A DIY guide to building and using a benchtop centrifuge force microscope

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Abstract
The ability to apply controlled forces to individual molecules or molecular complexes and observe their behaviors has led to many important discoveries in biology. The centrifuge force microscope (CFM) is a low-cost and easy to use instrument that enables high-throughput single-molecule studies. By combining the imaging capabilities of a microscope with the force application of a centrifuge, the CFM enables simultaneous probing of hundreds of single-molecule interactions using tethered particles. Here we present a comprehensive set of instructions for building a CFM module that fits inside a commercial benchtop centrifuge. The CFM module uses a 3D printable housing, off-the-shelf optical and electrical components, and can be built for less than $1000 in about one day. We also provide detailed instructions for setting up and running an experiment to measure force-dependent shearing of a short DNA duplex, complete with software for CFM control and data analysis. In a field where >$100,000 instruments are the norm, it is our hope that this CFM protocol can help bring single-molecule research into the hands of more labs.

Introduction
The ability to probe and observe individual biomolecules in solution has led to the field of single-molecule biophysics. The field has been largely driven by technological advancements over the past few decades including many advances in optics, computers, electronics, molecular design, and mechanical manipulation. This history of the field has already been well documented in several outstanding review articles [1,2]. Our understanding of biology has benefitted immensely from single-molecule experiments, from the detailed movement of molecular motors [3-5] to the folding and unfolding of proteins and other molecules [6-8], to the mechanical properties and genomic processes of nucleic acids [9-12].

One common class of experiments in the field is single-molecule pulling experiments, whereby a molecule or group of molecules is observed as an external force is applied. This experimental approach is valuable for reasons both obvious and subtle. The most obvious reason is that forces exist everywhere in biology (gravity, muscle tension, blood flow, etc.), and biological systems (at scales from molecular to organismal) can be affected by mechanical forces. At the molecular level, force can accelerate dissociation between molecules [13], influence enzymatic activity [14], provide signaling mechanisms [15], and prompt structural transitions [16], among a few examples. Another more subtle reason is that force provides a convenient way to measure biophysical properties of molecules or molecular complexes. Analogous to mechanical testing of bulk materials, observing responses to stress can allow us to understand material properties that can predict behavior in many conditions. Unlike bulk materials, weak biological interactions are influenced by thermal energy which causes their “strength” to be dynamic – influenced by both force and time [13]. Still, force can be used

to accelerate biochemical kinetics to allow observation on convenient experimental timescales, while still enabling extrapolation to other forces and force-free (solution) kinetics.

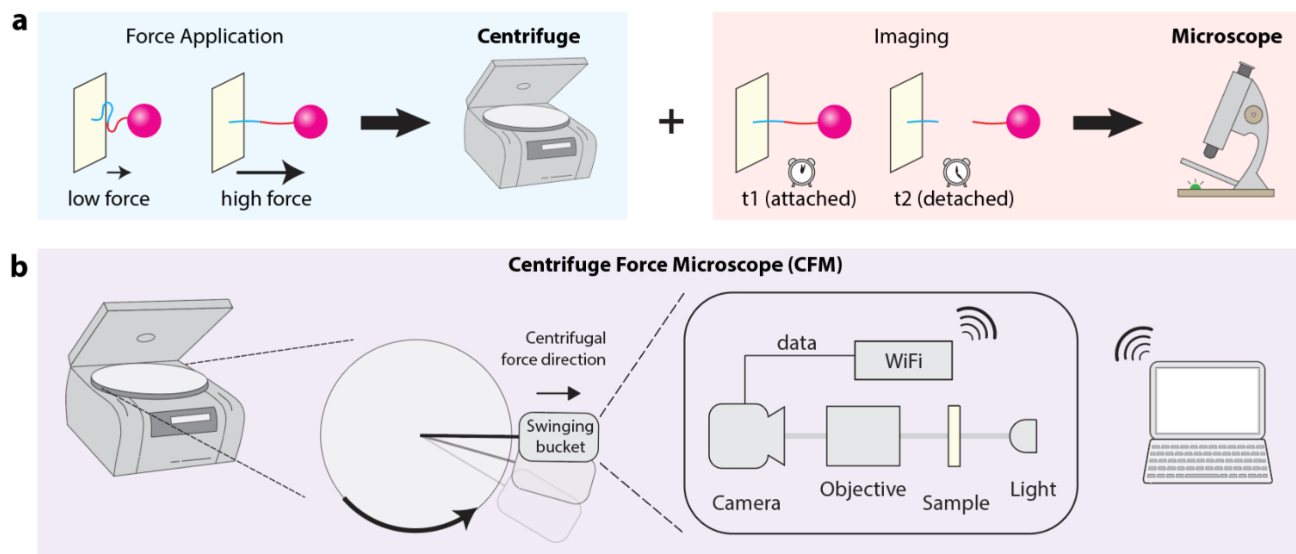

Figure 1 – Conceptual overview of the Centrifuge Force Microscope and its basic elements. A) The centrifuge aspect provides a mechanism for force application, while the microscope aspect enables observation of tethered particles. B) The CFM uses a benchtop swing-bucket centrifuge with the microscope housed inside the bucket and transmitting video to an external computer.

Several tools have been developed and refined for single-molecule pulling experiments [17], most popularly optical tweezers [18-20], magnetic tweezers [21-23] and atomic force microscopy (AFM) [24-26]. These methods differ in the mechanism of force application and measurement approach. Besides these three main methods, there are several other methods which have been demonstrated for such applications. These include nanopores [27], acoustic trapping [28], biomembrane force probe [29], DNA-based force probes [30], flow stretching assays [31,32], and the centrifuge force microscope (CFM) [33].

The Centrifuge Force Microscope (CFM) was developed as a single-molecule tool, to address both the low throughput and high cost of more conventional techniques such as optical tweezers. As the name suggests, the instrument combines the imaging capabilities of a microscope with the controlled force application of a centrifuge (Figure 1). This combined force and microscopy allows for the simultaneous probing of hundreds to thousands of single-molecule interactions using tethered particles. The method has evolved substantially from the initial proof of concept design using a custom open-air centrifuge to a user-friendly wireless module that can be used inside a commercial swing bucket centrifuge (Figure 1B) [33-36]. Recently, the method was used to measure individual base-stacking interactions in DNA [37], and other similar versions of the instrument have been used for measuring protein interactions [38], enzymatic cleavage [39], and colloidal interactions [40].

Here, we provide a comprehensive and detailed set of instructions for building a CFM, performing experiments, and analyzing data (Table 1 & Figure 2). We have optimized the protocol so that it can be followed successfully by researchers without prior single-molecule experience. It is our hope that providing this protocol will expand access of this powerful technique.

Overview of the technique

The protocol provided here consists of three main phases – CFM construction, experimental processing for a DNA shearing experiment, and data analysis. We provide a table outlining the main parts of the protocol (Table 1).

Table 1: Overview of the protocol.

	Process	Key features	Time	Steps	Figure(s)
CFM construction	3D printing of components	Components optimized for easy printing without supports, and minimal mass and printing time.	23 hr	1-4	Fig. 2, Fig. 3c-d
	Assembly of CFM optics	Optics selected for cost, weight, and simplicity of assembly. All off the shelf components.	30 min	5-22	Fig. 2, Fig. 3c-f Video S1
	Wiring and connections of CFM module	Electrical system simplified with minimal number of electrical connections.	1 hr	23-38	Fig. 2, Fig. 3g Video S2
	Set counterbalance	Simple counterbalance, easily configured.	15 min	39-43	Fig. 2
	Set up and test CFM	Clear instructions for configuring router, camera and centrifuge for routine CFM use.	30 min	44-52	Fig. 2 Fig. 3h
Experiment setup	Prepare DNA constructs	DNA constructs prepared using our modular self-assembly technique for versatility.	1.5 hr	53-59	Fig. 2, Fig. 4a-b
	Prepare beads with DNA	Simple attachment of DNA to beads.	30 min	60-68	Fig. 2, Fig. 4c
	Prepare/load chamber	Simple protocol for attaching DNA to cover glass and forming tethers with beads.	30 min	69-82	Fig. 2, Fig. 4d-e
Test and analyze	Force dependent dissociation	Use our Labview software for recording CFM images during centrifugation.	1 hr (varies)	83-91	Fig. 2, Fig. 5a-b
	Data analysis	Use our Matlab software to determine tether rupture times and determine kinetics.	2 hr (varies)	92-97	Fig. 2, Fig. 5c-d

