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Supporting Information

Cryo-EM-on-a-chip: custom-designed substrates for the 3D analysis of macromolecules

Nick A. Alden[a,c,e ‡], *A. Cameron Varano*[a,b,c,d,e ‡], *William J. Dearnaley*[a,c,d,e], *Maria J. Solares*[a,b,d,e], *William Y. Luqiu*[a], *Yanping Liang*[a], *Zhi Sheng*[a], *Sarah M. McDonald*[f], *John Damiano*[g], *Jennifer McConnell*[g], *Madeline Dukes*[g], *and Deborah F. Kelly*[a,c,d,e*]

a. Virginia Tech Carilion Research Institute, Virginia Tech, Roanoke, VA 24016, USA

b. Translational Biology, Medicine, and Health Graduate Program, Virginia Tech, Blacksburg, VA 24061, USA.

c. Department of Biomedical Engineering, Pennsylvania State University, University Park, PA, 16802, USA.

d. Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA, 16802, USA.

e. Center for Structural Oncology, Pennsylvania State University, University Park, PA, 16802, USA.

f. Department of Biology, Wake Forest University, Winston-Salem, NC, 27109, USA.

g. Application Science, Protochips Inc., Morrisville, NC 27560, USA.

‡ These authors contributed equally to the work.

*Correspondence to: Debkelly@psu.edu

Supplemental Materials and Methods.

Simian rotavirus DLPs. Simian rotavirus (strain SA11-4F) DLPs were prepared as described in previous work.[19] Briefly, transcription activation reactions were performed at 37°C using the following components: 1 μg DLPs prepared in 100 mM Tris-HCl pH 7.5, 6 mM MgAc, 4 mM DTT, 2 mM each of ATP, GTP, CTP, UTP, and 1μL RNasin (Promega Corp., Madison, WI). After an incubation period of 30 minutes, aliquots of the transcription reactions (2 - 3 μL each) were used for cryo-EM experiments.

Isolation of protein assemblies. The HCC1937 cells used in this study was purchased from ATCC and independently characterized by ATCC as mutated BRCA1^{5382insC}-BARD1. The HCC1937 cells were cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (ATCC) and 0.5× penicillin-streptomycin (Thermo Fisher Scientific). The U87MG cells were used this study cultured in DMEM (Life Technologies Corporation) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Inc.), 100 μg/mL of streptomycin, and 100 IU/ml of penicillin. Cell lines were incubated at 37°C in 5% CO_2. Immobilized metal affinity chromatography was used to enrich for either BRCA1^{5382insC}-BARD1 or p53 complexes. Approximately 1 million cells were collected using trypsin-EDTA (Thermo Fisher Scientific) and pelleted by centrifugation (500xg; 5 min). The nuclear fraction was extracted using the NE-PER kit (Thermo Fisher Scientific). The soluble nuclear material was incubated with 200 μL of Ni-NTA agarose beads (Qiagen) for 1 hour 1 hour at 4°C with gentle rotations. The beads with bound material were transferred to a 3ml column and washed with 3 mL of 20 mM HEPES buffer (pH 7.2; 140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM imidazole). The p53 complexes were eluted in the same HEPES buffer supplemented with 60 mM imidazole, while the BRCA1^{5382insC}-BARD1 complexes were eluted in the HEPES buffer supplemented with 150 mM imidazole. The concentrations of eluted proteins were determined using Bradford assays (Thermo Fisher).

SDS-PAGE and immunoblotting. Protein fractions were analyzed by SDS-PAGE stained and immunoblotting. Proteins were separated on 3 to 8% Tris-Acetate NuPAGE mini gels (Thermo Fisher Scientific). The gels were washed 3 times with ultra-pure water then stained with SimplyBlue SafeStain solutions (Invitrogen). Gels were washed with deionized water overnight on an orbital shaker then in 20% NaCl aqueous solution overnight to achieve maximum sensitivity. Antibodies against p53 (DO-1; Santa Cruz, sc-126) were used for immunoblotting.

