. The gels were then washed three times in PBS, each time for 5 min.
**Gel expansion, immobilization, and imaging for 2-round expanded samples**

The gels were trimmed into smaller pieces (~5 by 5 mm) while preserving the shape of the parallelogram. First, the gels were transferred (with the cell side facing down) into a rectangular 4-well dish that carries a glass slide in each well, and expanded in water three times, each time for 30 min. A glass-bottom 6-well plate was modified with poly-lysine, and the expanded gels were gently transferred to the poly-lysine modified coverslip surface for imaging, as previously described

**Expansion of HSV-1 virions (direct labeling and iterative expansion)**

**Immobilization and fixation**

Purified HSV-1 virion stock was prepared by the Viral Core Facility at the Massachusetts General Hospital (MGH) as previously described. The HSV-1 stock was diluted to a functional titer of 2.5 x 10⁸ functional virions/mL in PBS and kept on ice until immobilization. A #0 circular 12-mm coverslip was cleaned with a plasma cleaner (PDC-001, Harrick Plasma) for 1 min. Immediately after the plasma cleaning, 30 µL of the diluted HSV-1 solution was drop-cast onto the coverslip and incubated for 15 min at room temperature. The immobilized virions were fixed in 4% PFA in PBS for 10 min, and then washed with PBS twice, each time for 5 min.

**Oligo conjugation to envelope proteins**

Envelope proteins on the fixed virions were conjugated to DNA oligos with the SoluLink bioconjugation chemistry as previously described. The oligo provided a molecular handle for label anchoring, transfer, and amplification through the iterative expansion process. Briefly, a 22-bp oligo [sequence B1’ with a 5’ amine modification (Integrated DNA Technologies)] was purified with ethanol precipitation and reacted with Sulfo-S-4FB (S4FB) overnight in Buffer A (150 mM NaCl, 100 mM Na₂HPO₄, pH 7.4) at a molar ratio of 1:15. The S4FB-reacted oligo was purified with a size exclusion filter, and then stored at 4°C. Fixed virions immobilized on the coverslip were washed in Buffer A for 5 min, and then incubated with 100 µL of 160 mM S-HyNic in Buffer A for 2 hours at room temperature. The S-HyNic-reacted virions were washed with Buffer C (150 mM NaCl, 100 mM Na₂HPO₄, pH 6.0) twice, each time for 5 min. Oligo conjugation solution was prepared by first adding 50 nmol of purified S4FB-reacted oligo to 100 µL of Buffer C, and then adding an amount of 10x TurboLink Catalyst Buffer that equals to 1/9 of the combined volume. The S-HyNic-reacted virions were incubated in the oligo conjugation buffer overnight at room temperature in a humidified chamber. Next, the oligo-conjugated virions were washed three times with PBS, each time for 10 min, and then incubated in detergent-free hybridization buffer (10% (w/v) dextran sulfate, 1 mg/mL yeast tRNA, 5% (v/v) NDS, 2x SSC) for 3 hours at room temperature. The virions were incubated with 4 nmol of oligo B1-acrydite or oligo B1-azide for the subsequent gelation with TGs [or sodium polyacrylate/acylamide gels (PAAGs)], in 300 µL of the detergent-free hybridization buffer, overnight at room temperature. Finally, the virions were washed three times in PBS, each time for 10 min.

**Gelation and digestion**

As described in the previous section, cleavable TG gelling solution was prepared by mixing monomer 1 (200 mg/mL) and monomer 2 at a molar ratio of close to 1:1 and then adding water to adjust the final concentration of monomer 1 to ~3.3% (w/v). BAC-crosslinked cleavable PAAG gelling solution was prepared as previously described. A gelation
The chamber was constructed around the virus-immobilized coverslip using the following steps. First, the coverslip was transferred to the center of a glass slide. Spacers consisting of a stack of a #0 and a #1 coverslip were placed on either side of the virus-immobilized coverslip. 50 µL of freshly prepared TG or PAAG gelling solution was added to the coverslip, and the chamber was closed by placing a rectangular coverslip on top of the spacers. The gelling solution was further added from the side of the chamber until the chamber was completely filled. After gelation for 2 hours at 37°C, the chamber was partially opened using a diamond scribe to remove portions of the top cover glass that were not directly above the virus-immobilized coverslip. The chamber was then placed into a rectangular 4-well dish and incubated in digestion buffer with Proteinase K at 8 U/mL (New England Bio Labs; 1:100 dilution) overnight at room temperature with gentle shaking. After digestion, the top cover glass came off naturally and was removed from the solution. The diameter of the gel was measured for downstream estimation of the overall expansion factor. Regions inside of the circular 12-mm coverslip (i.e. regions with the immobilized viruses) were trimmed into a parallelogram, of which the side lengths were measured for downstream estimation of the overall expansion factor. Finally, trimmed gels were washed twice in PBS, each time for 10 min. To de-hybridize B1’ and B1 oligos, the gels were incubated in 80% formamide at room temperature with gentle shaking for 1 hr, and then washed three times in PBS, each time for 10 min.