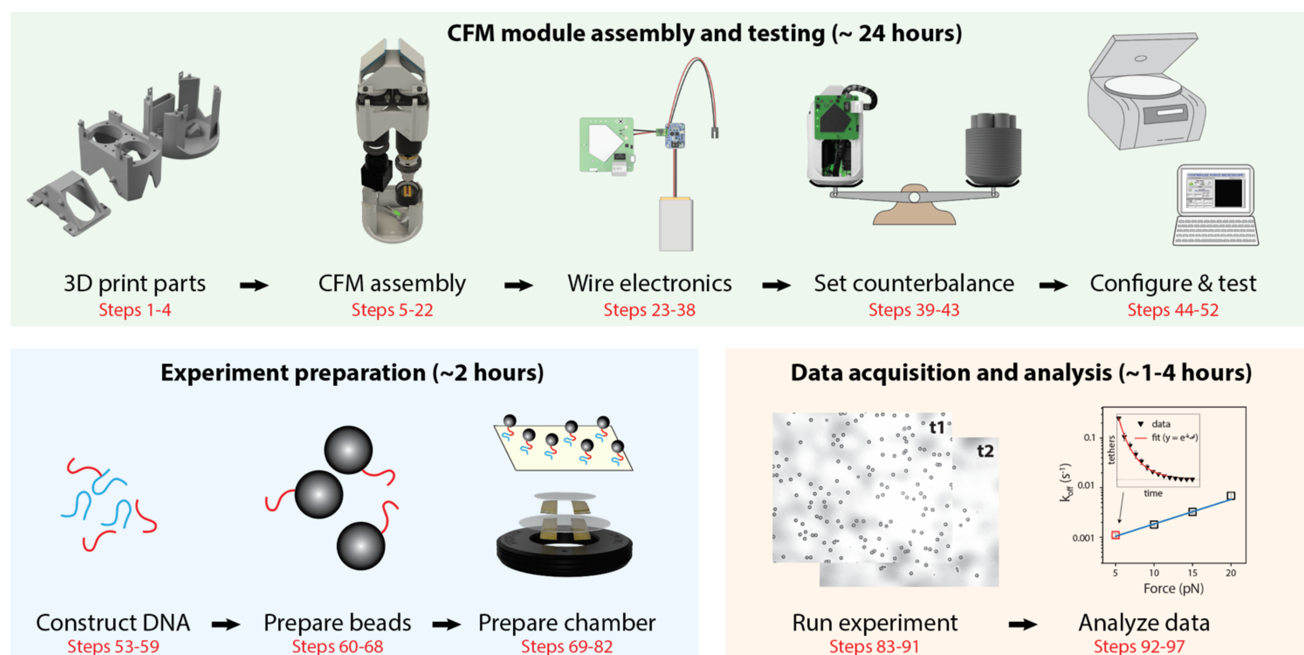

Figure 2 – Visual overview of the main steps of the protocol.

Development of the protocol

Since its introduction, the CFM has gone through many phases of development. The first instrument was a custom open-air centrifuge with an attached microscope imaging arm, which was used to demonstrate proof of concept [33]. Subsequent iterations have focused on improving safety, reducing cost, and improving the user experience. The second-generation instrument integrated the CFM design into a benchtop centrifuge which was modified to house a rotary fiber optic joint for data transmission [34]. The third generation provided the first fully wireless design that used a CFM module

with an onboard computer inside an unmodified benchtop centrifuge [35]. Our most recent fourth generation design is a wireless and live-streaming CFM, which enables real time control and data transmission over Wi-Fi [36]. Other groups have come up with their own CFM designs, most of which also converge around the use of a swing bucket centrifuge but vary in some of the optical and data transmission design choices [39-41].

With years of designs and iterations behind us, updates to our CFM designs have recently tended to converge, and changes have become progressively more minor. Hence, we thought it prudent to provide a detailed protocol, realizing that our design is now “battle tested”. The protocol presented here is based on the design first shown in *Biophysical Journal* [36] and more recently used in *Nature Communications* [37]. The core of the design (optical assembly and control components) is functionally identical to both of those models, but we have revised and optimized the current build to improve cost, construction, image quality, and ease of use leading up to this protocol. Specifically, we have re-engineered the 3D printed housing, improved illumination quality, reduced the number of parts (including eliminating all custom machining), replaced bulky turning mirrors, streamlined electrical components and wiring, and simplified design and implementation of a counterbalance. Together these improvements make it easier for general researchers to construct and use a CFM (Figure 3).

Once constructed, the protocol focuses on setting up and using the CFM. Detailed instructions are provided for executing an experiment to measure the forced dissociation of a short DNA duplex (Figure 4). This experiment reproduces part of our recent work on DNA base stacking [37], including instructions for making the DNA constructs, preparing the beads and cover glass, and running the experiment. Last, there are instructions for data analysis using our custom software (Figure 5). For convenience, we collected new data for this paper and provide the raw data to help users validate the analysis workflow.

Applications of the method

The CFM is best suited for performing many single-molecule pulling experiments in parallel, especially those for which dissociation is observed. The benefit of multiplexing is especially evident when measuring molecular interactions with slow kinetics, when it may not be feasible to collect hundreds of statistics in serial for an hours-long process, for example. We have applied our method to shearing, unzipping, and overstretching of DNA duplexes [33-36], and more recently to quantify base stacking energies [37]. Other groups have used the CFM (with similar but not identical design) to measure force-dependent enzyme kinetics [39], interactions between colloids and surfaces [40], and antibody affinities [38].

There are several application areas for CFM that have not yet been fully developed. One area would be the study of larger structures, such as cellular (or even organismal) biophysics. The CFM could be adapted for cell adhesion or compression studies, as well as for larger structures such *Drosophila* embryos or even whole organisms like *C. elegans*. Some versions of the CFM have already demonstrated fluorescent imaging [40] which could prove useful in such studies of mechanobiology. The CFM could also be used for molecular unfolding studies, despite limited studies so far. While the CFM may not achieve the resolution of optical tweezers, nanometer level tracking has already been demonstrated [34,41] and other imaging modifications such as reflection interference [42] or stereo darkfield interferometry [43] could potentially improve the axial resolution. Investigations into molecular heterogeneity are also well suited, due to the ability to repeatedly probe many individual molecules in a population, as demonstrated in some previous studies [34,38].

Comparison with other methods
For single-molecule pulling experiments, optical tweezers have arguably been the dominant technology for the last two decades. The method uses one or more lasers to trap and manipulate particles and can typically achieve sub-nm and sub-pN length and force resolutions, respectively. Other popular choices are AFM and magnetic tweezers with AFM generally having a higher force range and magnetic tweezers generally having a lower force range. Most single-molecule studies using these instruments pull molecules one at a time, which has given these experiments an appropriate reputation of being painstaking and laborious.

Recently, there has been an effort to multiplex single molecule pulling experiments, driven in part by the advent of the CFM as an inherently multiplexed instrument. Among the major single-molecule tools, magnetic tweezers are the most suitable for multiplexing as evidenced by several developments [44-46] including a few that pre-date the CFM. More recent efforts in multiplexing single-molecule experiments have used acoustic forces [28], fluid flow [32,47], or DNA based probes [30].

Aside from throughput, some major obstacles of doing single-molecule biophysics come from the cost, complexity, and technical skill required to operate the instruments. Many studies use expensive and custom-built instruments, which has historically relegated single-molecule biophysics into relatively few physics-oriented labs that can build, maintain, and operate the equipment. Efforts to commercialize these instruments have had mixed results, often reducing the complexity and technical skill required for the instruments at the expense of even higher cost. There are now several options for commercial AFMs and optical tweezers, almost all of which fall in the low to mid six-figure USD price range. Acoustic force spectroscopy, a multiplexed method introduced in 2015 [28] was quickly commercialized for single molecule analysis [48] at a lower price point, but has been since discontinued.

We provide a summary table which compares the attributes of the various methods (Table 2).

Table 2 – Comparison of single-molecule pulling techniques.

Technique	Force mechanism	Force range [pN]	Spatial resolution [nm]	Cost	Throughput	Advantages	Limitations
Optical Tweezers	Light	0.1-100	0.1-2	$$$	Single (typ.)	High spatial, force resolution Commercially available	Sample heating Photodamage Complex instrumentation
Magnetic Tweezers	Magnetic field	0.01-100	2-10	$$	Single or multiplex	Ability to apply torque Can be multiplexed	Requires magnetic particles Field strength/uniformity tradeoff Limited spatial resolution
Atomic Force Microscopy	Cantilever	10-1000+	0.1-1	$$$	Single	High spatial resolution Commercially available	Stiff probe High minimum force
Biomembrane Force Probe	Red blood cell	0.1-1000	2-10	$$	Single	Useful force range	Requires fresh blood Limited time window for experiments
Flow cell	Fluid flow	0.1-10	~10	$	Multiplex	Inexpensive Inherent multiplexing Simple instrumentation	Potential surface interactions Limited spatial resolution Difficult force calibration
Acoustic Force Spectroscopy	Sound waves	1-100	2-10	$$	Multiplex	Inherent multiplexing	Custom fabricated chip Complex force field/calibration
Centrifuge Force Microscope	Centrifugal force	0.1-1000	2-10	$	Multiplex	Inexpensive Inherent multiplexing Uniform/stable force field Calibration-free Easy to use	Limited spatial resolution Limited temporal resolution

*Partly adapted from [17, Neuman et al 2007]
$$$ - > $100,000 USD
$$ - $10,000 - $100,000
$ - < $10,000

Experimental design
The experiment described in this protocol probes the force-dependent dissociation of a short DNA duplex in the 5-20 pN force range. This sample experiment is meant to be a starting point for new users, and one that has ample supporting data from our recent paper [37]. It should build confidence in the design and operation of the technique. However, it should not be considered limiting or necessary as there are many similar experiments that could be performed. Other experiments that could provide good starting points are DNA overstretching or DNA unzipping experiments, which have been detailed for CFM as well [33,34].