EM specimen preparation and data collection. Aliquots of purified proteins or virus assemblies (~300 μL each of 0.02 mg/mL) were added to 96-well microtiter plates. Clean SiN microchips were either glow-discharged or functionalized with 20% Ni-NTA lipid monolayers as previously described.[20] Following an appropriate incubation period (10 seconds to 1 minute), the excess aqueous solution was blotted away and the microchip was loaded into a FEI Mark III vitrobot (ThermoFisher Scientific) for plunge freezing. Blotting times were typically 4 - 6 seconds or ~2 seconds/sample volume. Alternatively, ~2-μL aliquots of biological material may be added direct to the microchip surface prior to plunge-freezing. In parallel, holey carbon EM grids (Protochips, Inc.) were prepared by adding 2-μL aliquots of each sample directly to the grid and incubating for one minute. Grids were loaded into the same freezing device prepared under the same blotting conditions. Frozen-hydrated specimens were examined using a FEI Spirit BioTwin TEM (Thermo Fisher Scientific) equipped with a LaB_6 filament and operating at 120 kV under low-dose conditions (<5 electrons/$Å^2$). Images were recorded using an Eagle 2k HS CCD camera having a pixel size of 30-μm (Thermo Fisher Scientific) at various magnifications. For the p53 protein assemblies, a nominal magnification of ~68,000x was used for a final sampling of 4.4 Å / pixel.

Image processing and movie production. For the p53 reconstruction, particles were selected from EM images using automated routines implemented in the RELION software package. A filtered (30 Å) structure of the p53 core bound to DNA (pdb code, 2AC0) was used as an initial model. Class averages were calculated for the data and the density map contained 12,144 particles. The initial model assisted in the first round of 3D refinement to assign orientation values to each particle. Up to 25 subsequent refinement iterations were executed in RELION implementing a regularization parameter of *T=4,* a pixel size of 4.4 Å, and a mask value of 80 Å. Following the refinement procedures, particle data was divided into two halves and the resolution for each half converged to a common numerical value. The 0.5-FSC criteria provided an estimate of the resolution in RELION, which was verified using the RMEASURE program to be approximately 10 Å. During the refinement procedures, we imposed the C2-symmetry operator, bringing the total particle equivalency to 24,288. The final map was masked at ~80 Å in diameter and the Chimera software package was used to visualize all density maps. Threshold values are included as part of the accompanying information for the EM map deposition.

To highlight components of the p53 structural assemblies and the molecular models, we used the Chimera software package to produce movies of the data. Each movie contained the p53 EM reconstruction and models that were imported and manually aligned. The density map with the atomic models in place were rotated and cross-sectioned during the movie production procedures. To generate cross-sectional views, slices through the output were generated at 110 frames, then reversed as frame slices were replaced. The structure was rotated about the x– or y– axis by ~1 degree per frame using up to 90 frames in total. Movies output was .mov format.

Supplemental Figures.