**Re-embedding into a BAC-crosslinked non-expanding 2nd gel**

The gels were transferred (with the virion side facing down) into a rectangular 4-well dish that carries a glass slide in each well, and expanded in water three times, each time for 30 min. The gels were then incubated in 3 mL of BAC-crosslinked non-expanding gelling solution [10.4% (w/v) acrylamide, 0.2% (w/v) BAC, 0.05% (w/v) TEMED, 0.05% (w/v) APS] for 5 min with gentle shaking. After the incubation, the non-expanding gelling solution was removed from the well, and the glass slide carrying the expanded gel was transferred to a gelation chamber. Spaces consisting of a stack of #1.5 cover glasses were placed on either side of the gel, and the chamber was closed with a rectangular cover glass. The non-expanding gelling solution was added from the side of the chamber until the chamber was completely filled. The gelation chambers were incubated for 2 hours at 37°C. After gelation, the chambers were opened by removing the top cover glass. Side lengths of the parallelogram were measured. The gels were trimmed to leave only the portion inside the parallelogram, while preserving the shape of the parallelogram. Side lengths of the trimmed gels were measured. Finally, the trimmed gels were washed twice in PBS, each time for 10 min.

**1st Linker hybridization**

The gels were incubated in hybridization buffer (4x SSC + 20% (v/v) formamide) for 30 min at room temperature. For readout after 2-round expansion (~10-20x expansion factor), the gels were incubated with 1 nmol of oligo 5’Ac-B1’-4xB2’ in 500 µL of hybridization buffer overnight at room temperature. For readout after 3-round expansion (~40-80x expansion factor), the gels were incubated with 1 nmol of oligo 5’Ac-B1’-A2’ in 500 µL of hybridization buffer overnight at room temperature. After incubation, the gels were washed in hybridization buffer three times, each time for 1 hour, and then overnight, all with gentle shaking. The gels were then washed three times in PBS, each time for 5 min.

**Re-embedding into a DATD-crosslinked expanding 3rd gel**


The gels were incubated in DATD-crosslinked expanding gelling solution [8.6% (w/v) sodium acrylate, 2.6% (w/v) acrylamide, 0.5% (w/v) DATD, PBS, 2M NaCl, 0.01% (w/v) 4-HT, 0.2% (w/v) TEMED, 0.2% (w/v) APS] for 30 min at 4°C. The gels (with the virion side down) were enclosed in gelation chambers, incubated for 2 hours at 37°C, size-measured, trimmed, size-re-measured, and washed as described in “Re-embedding into a BAC-crosslinked non-expanding 2nd gel”.

Cleaving BAC-crosslinked 1st and 2nd gels
The gels were incubated in BAC-cleaving buffer (0.25M TCEP-HCl, 0.75M Tris-HCl, pH 8.0) overnight at room temperature. The gels were then washed four times in PBS, each time for 30 min. For samples designated for 3-round expansion, the gels were incubated in thiol-blocking buffer (100 mM maleimide, 100 mM MOPS, pH 7.0) for 2 hours at room temperature. The thiol-blocked gels were washed three times in PBS, each time for 10 min.

LNA hybridization for readout after 2-round expansion
For samples designated for 2-round expansion, the gels were incubated with 1 nmol of LNA_B2-Atto647N in 500 µL of hybridization buffer. The LNA-hybridized gels were washed in hybridization buffer three times, each time for 1 hour, and then overnight, all with gentle shaking. The gels were then washed three times in PBS, each time for 5 min.

Gel expansion, immobilization, and imaging for 2-round expanded samples
The gels were trimmed into smaller pieces (~5 by 5 mm) while preserving the shape of the parallelogram. First, the gels were transferred (with the virion side down) into a rectangular 4-well dish that carries a glass slide in each well, and expanded in water three times, each time for 30 min. A glass-bottom 6-well plate was modified with poly-lysine, and the expanded gels were gently transferred to the poly-lysine modified coverslip surface for imaging, as previously described.