In designing other experiments, the user should consider several aspects including attachment strategy, force range, required magnification/throughput and time resolution. Here we use simple adsorption of streptavidin to glass to anchor biotinylated DNA to the chamber, and streptavidin coated beads to anchor biotinylated DNA to the beads. Various covalent and non-covalent linking strategies can also be used with a variety of linking molecules [49]. The main adaptations in switching to other molecules would be empirically determining the density of tethers to achieve single-molecule attachments, typically done by varying concentration and/or time of incubation. For force range, the main considerations are bead size, bead density, and rotational speed (Supplementary Note 1). Tweaking these parameters, it is theoretically possible to achieve a very broad range of forces from fN to μN, which have so far been experimentally demonstrated in the range of ~ 10^{-1} to 10^{4} pN. Small (1 μM) polymer beads are best for very low forces below 1 pN, while larger (2-10 μM) iron core or metallic beads would be a better choice to extend into the 10-1000 pN range. It is worth noting that choice of beads may also affect coupling strategy and density, since surface properties and surface area are both being changed. The magnification requirements may also change with bead choice as well as desired throughput and resolution – generally achieving higher throughput (number of beads) at the expense of optical resolution. Similarly, the throughput limitations impart a tradeoff between time resolution and frame size such that increasing time resolution requires decreasing frame size. These parameters can generally be decided on a case-by-case basis depending on the experimental requirements.

Advantages and Limitations
The authors categorize the main advantages of the CFM into three main aspects: high throughput, low entry barrier, and simplicity of use. Achieving high-throughput single-molecule manipulation was the original goal of the CFM concept and was initially demonstrated by performing thousands of pulling experiments in parallel on an antibody-antigen complex [33]. Since then, other multiplexed methods have been developed or improved, including acoustic force spectroscopy [28], multiplexed magnetic tweezers [44], flow-based systems [32], and combined magnetic/flow systems [47]. These methods all use camera-based detection, resulting in similar spatial resolutions at the same magnifications. They differ mainly in force application method, calibration of forces, physical set up of experiments, and analysis. Importantly for multiplexing, centrifugation provides a precise, calculable, and macroscopically uniform force field. Practically speaking, this enables calibration-free operation and eliminates concerns about force drift and alterations in force due to nearby objects.

The other two advantages, low entry barrier and ease of use, go hand in hand. To our knowledge there is no other single-molecule pulling technique that could be implemented for under $1000 in less than 24 hours without specialized skill or equipment. The low cost is intrinsic to the technique since the method of force application is handled by commercially available centrifuges that are already ubiquitous in most life science laboratories. To push the cost under $1000, we reconsidered the design and reduced the number of parts and the cost of major components. To minimize time and difficulty of construction, we eliminated all custom machined components and reduced electrical connections. Once built, the instrument is relatively simple to use. Material preparation is straightforward, the centrifuge can be operated normally with push button operation, and the force

does not require any calibration. In fact, our lab now routinely has undergraduate researchers collecting publication quality data on the instrument without assistance [36,37].

The CFM is not without limitations, some of which are inherent to the technique and some of which are particular to this protocol. Inherent to the technique is centrifugation, which poses several challenges. Centrifugation causes the experiment to be essentially cut off from the outside world, so that everything must either be pre-set or remotely controllable during the experiment. It also imposes strict requirements on space, weight and structural stability that must be dealt with. Among the difficulties typically encountered are mechanical shifting from centrifugation, data bandwidth limitations between the rotating microscope and non-rotating computer, and relatively slow spin-up and spin-down times.

The protocol as written also has some limitations, though many of these can be overcome with tweaks to the instrument or experimental protocol. The instrument is designed for a Thermo Fisher Legend XR1 centrifuge and may require adaptations to fit other centrifuges. A smaller centrifuge bucket would likely take significant redesign, but larger buckets can be accommodated with a simple external ring as we have shown in another centrifugation project [50]. The chosen Wi-Fi router imposes a data bandwidth limitation of ~100 Mb/s (~12.5 MB/s), equivalent to around 2 fps for 6-megapixel resolution or 40 fps for VGA resolution with an 8 bit depth. It is common to incur some data loss as the throughput approaches the theoretical limit, in the form of blackened areas of some images. Our bandwidth is limited by the router's LAN port, and previously we demonstrated faster bandwidth using a different router [36]. In this protocol, we used 1 fps for the experiment, but bandwidth should be considered when choosing a camera and designing experiments. Faster frame rates can be accomplished with smaller images which could make a lower resolution camera a good choice if higher rates are important. Alternatively, commercially available action cameras such as GoPro are small and contain their own hardware for fast high-resolution video transfer and have been used in another CFM design [40]. Different cameras can be used, but cameras exceeding a cube of about 30 mm per side or exceeding ~50 g may require some rearrangement or additional testing. Similarly, a wide range of finite and infinite conjugate objectives can be used, but objectives larger than 25 mm in diameter or heavier than ~100 g could pose challenges.

For the experiment itself, the protocol written here probes the forced dissociation of a short duplex in a shearing configuration. We use a self-assembly technique for our DNA tethers, but there are many tethering strategies for single-molecule experiments that could be used. DNA handles can be made from PCR products or derived from other biological sources like lambda DNA. Our protocol uses magnetic beads, but this is not a requirement. Magnetic beads are convenient for three reasons: 1) high density compared to polystyrene beads giving a good force range, 2) very high uniformity, and 3) sample prep is simplified with magnetic washing. If using other beads, force should be recalculated, concentrations and incubation times may need adjustment for proper molecular attachment, and washing steps would be done by centrifugation instead of magnetic separation. Our experimental protocol specifies centrifuge speeds up to 1500 rpm, and we have tested our design up to 2000 rpm. Higher speeds would require testing, but another design went to 5000 rpm without issue [40]. It is also important to consider that our design has a fixed focus, which must be adjusted and locked in place before the experiment starts. Higher spinning speeds can cause a shift in focus which must be considered. Lastly, we have provided our software tools to analyze the experiment but acknowledge that many different types of analysis could be used to achieve the same results.

Expertise needed to implement the protocol

This protocol requires minimal experience. It is helpful but not required to have some experience with optics, 3D printing, and basic wiring and soldering of electronics to facilitate the construction of the CFM. Basic molecular biology skills and general laboratory skills are useful in the execution of the

experiment as we’ve described it. The protocol is meant to be accessible to a wide range of researchers in biology, chemistry, engineering, or physics.

Safety considerations
Many aspects of building and using the CFM can be hazardous without proper precautions. We have refined this method to be as safe as possible, and we have used the method described below without issue. However, we cannot guarantee safety or take liability for injuries obtained while following the protocol. The construction process employs 3D printing which poses burn and pinch risks, as well as cut and bruise risks when removing the prints. Several steps of the construction process use sharp tools including knives, scrapers, and pliers. Connecting the electronics requires use of a soldering iron, posing both a burn risk and a potentially toxicity risk if using lead-based solder. Working with electrical parts always poses a shock risk as well.

Use of the instrument also poses some risks, due to the kinetic energy of centrifugation. It is critical that parts inside the centrifuge buckets are secure and have clearance to swing, that the centrifuge is well balanced (with matched mass and center of mass), at a speed that the CFM can support, and that the centrifuge is in a safe area. The CFM module includes a lithium-polymer battery subjected to an unusual amount of force. Since lithium batteries can be prone to explosion or fire (with toxic fumes), care must be taken to inspect batteries prior to use. Again, we have used this build of the CFM safely for over a year, but we warn users about potential safety issues that could arise. We recommend not leaving the CFM unattended while in operation for these reasons.