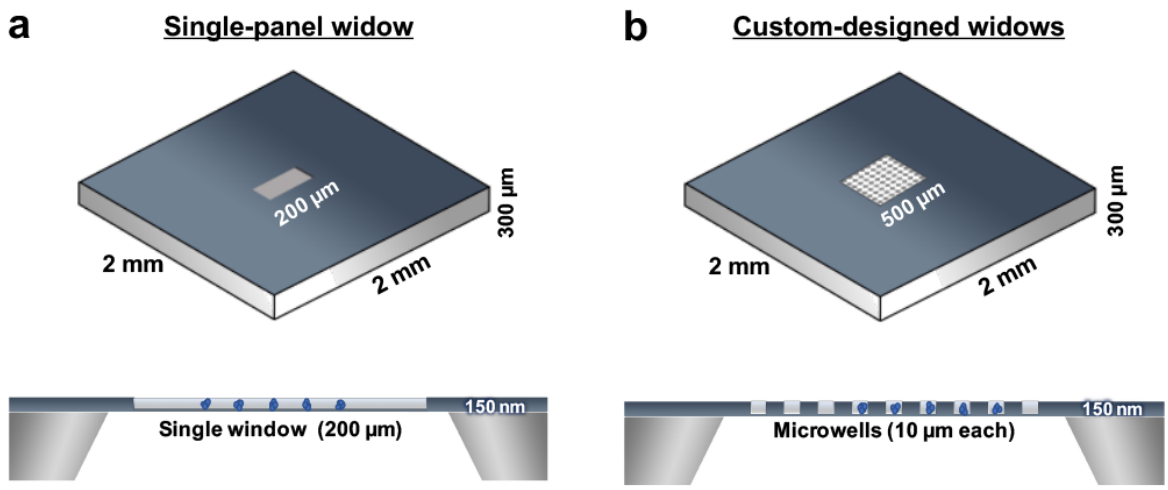

Figure S1. Comparion of SiN microchips used for cryo-EM applications. (a) Previously used SiN microchips contained a single-panel imaging window that may vary from 200 – 500 µm in x- and y- dimensions. **(b)** New custom-designed microchips contain patterned micro-wells or micro-posts that can vary in dimension based on the desired application or specimen needs. Top and side views of the chips designs are noted. Micro-wells used in the current work were ~10 µm in x- and y- dimensions and the z-spacers were 150 nm.

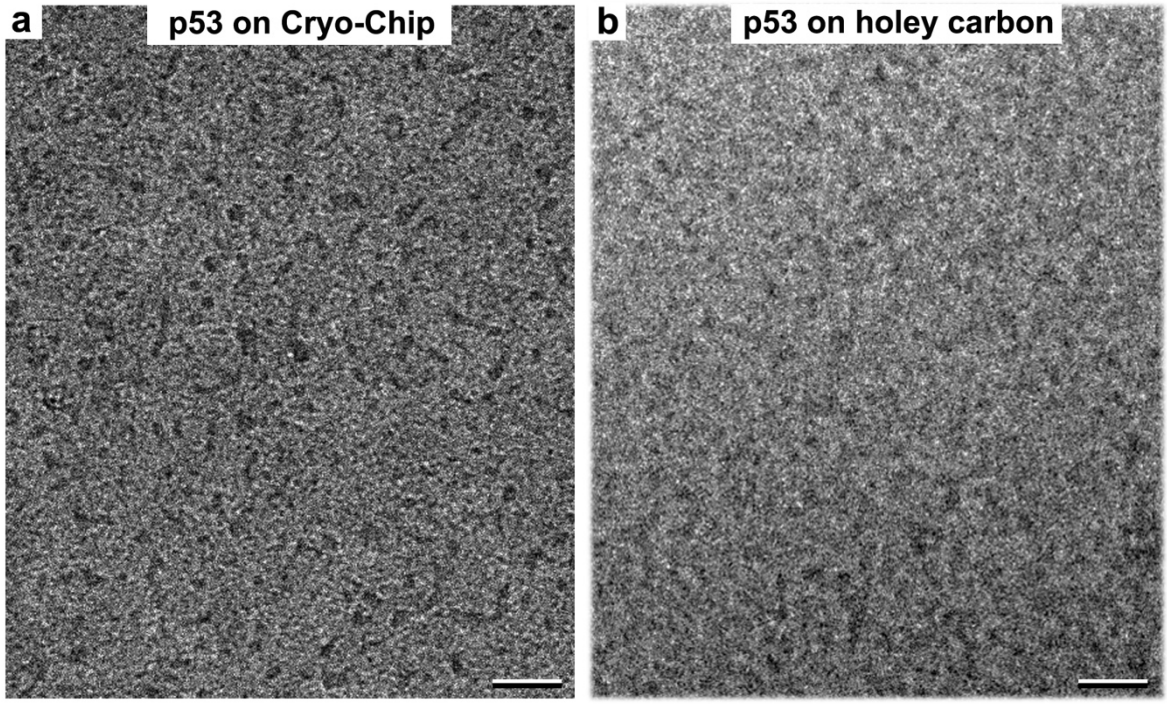

Figure S2. Comparison of p53 assemblies prepared on Cryo-Chips and holey carbon film. **(a)** Samples of p53 assemblies prepared on Cryo-Chips revealed particles that were more easily distinguished for image processing procedures. **(b)** p53 assemblies prepared on holey carbon film were non-optimal in comparison to Cryo-Chip specimens. Scale bar is 100 Å.

Movie descriptions.

Movie S1 (.mov). Video to demonstrate the SiN microchip specimen preparation technique.

Movie S2 (.mov). Video to demonstrate the transfer of SiN microchips into a Gatan 626 specimen holder for cryo-EM imaging analysis.

Movie S3 (.mov). Rotational movie of the p53 EM structure with molecular models in place.

Movie S4 (.mov). Cross-sectional movie of the p53 structure with molecular models in place.