Re-embedding into a DATD-crosslinked non-expanding 4th gel (for 3-round expansion)
Thiol-blocked gels in “Cleaving BAC-crosslinked 1st and 2nd gels” were subsequently trimmed into smaller pieces (~5 by 5 mm) while preserving the shape of the parallelogram. The gels were transferred (with the virion side down) into a rectangular 4-well dish that carries a glass slide in each well, and expanded in water three times, each time for 30 min. The gels were transferred onto a slide glass and trimmed in the z-direction into a thickness of 1 mm. Briefly, the glass slide (with 1-mm thickness) carrying the expanded sample was placed between two stacks of 1-mm-glass slides, and a cryostat blade was pushed slowly through the expanded gel. The bottom gel, which carries the virus at the bottom side, was transferred back to the 4-well plate. The z-trimmed gels were then incubated in DATD non-expanding gelling solution [10.4% (w/v) acrylamide, 0.5% (w/v) DATD, 0.05% (w/v) TEMED, 0.05% (w/v) APS] for 30 min at 4°C. The gels were enclosed in gelation chambers, incubated for 2 hours at 37°C, size-measured, trimmed, size-re-measured, and washed as described in “Re-embedding into a BAC-crosslinked non-expanding 2nd gel”.

2nd Linker hybridization
The gels were incubated in hybridization buffer (4x SSC + 20% formamide) for 30 min at room temperature. The gels were incubated with 0.5 nmol of oligo 5’Ac-A2-4xB2’ in 1 mL of
hybridization buffer overnight at room temperature. After incubation, the gels were washed in hybridization buffer three times, each time for 1 hour, and then overnight, all with gentle shaking. The gels were then washed twice in PBS, each time for 30 min.

Re-embedding into a bis-crosslinked expanding 5th gel
The gels were incubated in bis-crosslinked expanding gelling solution [8.6% (w/v) sodium acrylate, 2.6% (w/v) acrylamide, 0.15% (w/v) N,N-methylenebisacrylamide (bis), PBS, 2M NaCl, 0.01% (w/v) 4-HT, 0.2% (w/v) TEMED, 0.2% (w/v) APS] for 30 min at 4°C. The gels (with the virus side down) were enclosed in gelation chambers, incubated for 2 hours at 37°C, size-measured, trimmed, size-re-measured, and washed in the same way as described in “Re-embedding into a BAC-crosslinked non-expanding 2nd gel”.

Cleaving DATD-crosslinked 4th and 5th gels
The gels were incubated in DATD-cleaving buffer (20 mM sodium periodate, PBS, pH 5.5) for 30 min at room temperature. The gels were then washed three times in PBS, each time for 30 min, and then overnight with gentle shaking.

LNA hybridization for readout after 3-round expansion
The gels were hybridized with LNA_B1_Atto647N as described in “LNA hybridization for readout after 2-round expansion”.

Gel expansion, immobilization, and imaging
The gels were trimmed, expanded, immobilized and imaged as described in “Gel expansion, immobilization, and imaging for 2-round expanded samples”.

Expansion factor estimation
Side lengths of the gels were recorded before and after each trimming step (for example, after every re-embedding step and before every immobilization step) and immediately before imaging. Single-stage expansion factor was calculated by taking the averaged quotient between the pre-trimming size of the current step and the post-trimming size of the previous step. Overall expansion factor was calculated from the product of all the previous single-step expansion factors until the final step.

Imaging
Unless otherwise noted, all the expanded samples were imaged with an Andor spinning disk (CSU-W1, Yokogawa) confocal system on a Nikon Eclipse Ti-E microscope body with a CFI Apo LambdaS LWD 40x, 1.15 NA water-immersion objective (Nikon). The two-color HeLa cell tubulin and clathrin images and the HSV-1 virion images were deconvolved with the theoretical point-spread functions (PSFs) (Huygens Essential, SVI) before visualization and image analysis.

Visualization
Unless otherwise noted, all 3D renderings were generated using Imaris x64 8.3 (Oxford Instruments).

Averaged HSV-1 virion images
An averaged HSV-1 virion particle was generated using a semi-automated image analysis pipeline implemented on MATLAB ("Particle Analysis Assistant"). The "Particle Analysis Assistant" is available for download\textsuperscript{45}. First, within an acquired image z-stack, all round objects with a local minimum inside the object were identified as virion particles. The center of each virion was then determined manually within the image z-slice that had the largest virion diameter. Next, the center of the virions was re-inspected and re-aligned once more. During the second inspection, a small portion (<10%) of the virions, which had significant overlaps with the neighboring virions, were rejected from the averaging. Finally, the single virion images around each virion center were automatically cropped, calibrated with the expansion factor, and arithmetically averaged.