Materials

Equipment
- 3D Printer (Ultimaker 3)
- Swing bucket centrifuge (Thermo Fisher Scientific, Sorvall Legend X1R)
- Computer with Wi-Fi adapter and external antenna (Dell Optiplex 7070 & Intel AC9560 adapter)
- Thermal Cycler (Bio-Rad T100)
- Vortex mixer (Ohaus VXMNDG)
- Magnetic tube rack – DynaMag Spin Magnet (Invitrogen, 1232D)
- Single channel pipette set (Corning, LambdaPlus)
- Scale/Balance, 400 g capacity, 0.1 g readability (Ohaus, Scout pro)

Tools and accessories
- Tough PLA filament (Ultimaker, 2.85mm, white)
- Soldering station (Weller, WE1010)
- Desoldering tool (Jonard Tools, DP-100)
- Lead free solder (Adafruit, 2473) *Note leaded solder can be used, easier but more toxic.
- Solder tip tin (Digikey, SMDTCLF-ND)
- Precision knife (Weller, Xcelite XN100)
- Scraper/Putty knife (Warner, 10997)
- Hot glue gun (Surebonder, Pro2-100L)
- Hot glue (Surebonder, DT-8)
- Hex ball end drivers, 2.5mm and 1.5mm (Thorlabs, BD-2.5 & BD-1.5)
- Precision flathead screwdriver (Adafruit, 424)
- Diagonal cutters (American Hakko Products, CHP-170)
- Wire strippers (American Hakko Products, CSP-30-1)

- Ruler (Grainger, 41N622)
- Tweezers (Adafruit, 421)
- Battery charger (Adafruit, 1904)
- Scissors (Wescott, Kleenearth)

Optional tools (useful but not strictly necessary)
- Soft-jaw pliers – for easier disassembly of lens tubes (Thorlabs, TGP1)
- Spanner wrenches – for easier installation of retaining rings (SPW602 & SPW603)
- Digital Calipers – for easier measurements (Mitutoyo, CD-6” ASX)
- Multimeter – for electrical testing/troubleshooting (Fluke electronics, 175 multimeter)
- 3rd hand or vise – for holding parts during soldering (e.g. Digikey, 243-1018-ND)
- Cotton gloves – for handling optics (Thorlabs, MC6-M)
- Digital dry bath or hotplate - for removal of Olympus objective casing (Labnet, Accublock)
- Tongue and groove pliers (x2) – for removal of Olympus objective casing (Grainger, 46MW60)

Consumables and reagents
- Parts to construct the CFM (see Table 3 and Table S1)
- Kimwipes (VWR, 21905-026)
- Double sided tape (Bertech, PPTDE-1/4)
- 12mm diameter coverglass #1½ (Electron Microscopy Sciences, 72230-01)
- 18mm diameter coverglass #1½ (Electron Microscopy Sciences, 72222-01)
- High vacuum grease (Dow Corning, 2021854-0313)
- Pipette tips (VWR, 76322-528, 76322-150, 7322-154)
- Microcentrifuge tubes (VWR 10025-716, 10025-724)
- 0.2 mL PCR tubes (VWR, 20170-012)
- Acetone (Fisher Scientific, 32201)
- Nuclease free water (Cytiva HyClone Laboratories, SH30538.01)
- PBS 10X, USP Sterile (VWR amresco, K813-500ML)
- Tween detergent (Millipore Sigma, P1379-25ML)
- Invitrogen Dynabeads M-270 Streptavadin (Thermo Fisher Scientific, Cat. No. 65305)*
- M13mp18 single-stranded DNA (New England BioLabs, N4040S)**
- BtsCI enzyme (New England BioLabs, R0647S)**
- 10X CutSmart Buffer (New England BioLabs, included with BB7204S)**
- Streptavadin 1mg/mL (VWR, E497-1MG)**
- Cut site oligonucleotide (IDTDNA, TAGTAGCATTAACATCCAAT)**
- Oligonucleotides for backbone mix (IDTDNA, See Table S2)** ^^
- Tether “A” oligonucleotides “OH-A” & “A: G-T” (IDTDNA, See Table S2)**##
- Tether “B” oligonucleotides “OH-T” & “T: Sp-A-C” (IDTDNA, See Table S2)**##

* stored at 4C
** stored at -20C
^^ aliquots provided upon request with material transfer agreement
PAGE purification preferred for comparison against ref [37]

Software
- Ultimaker Cura (version 5.4)
- Origin analysis software (Originlab, version 2019)
- Labview (National Instruments)
- MATLAB (Mathworks)

Reagent Setup

1) Prepare 1x PBST buffer. Mix 0.5mL of 10x PBS and 4.5mL of nuclease free water. Add 5uL of Tween to achieve 0.1%. Shake well to mix. Can be stored at room temperature for a week.
2) Prepare oligonucleotide mix. Mix equal volumes of the 122 individual oligos (1-122 in Table S2) into a single tube (5 uL each recommended). Can be stored frozen for several years.
3) Prepare streptavidin working solution of 0.1 mg/mL in 1x PBS. Can be stored at 4˚C for a week.

Procedure

3D printing of parts
Timing 23 h

1) Ensure 3D printer is properly calibrated, with flat and clean printing surface. Load sufficient filament to complete the print.
   ***Caution** – 3D printing can pose risks. There are moving parts with pinch points, high temperature nozzles, and potentially dangerous fumes. Ensure you work in a well-ventilated area with no obstructions to the printer.
   ***Critical step** – The type of filament used is important. We suggest Tough PLA (or stronger material) as regular PLA is typically too brittle for structural elements.
2) Using Cura or other 3D printing software configured for your printer, load 3D STL models for printing (see Supplementary Files). Modifiable 3D models are also provided in the Fusion 360 format, which can be exported directly to Cura for printing. Arrange the models on the Cura software, and slice to prepare the .gcode required by the printer.
   ***Critical step** – print settings can be important. We use “default” settings for our Ultimaker 3 of 0.15 mm profile (0.2 mm for counterbalance), 20% infill, and adhesion on. The only non-default setting is to turn supports “off”, since we have designed parts to be printable without supports.
3) Print the parts. They can be printed in groups or individually.
   ***Pause point** – other aspects of the protocol can be continued as parts become available
   ***Troubleshooting**
4) Once the print completes and the build plate is fully cooled, the prints can be removed using a scraper. Finish the prints by scraping excess build material and cleaning the edges of holes.
   ***Troubleshooting**

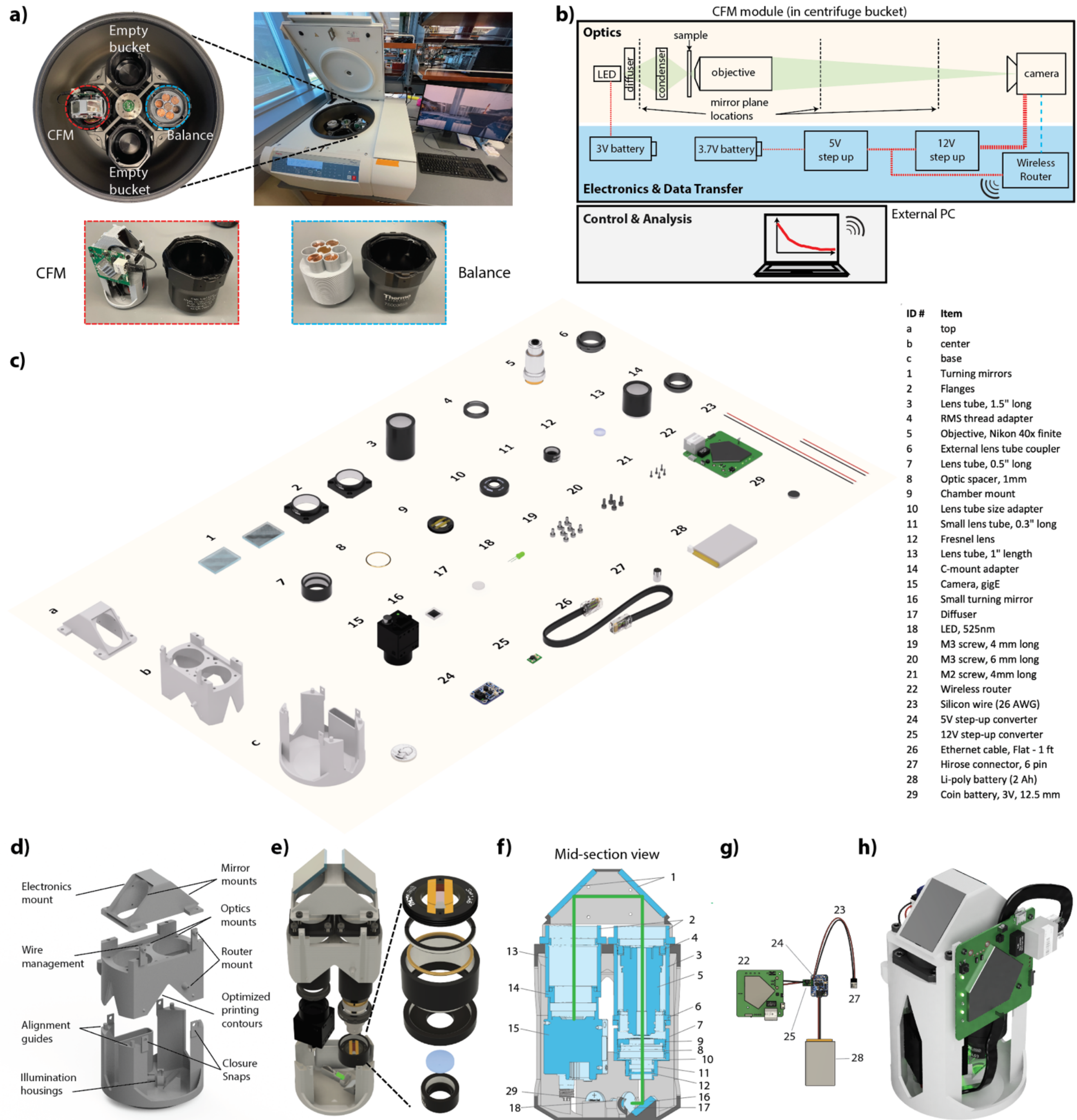

Figure 3: Details and construction of the CFM module. A) Images of the CFM and its balance within and outside of a benchtop centrifuge. B) a schematic showing the major optical and electrical components of the CFM. C) 3D rendering of parts and the associated parts list. US Quarter for scale. D) Detailed view of the 3D printed housing elements and main features. E) Exploded view of the CFM module optical components. F) Section view of the assembled CFM module with green line illustrating the light path, plastic housing in grey, and optical components in blue. G) Rendered view of the electrical system. H) Full 3D rendering of the CFM module.