**HSV-1 virion envelope protein layer roundness analysis**

Roundness (deviation from the perfect circle) of the HSV-1 virion envelope protein layer was measured by the standard deviation of the radii within each virion particle, using the semi-automated image analysis pipeline implemented on MATLAB ("Particle Analysis Assistant")\textsuperscript{45}. All HSV-1 virion particles that passed the second inspection in the previous section were analyzed with the following semi-automated procedure. For each particle, the z-plane that corresponded to the vertical center of the particle was manually identified by selecting the one with the maximum contour diameter of the envelope layer. From the centerline z-plane image, the radii in 8 directions (45 degrees apart) were algorithmically measured by computing the distance from the particle centroid to the Gaussian-fitted center of the envelope profile. Manual inspection was carried out to exclude portions of the 8 radii that were measured either incorrectly or with low confidence, based on the following criteria for rejection: (1) when the line intensity profile contained peaks that belonged to other virion particles, (2) when the envelope layer in the particular direction was not present or was significantly dimmer than in other directions, or (3) when the automated Gaussian fitting failed. Standard deviation of all the accepted radii within the same particle was reported as population statistics. For normalization, the accepted radii were divided by their mean (such that the mean of all the normalized radii was equal to 1).
Supplementary Fig. 1. Synthesis of monomer 1.
Supplementary Fig. 2. Expansion and two-color imaging of herpes simplex virus type 1 (HSV-1) envelope proteins (white) and DNA (blue). The virions were expanded by TG-based 2-round iterative expansion with direct labeling of the envelope proteins. Expansion factor, 10.7x. Scale bar, 1 µm (10.7 µm).
Supplementary Fig. 3. Expansion of vesicular stomatitis virus (VSV) virions. The virions were expanded by TG-based 2-round iterative expansion with direct labeling of the envelope proteins. Expansion factor, 10.9x. Scale bar, 1 µm (10.9 µm). Inset, magnified view of the boxed region on a single xy-plane. Scale bar, 100 nm (1.09 µm).
Supplementary Fig. 4. Expansion of HSV-1 virions. a, HSV-1 virions with directly labeled envelope proteins, expanded by TG- (left) and sodium polyacrylate/acrylamide gel (PAAG)-based (right) 2-round iterative expansion. Scale bar, 1 µm (TG, 10.3 µm; PAAG, 15.3 µm). Expansion factors, 10.3x (TG) and 15.3x (PAAG). b, Averaged single-particle images of HSV-1 virions after TG- (top) and PAAG-based (bottom) 2-round iterative expansion (TG, n = 405; PAAG, n = 357 virion particles; virion particles from the same single batch of live HSV-1 preparation). Scale bars, 100 nm.
Supplementary Fig. 5. 3D rendered images of the envelope proteins of two HSV-1 virions expanded by TG-based 3-round iterative expansion with direct labeling. Overlaid images of the deconvolved puncta (white) and the fitted centroids (red) are shown on the left, and the extracted centroids (colored) are shown on the right. Expansion factors, 38.8x (virion a) and 38.3x (virion b). Scale bars, 100 nm (virion a, 3.88 µm; virion b, 3.83 µm). Insets (dotted boxes), maximum intensity projection (MIP) of the same virions over a ~65 nm range around the particle center (between the dotted lines in the 3D rendered images). Scale bars, 100 nm (virion a, 3.88 µm; virion b, 3.83 µm).
### Supplementary Table 1. DNA oligo sequences.

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Purpose</th>
<th>Sequence (IDT format)</th>
<th>Modification</th>
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<tbody>
<tr>
<td><strong>For iterative expansion of HeLa cells (Fig. 3)</strong></td>
<td></td>
<td></td>
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<tr>
<td>5’Amine-B1’</td>
<td>Conjugation to secondary antibody for pre-G1 immunostaining</td>
<td>AAT ACG CCC TAA GAA TCC GAA C</td>
<td>5’ Amino Modifier C6</td>
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<td>5’Amine-A2’</td>
<td>Conjugation to secondary antibody for pre-G1 immunostaining</td>
<td>AAG GTG ACA GGC ATC TCA ATC T</td>
<td>5’ Amino Modifier C6</td>
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<td>5’Azide-B1</td>
<td>Pre-G1 adaptor for TG-based iExM</td>
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<td>5’ Azide</td>
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<tr>
<td>5’Azide-A2</td>
<td>Pre-G1 adaptor for TG-based iExM</td>
<td>AGA TTG AGA TGC CTG TCA CC</td>
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<td>5’Acrydite-B1’-4xB2’</td>
<td>Post-G2 linker</td>
<td>TAC GCC CTA AGA ATC CGA ACA TGG ATT ACA GCC CTC AAT GCA TTA CAG CCC TCA ATG CAT TAC AGC CCT CAA TGC ATT ACA GCC CTC A</td>
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<td>5’Acrydite-A2’-4xA1’</td>
<td>Post-G2 linker</td>
<td>GGT GAC AGG CAT CTC AAT CTA TTA CAA AGC ATC AAC GAT TAC AAA GCA TCA ACG ATT ACA AAG CAT CAA CGA TTA CAA AGC ATC AAC G</td>
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<td>LNA_B2-Atto647N</td>
<td>Post-G3 readout</td>
<td>TGAGGGCTGTAATGC</td>
<td>3’ Atto 647N, LNAs (underlined)</td>
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<td>LNA_A1-Atto565</td>
<td>Post-G3 readout</td>
<td>CGTTGATGCTTTGTA</td>
<td>3’ Atto 565, LNAs (underlined)</td>
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<tr>
<td><strong>For iterative expansion of HSV virions (Fig. 4)</strong></td>
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<td></td>
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<td>5’Amine-B1’</td>
<td>Pre-G1 conjugation to envelope proteins</td>
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<td>5’ Amino Modifier C6</td>
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<td>5’Acrydite-B1</td>
<td>Pre-G1 adaptor for PAAG-based iExM</td>
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<td>5’ Acrydite</td>
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<tr>
<td>3’Azide-B1</td>
<td>Pre-G1 adaptor for TG-based iExM of virions</td>
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<td>3’ Azide</td>
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<td>5’Acrydite-B1’-4xB2’</td>
<td>Post-G2 linker for 2-round iExM</td>
<td>TAC GCC CTA AGA ATC CGA ACA TGG ATT ACA GCC CTC AAT GCA TTA CAG CCC TCA ATG CAT TAC AGC CCT CAA TGC ATT ACA GCC CTC A</td>
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<tr>
<td>5’Acrydite-A2-4xB2’</td>
<td>Post-G4 linker for 3-round iExM</td>
<td>AGA TTG AGA TGC CTG TCA CCA TGC ATT ACA GCC CTC AAT GCA TTA CAG CCC TCA ATG CAT TAC AGC CCT CAA TGC ATT ACA GCC CTC A</td>
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<td>LNA_B2-Atto647N</td>
<td>Post-G3 or Post-G5 readout</td>
<td>TGAGGGCTGTAATGC</td>
<td>3’ Atto 647N, LNAs (underlined)</td>
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**Supplementary Table 2. Recipes of hydrogel gelling solutions.**

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<th>Gel Name</th>
<th>Purpose</th>
<th>Recipe (w/v, unless otherwise noted)</th>
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<tr>
<td>Tetra-gel (TG)</td>
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<td><strong>Monomer 2′, 2′, or 2‴ (200 mg/mL DMSO)</strong></td>
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<tr>
<td>Non-cleavable TG (Monomer 2′ or 2″)</td>
<td>Single-round expansion</td>
<td>2 parts (2′ or 2″)</td>
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<tr>
<td>Cleavable TG (Monomer 2‴)</td>
<td>1st Gel for TG-based iExM</td>
<td>2 parts (2‴)</td>
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<tr>
<td>Sodium polyacrylate/acrylamide gel (PAAG)</td>
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<td></td>
</tr>
<tr>
<td>BAC-crosslinked expanding gel</td>
<td>1st Gel for PAAG-based iExM</td>
<td>2.6%</td>
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<tr>
<td>BAC-crosslinked non-expanding gel</td>
<td>2nd Gel</td>
<td>10.4%</td>
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<tr>
<td>DATD-crosslinked expanding gel</td>
<td>3rd Gel</td>
<td>2.6%</td>
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<tr>
<td>DATD-crosslinked non-expanding gel</td>
<td>4th Gel</td>
<td>10.4%</td>
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<tr>
<td>Bis-crosslinked expanding gel</td>
<td>5th Gel</td>
<td>2.6%</td>
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</table>
REFERENCES

43. Park, Y. G. et al. Protection of tissue physicochemical properties using polyfunctional


45. Yu, C.-C. Particle Analysis Assistant. Available at: https://github.com/jayyu0528/Particle_Analysis_Assistant.