ID #	Item	Supplier	Part number	Qty	Total cost	Mass (g)	Notes
1	Turning mirrors	Edmund Optics	43-875	2	$ 50.00	12.8	1
2	Flanges	Thorlabs	SM1F1	2	$ 43.80	20.4	
3	Lens tube, 1.5" long	Thorlabs	SM1L15	1	$ 16.98	21.1	
4	RMS thread adapter	Thorlabs	SM1A3	1	$ 19.43	2.8	
5	Objective, Nikon 40x finite	Edmund Optics	59-936	1	$ 165.00	71.2	2
6	External lens tube coupler	Thorlabs	SM1T4	1	$ 29.06	5.8	
7	Lens tube, 0.5" long	Thorlabs	SM1L05	1	$ 13.62	8.1	
8	Optic spacer, 1mm	Thorlabs	SM1S1M	1	$ 9.20	0.7	3
9	Chamber mount	Thorlabs	SM1A6	1	$ 22.37	3.0	
10	Lens tube size adapter	Thorlabs	SM1A1	1	$ 22.95	3.2	
11	Small lens tube, 0.3" long	Thorlabs	SM05L03	1	$ 14.92	2.9	
12	Fresnel lens	Thorlabs	FRP0510	1	$ 20.27	0.2	4
13	Lens tube, 1" length	Thorlabs	SM1L10	1	$ 15.41	14.4	
14	C-mount adapter	Thorlabs	SM1A39	1	$ 22.27	6.1	
15	Camera, gigE	FLIR	BFS-PGE-63S4M-C	1	$ 399.99	37.7	5
16	Small turning mirror	Thorlabs	ME05S-G01	1	$ 24.05	1.3	6
17	Diffuser	Thorlabs	DG05-120	1	$ 14.45	0.6	4
18	LED, 525nm	Thorlabs	LED525E	1	$ 4.52	0.3	4
19	M3 screw, 4 mm long	Grainger	4CE47	8	$ 0.34	4.5	1
20	M3 screw, 6 mm long	Grainger	6CE49	4	$ 0.17	2.8	1
21	M2 screw, 4mm long	Grainger	6CE30	5	$ 0.20	1.0	1
	optics subtotal				**$ 908.99**	**220.8**	
	Electronics						
22	Wireless router	TP-Link	TL-WR902AC	1	$ 39.99	25.2	
23	Silicon wire (26 AWG)	Adafruit	1881 & 1877	1	$ 1.90	1.5	1
24	5V step-up converter	Adafruit	1903	1	$ 9.95	3.0	
25	12V step-up converter	Pololu	U3V16F12	1	$ 4.95	0.6	
26	Ethernet cable, Flat - 1 ft	Monoprice	43073	1	$ 1.25	9.5	1
27	Hirose connector, 6 pin	Digikey	HR1588-ND	1	$ 15.62	1.7	1
28	Li-poly battery (2 Ah)	Adafruit	2011	1	$ 12.50	36.0	7
29	Coin battery, 3V, 12.5 mm	Digikey	P183-ND	1	$ 1.38	0.9	1
	electronics subtotal				**$ 87.54**	**78.4**	
	Printed parts						
a	top				$ -	10.7	
b	center				$ -	30.7	
c	base				$ -	34.5	
	3D part subtotal				$ -	**75.8**	
	Total				**$ 996.53**	**375.0**	
	Notes						
1	Many supplier options						
2	Many objective options. See Supplemental Note 1						
3	Spacer optimized for this build to give best range of focus						
4	Many alternatives, illumination results may be altered						
5	Many camera options in similar form factor						
6	Alternative suppliers, thickness changes will affect optical path						
7	Alternative suppliers but check dimensions for housing fit						

Table 3. Detailed parts list for the CFM

Assembling the CFM module (Supplementary Video 1): Timing 30 min

5) Carefully push mirrors (ID-1) into mirror housing (ID-a). These should be a close fit but should not require force. Be sure to have the correct mirror face (with the protective film removed) toward the inside of the part. While applying light pressure to each mirror, dab the corners with hot glue to secure them. Set mirror housing (ID-a) aside for later.

6) Screw the flanges (ID-2) into the center piece (ID-b) with the flat sides down using eight M3-4 screws (ID-19) and 2.5 mm hex driver. Finger tighten.
   ***Note:** if the holes are well printed the screws should self-thread into the plastic without much difficulty. Avoid overtightening screws or the plastic threading can slip.
7) Remove retaining ring from 1.5" long lens tube (ID-3) and screw the tube from the bottom into the right-side flange (with the router mounts facing toward you). Hand tighten.
8) Screw the RMS thread adapter (ID-4) onto the objective (ID-5). It is recommended to unscrew and remove the outer casing of the objective to reduce weight.
   ***Critical step** - This step may vary if using a different objective (see Supplementary Note 2)
9) Screw the objective (ID-5) with the RMS thread adapter (ID-4) into the 1.5" long lens tube (ID-3) until it hits the back. Hand tighten.
   ***Critical step** – the objective is the heaviest part and most likely to cause problems. Ensure a tight fit that requires some effort to dislodge. If necessary, an additional retaining ring can be screwed in for extra security.
10) Screw the external lens tube coupler (ID-6) into the 1.5" long lens tube (ID-3) with the hard stop facing the tube.
11) Remove the retaining ring from the 0.5" long lens tube (ID-7) and drop in the optic spacer (ID-8). Secure the optic spacer with the removed retaining ring.
12) Screw the chamber mount (ID-9) into the 0.5" long lens tube (ID-7) with the label side up until it hits the retaining ring. Tweezer tips or a paper clip can be used with the holes to ease the process. The top surface will later house the chamber.
13) Screw the 0.5" long lens tube (ID-7) onto the exposed end of the external lens tube coupler (ID-6) and finger tighten.
   ***Critical step** – always be aware of the distance to the objective to prevent the chamber from hitting the front end of the objective.
14) Attach the lens tube size adapter (ID-10) to the exposed end of the 0.5" long lens tube (ID-7).
15) Remove the retaining ring from the small lens tube (ID-11) and place the Fresnel lens (ID-12) into the tube with the smooth side down. Use the retaining ring to lock the lens into place.
16) Attach the small lens tube (ID-11) to the lens tube size adapter (ID-10) at the end of the chamber assembly and finger tighten.
17) Remove the retaining ring from the 1" long lens tube (ID-13) and screw the tube into the empty flange (ID-2) from the bottom side.
18) Screw the c-mount adapter (ID-14) into the end of the SM1-1" tube (ID-13).
19) Screw the camera (ID-15) into the c-mount adapter (ID-14) and finger tighten.
20) Push small turning mirror (ID-16) into mirror housing on base (ID-c) with the mirrored side up, being careful to not smudge the mirror. Dab hot glue in the corners for extra security.
21) Push diffuser (ID-17) into diffuser housing on base (ID-c) with the rough side facing away from the mirror.
22) Trim LED (ID-18) leads to ~10mm, and push LED into LED housing on base (ID-c). Tuck the LED leads into the grooves in the coin cell holder.

Attach and connect the CFM electronics (Supplementary Video 2):
Timing 1 h

23) Disassemble the housing of the wireless router (ID-22) and remove the circuit board. Record the SSID and wireless password for future use in step 46.
24) Disconnect the large USB port from the wireless router (ID-22) using the diagonal cutters. Desolder the 2 outer connections of the 4 in-line USB connections.
   ***Caution** - Soldering can pose burn risks as well as exposure to lead and other toxic metals. Ensure proper ventilation and personal protections to avoid harm.
25) Cut 3" and 7" lengths of red and black silicon wire (ID-23) and use the wire strippers to strip about 1/8" of insulation off the ends.

26) Insert the ends of the 3” wires into the positive (red) and negative (black) USB terminals from the back side of the wireless router circuit board (ID-22), and solder in place. The positive is toward the ethernet connection.

27) Insert the other ends of the 3” wires into the positive (red) and negative (black) terminals on the back side of the 5V step-up converter (ID-24), and solder in place. At this point, a fully charged lithium polymer battery (ID-28) can be plugged into the 5V step-up (ID-24) to test power to the router. Lights on the step-up converter and router should be visible.
***Caution** – working with electricity and with lithium-ion batteries can pose shock and fire hazards. Use caution to avoid electrical shorts and to avoid puncturing or otherwise damaging the battery.
***Troubleshooting**

28) Break one pin off of the straight 3 pin header included with the 12V step-up converter (ID-25), and push the short end into the 5V and GND holes from the top of the 5V step up (ID-24). Solder in place.

29) Push one end of the 7” red wire through the VOUT hole in the back of the 12V step-up (ID-25). Solder in place and trim excess.

30) Push the two header pins into the 12V step-up (ID-25) from the back, aligning the 5V with VIN and GND with GND between the 5V step-up (ID-24) and 12V step-up (ID-25), respectively. Solder both in place and trim excess.

31) Push one end of the 7” black wire through the GND hole in the 5V step-up (ID-24) from the back. Solder in place and trim excess. Optionally, at this point you can test the exposed red and black wires for 12V with a multimeter when the battery is plugged in.

32) Screw the 5V step-up (ID-24) into the top mirror housing (ID-a) using two M2 screws (ID-21) and 1.5 mm hex driver. The holes should self-thread with a little bit of force. Four screws can be used if desired, but the top two are sufficient.
***Troubleshooting**

33) Tuck the wires connecting the wireless router (ID-22) and step-up converters (ID-23 & ID-24) between the flanges (ID-2) that will sit beneath the mirror housing (ID-a) when assembled. Push the hanging ends of the 7” red and black wires through the hole in the center housing (ID-b) between the flanges.

34) Attach mirror housing (ID-a) (from step 5) to the center housing (ID-b) using four M3-6 screws (ID-20) and 2.5 mm hex driver. The step-up converters and the wireless router should be on opposite sides of the housing.

35) Plug the ethernet cable (ID-26) into the wireless router (ID-22), stow cable behind the wireless router, and screw it to the housing (ID-b) with three M2 screws (ID-21) and 1.5mm hex driver.

36) Unscrew the plug end of the Hirose connector (ID-27) from the connector housing. Attach the open ends of the black and red wires to the correct terminals on the 6-pin Hirose connector (ID-27) and solder in place. When looking at the connector side, the positive and negative terminals are the first clockwise and counter-clockwise positions (respectively) from the thickest metal tab. A large dab of hot glue can help secure these after soldering.
***Critical Step** – these connections are the most difficult to solder and may take some practice.

37) Plug the Hirose connector (ID-27) and the ethernet cable (ID-26) into camera and stow the wires as best as possible.
***Troubleshooting**

38) Place the lithium polymer battery (ID-28) and coin battery (ID-29) into their compartments in base housing (ID-c). Correct placement of coin battery (ID-29) will illuminate the LED (ID-18). The lithium polymer battery (ID-28) gets plugged into the 5V step-up (ID-24) to power the main electronics. Correct power should turn on indicator lights on the 5V step-up, the wireless router, and the camera. Attach the base (ID-c) to the rest of the CFM and the build is complete.
***Troubleshooting**

Set counterbalance:
***Note** – this section can be skipped if an identical duplicate CFM module is used as a counterbalance
Timing 15 min

39) Weigh the constructed CFM module. For reference, our “as built” model has a mass of 374.7 grams. If the instructions and parts list were followed exactly, the build should be within a few grams of this ~375 gram weight.
40) Place the counterbalance on a scale and add US pennies (or similarly sized and weighted coin) to the scale until the mass is as close as possible to the CFM mass. For our build, we used 96 pennies to reach 375 grams.
    ***Critical step** – matching the mass is one important part of making sure the centrifuge is well balanced.
41) On a flat surface, use the constructed CFM and balance sideways on a thin edge or cylindrical object (a ruler edge, or dowel or pen all work fine) until you find the approximate center of mass in the Z direction. Mark this location on the housing with pen or marker. For reference, our “as built” model has a center of mass ~67 mm from the bottom surface.
42) On the counterbalance housing, mark the target center of mass based on the results from the previous step (~67 mm from the bottom in our case). Distribute the pennies among the 7 compartments of the counterbalance to match the center of mass of the CFM. For our build, we used 14 pennies in each of the 3 shallowest compartments, 19 pennies each in the next 2 shallowest compartments, and 8 pennies each in the 2 deepest compartments. Temporarily secure the pennies in place with tape or glue and repeat the center of mass measurement from step 41 for the loaded counterbalance. Note if the measured center of mass aligns with the desired center of mass.
43) Make fine adjustments. If the measured center of mass was higher than the target, the mass should be lowered by rearranging pennies from higher to lower positions. If the measured center of mass was lower than the target, the mass should be raised by rearranging pennies from lower to higher positions. Repeat as necessary until the measured and target centers of mass are within 1-2 mm of each other.
    ***Critical step** - This step matches the center of mass and finalizes the counterbalance. An improperly balanced centrifuge can be dangerous. For significant design alterations or greater adjustment capabilities, we include an optional adjustable counterweight design described in Supplementary Note 3.

Setup and test run:
Timing 30 min

44) Set the router switch to the access point position nearest the ethernet port. Power up the CFM by connecting the battery and ensure that lights on the router board illuminate.
45) Connect the router to the PC and configure the router. First disconnect any existing ethernet connections and then connect the router to the PC by ethernet cable. Open a web browser and navigate to tplinkwifi.net. The login screen will either ask for login and password (“admin” without quotes is default for both), or you will be prompted to create a new password. Navigate to the operation mode tab and adjust the router settings to “access point”. Navigate to the wireless 2.4GHz tab and disable the wireless 2.4 GHz. Click “save” to finish and then log out of the router and unplug the ethernet connection.
    ***Troubleshooting**
46) Setup the wireless connection with the router. Find the Wi-Fi signal for your router, labeled “TP-Link_XXXX_5G”, where XXXX designates your specific router. This can be found through the network icon on the taskbar. Select the correct connection and enter the password (recorded in step 23 and found on the router housing). Once connected, bridge the wireless and ethernet connections by navigating to Settings → Network and Internet → Network and Sharing Center

→ Change Adapter Settings. Select both the "Ethernet" and "Wi-Fi" icons (holding ctrl for the second selection) and choose "Bridge Connections" from the right-click dropdown list.
***Critical step** - The ethernet connection used for the bridge should be free/open and not connected to the internet or a local network. A second dedicated ethernet card can be used on the computer to allow connection with CFM and another network.

47) Setup the camera. Open NI MAX and navigate to My System → Devices and Interfaces → Network Devices. The camera should appear here. Select the camera and use the camera attributes tab at the bottom to setup the camera parameters. A more complete discussion of camera parameters and WiFi bandwidth is provided in Supplementary Note 4 Important parameters are listed below and our complete parameter file is included in the Supplementary Files:
    - AcquisitionAttributes: PacketSize: 1500 bytes
    - AcquisitionAttributes: ReceiveTimestampMode: System Time
    - CameraAttributes: ExposureAuto: Off
    - CameraAttributes: AcquisitionFrameRate: 1 (Maximum frame rate depends on frame size and bit depth, 1 fps is a good starting point for testing)
    - CameraAttributes: AcquisitionFrameRateEnable: True
    - CameraAttributes: AnalogControl: GainAuto: Off

    Save these settings in NI MAX.
    ***Troubleshooting**
48) Use the play button in NI MAX to initiate an image grab, to make sure camera is working and live images are transferred. This live feed can be kept open for the subsequent testing steps.
49) Prepare the centrifuge. Ensure that the centrifuge is on a stable bench in a safe location, and with a computer nearby. Position the computer's Wi-Fi antenna over the central window on top of the centrifuge lid and secure with tape.
50) Load the CFM module and its properly arranged counterbalance (from step 43) in opposing buckets. Keep the other two empty buckets in the centrifuge. Swing the buckets manually by 90 degrees to ensure there is clearance and no obstructions. Close the lid.
    ***Critical step** – any obstructions or incorrect loading can cause damage. The tab on the CFM housing must align properly in the bucket for proper seating of the CFM.
51) Turn on the centrifuge. For the first time, it is recommended to start at the lowest speed (300 rpm) with lowest acceleration (1) and fastest deceleration (9). If everything is smooth, keep increasing speed up to 1500 RPM in 100 RPM steps. Check that the live feed is active in NI MAX. The centrifuge shouldn't vibrate any more than it would for well-balanced centrifuge tubes, if the counterbalance was prepared correctly.
    ***Troubleshooting**
52) Stop the centrifuge and remove the CFM module. Disconnect the batteries (and optionally recharge) and remove the chamber mount to prepare for experiment.

Prepare DNA constructs (Figure 4b)
Timing 1.5 h

53) In a PCR tube, combine 5 µL of stock M13mp18 ssDNA, 2.5 µL of NEB 10X CutSmart buffer, 1 µL of 100 µM cut site oligonucleotide, and 16.5 µL of nuclease free water.
54) Place tube in thermal cycler and run a linearization program: 95˚C for 30 seconds, 50˚C for 15 minutes, 95˚C for 1 minute, ending on a 4˚C hold.
55) When the program reaches 50˚C, add 1 µL of the NEB BtsCI (20 U/µL) enzyme. Mix well by pipetting 10 µL up and down a few times and return to thermal cycler for completion.
    ***Critical step** – Incomplete mixing is a common mistake that can result in incomplete linearization.
    ***Pause point** – this linearized M13 can be stored frozen for at least 1 month.

56) Make the total mix for tether “A” by mixing 2.5 μL of the backbone mix (Reagent 2), 0.3 μL of 100 μM “OH-A” oligonucleotide, and 1 μL of 100 μM “A: G-T” oligonucleotide.
57) Make the total mix for tether “B” by mixing 2.5 μL of the backbone mix (Reagent 2), 0.3 μL of 100 μM “OH-T” oligonucleotide, and 1 μL of 100 μM “T: G-T” oligonucleotide.
58) In two separate PCR tubes labeled “DNA A” and “DNA B”, combine 5 μL of the linearized ssDNA (from step 55) with 1.2 μL of the total mix for either tether “A” or “B”.
59) Place both tubes in the thermal cycler and run the annealing protocol: 90˚C to 30˚C cooled by 1˚C per minute, followed by hold at 12˚C.
***Pause point** – This can be stored at 4˚C for 1-2 weeks.

Attach DNA construct “A” to beads (Figure 4c)
Timing 30 min

60) Prepare a working solution of DNA “A” by mixing 1μL of DNA “A” (from step 59) with 39 μL of PBST (Reagent 1).
61) In a 1.5 mL tube, add 20 μL of well mixed dynabeads.
62) Place the tube in the magnetic holder and wait until pellet forms and liquid clears. Using a micropipette, remove and dispose of supernatant without disturbing beads.
***Critical step** – careful and complete washing of beads is important for moving excess reagents that can compete with DNA attachment and tether formation in subsequent parts of the protocol.
63) Remove from magnet, add 100 μL of 1X PBST and mix well.
64) Repeat wash (steps 62-63) twice more (three washes total) to remove any free streptavidin.
65) Resuspend in 40 μL of PBST and add 40 μL of diluted DNA “A” from step 60.
66) Incubate with agitation on a vortex mixer at 1000 rpm for 20 minutes.
***Critical step** – beads should not settle out of solution or reactions will be inefficient or cause non-uniform bead coatings. Rotating end over end also works as an alternative to vortexing. The tween in PBST is important for preventing sticking to the surface of the tubes.
67) Wash beads three times (steps 62-63).
68) Resuspend in 40 μL of 1X PBST. These can be stored at 4˚C for up to a week.

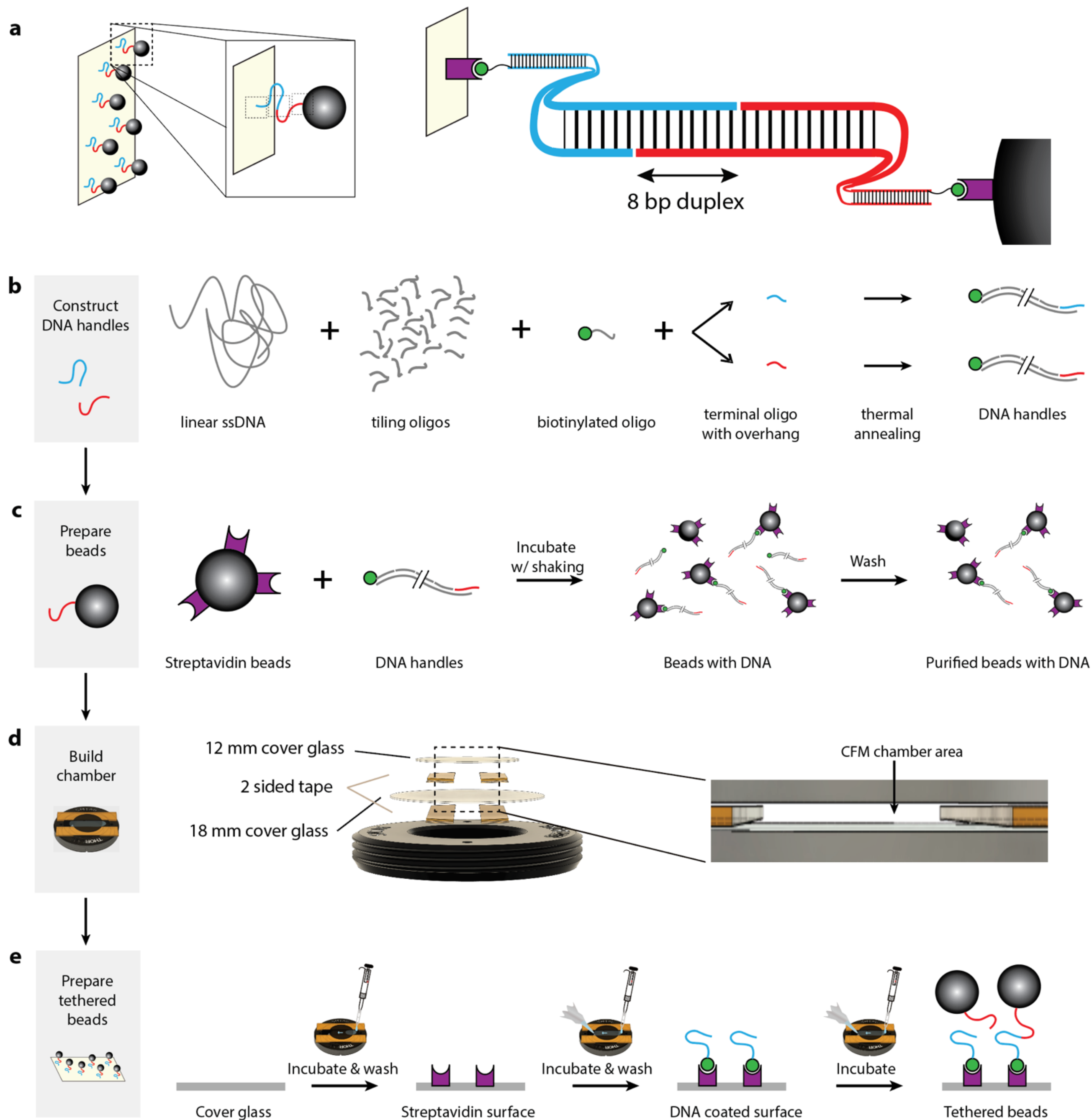

Figure 4: Preparing materials for a CFM experiment. A) Cartoon showing the desired outcome of beads tethered to a surface, with a zoom in cartoon showing the 8bp duplex being probed, as well as the biotinylated ends anchoring the tethers to streptavidin surfaces. B) DNA handles are constructed from DNA self-assembly of a genomic ssDNA with a cocktail of hybridizing oligonucleotides. C) DNA handles are bound to beads. D) The experimental chamber is built from sandwiching two cover glasses with double stick tape. E) Beads are tethered to the surface after coating with streptavidin and DNA handles.

Prepare and load the chamber (Figure 4d-e)
Timing 30 min

69) Cut 4 strips of the double-sided Kapton tape to a length of ~ 20 mm.

70) Stick 2 parallel strips of tape to the chamber platform (ID-9), roughly 1/8” (~3 mm) separated with the tightening holes on the platform exposed in the center (refer to Figure 4d). If tape extends beyond the circular platform, trim back with precision knife. Remove the solid backing on the top side of the tape.
71) Center a clean, dust-free 18 mm cover glass (air dusting or gently wiping with lint-free tissue) and press to adhere, without leaving residue on the glass surface. We typically use tweezers to help hold, position, and adhere the cover glass.
72) Repeat step 70 with a second layer of tape positioned similarly.
73) Center a dust-free 12 mm cover glass and press to adhere, without leaving residue on the glass surface.
***Pause point** – Dry chambers can be prepared and stored at room temperature. We often make many chambers at a time to prepare for several experiments.
74) Add the streptavidin working solution (Reagent 3) to the chamber by placing a 5 μL drop at the edge. The solution will travel by capillary action to occupy the whole chamber. Incubate for 1 minute.
75) Wash the chamber 3x with 100 μL of 1x PBST by pipetting slowly on one side while wicking excess solution with a lab wipe on the other.
***Critical step** – make sure bubbles are not formed and that the sample does not dry out.
76) Prepare a working solution of DNA “B” by mixing 1μL of DNA “B” (from step 59) with 39 μL of PBST (Reagent 1).
77) Add 5 μL of the diluted DNA construct “B” (from above step) to the chamber and incubate for 10 minutes.
78) Repeat chamber washing 3x with 100 μL 1x PBST (step 75).
79) Load 5 μL of the bead mixture from step 68.
80) Seal the chamber by applying a small dab of vacuum grease to both entrances. We use a plastic pipette tip as an applicator.
***Critical step** – Improperly sealed chamber can cause bubbles or leakage during centrifugation. It can take some practice to seal chambers effectively.
81) Screw the chamber mount into the SM1-05” tube (ID-7) until it stops. Finger tighten.
82) Set a timer for 10 minutes and invert the chamber assembly to allow beads to make contact and form tethers with the 12 mm cover glass.
***Note** that steps 83-87 will be completed during this incubation time.

Prepare and run the CFM
Timing 1h

83) Connect the CFM to the battery and wait for the router to turn on. Connect the PC to the wireless network “TP-Link_XXXX_5G”, where XXXX designates your specific router. Open NIMAX and check that the camera appears, and that it has the desired settings (from step 47 or as you prefer to configure). Save the settings and close NIMAX. These settings will be carried over into the Labview control program.
84) Open the Labview control program (“CFM control.vi”). Navigate to the wiring diagram and select the camera. On the front panel, set the file path for saving the images. Press the play button to start the program, flip the front panel switch to turn the camera on. Images from camera should appear on the screen.
***Troubleshooting**
85) Invert the CFM so that the objective tip faces upward (use our optional stand – see Supplementary Files). Screw the entire chamber assembly onto the end of the objective housing until the beads come into focus. Good overhead lighting or a task light can help for this step. Secure the focus position with the locking ring on the external coupler (ID-6).

***Critical step** – Be careful to not contact the cover glass to the objective, which can damage the chamber or the objective and get vacuum grease on the optics. Proper focus takes some practice, and some slight shifting may occur during run.
***Troubleshooting**

86) Place the coin cell battery (ID-29) for the LED (ID-18) in the slot provided in the CFM base (ID-c). The LED lamp should illuminate.
87) Once the 10 minutes has elapsed from step 82, attach the base to CFM and place the lithium-ion battery in its holder and place it in the centrifuge bucket. Ensure the already configured counterbalance is in the opposing bucket and that the other two buckets are in place but empty.
***Critical step** – double check that buckets can rotate freely without obstruction.
88) Push the "record" button to initiate saving of images. The timing of this and the next step can be altered depending on when you want to start the recording with respect to the centrifuge starting.
89) On the front panel of the centrifuge, set the desired RPM and run time (see Supplementary Note 1 for more information about selecting the force). For this protocol, the rotation radius is 119 mm, and the beads are 2.8 μm diameter with 1.6 g/cm^3 density. To achieve forces of 5, 10, 15, and 20 pN would require speeds of 745, 1054, 1290, and 1491 RPM, respectively. Based on the previously measured lifetimes of the interaction, a run time of ~1 hour should ensure most or all of the interactions have dissociated. Close the lid and start the centrifuge.
***Troubleshooting**
90) Bask in the glory of your first CFM experiment. Become entranced by the beads disappearing from view as unseen molecular tethers release their grasp and reveal their biophysical secrets.
91) To stop the experiment, the stop buttons in Labview and on the centrifuge can be pressed to stop recording and spinning, respectively. It is also okay to let it go until the CFM battery dies; the data will be preserved but an error in Labview will come up at the end.

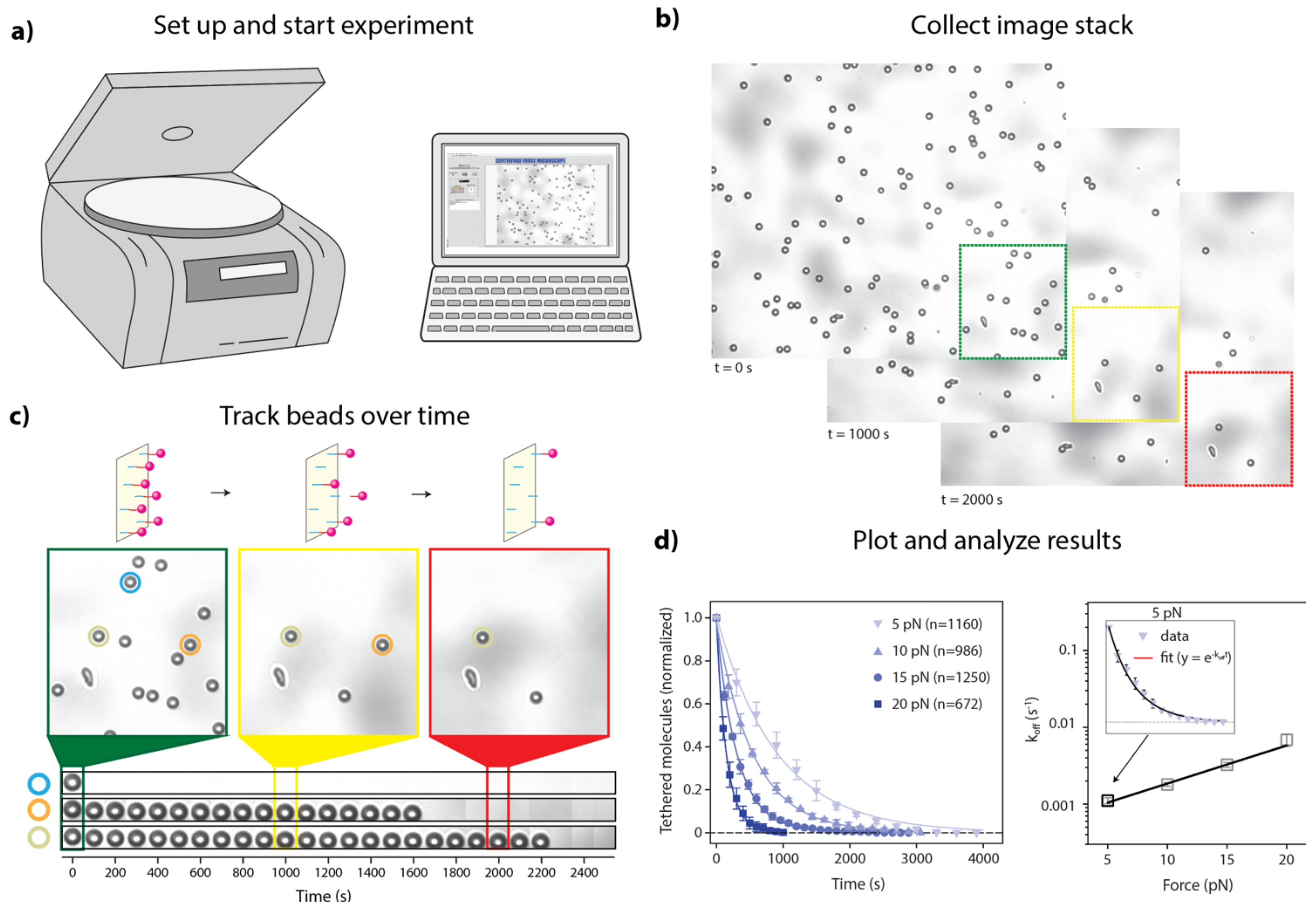

Figure 5 – Running the experiment and analyzing data. A) Initiate the experiment using computer control and the front panel of the centrifuge. B) Individual images will be streamed and saved at the specified frame rate. C) Our MATLAB analysis program aids in identifying and tracking tethered beads over time. D) Bead rupture times are collected and plotted to determine underlying biophysical properties.

Data Analysis

Timing ~1h

92) On the PC, open our analysis program on MATLAB (“CFM dissociation analysis.m”). The code is divided into four sections which can be run in sections.
***Troubleshooting**
93) Run the first section of code to load images. The user will be prompted to choose the folder containing the data and input the first and last frames of analysis. The first frame is typically after full speed has been reached, and this must be identified from scrolling through the first few frames or by noting the time to reach full speed.
94) Run the second section of code to identify beads. The program will show the automatically found beads using different sensitivity values for the algorithm. The user is prompted to enter a sensitivity value for further analysis. The user is prompted to select a reference bead, which is done by drawing a rectangular box around a bead which is putatively tethered and selecting “crop”. The program will automatically identify the clustered beads and the ones close to the edge of the frame as bad beads. Further, the program will then go through all the beads found in the circle finding algorithm and prompt the user to identify each bead as “good” or “bad”. Good beads should look similar to the reference bead and be isolated from other beads. These will be included in the analysis. Bad beads can consist of obvious non-beads (dirt, etc.), beads which

are significantly different in size, beads in the wrong focus, or beads with other flaws. These beads will be excluded from analysis.

95) Run the third section of code to track beads over time. The user will be prompted to enter the desired time resolution for analysis, which essentially bins the data.

96) Run the fourth section of code to calculate the dissociation times. This code will produce an array of all the dissociation times for the run. There are two error codes which can also occur: “100000” which means the bead did not dissociate, and “100001” which means that it was indeterminate and requires manual checking. Usually the 100001 error occurs due to either shifting of a bead in the region of interest, or dropped frames or frame segments in the video transmission.
***Troubleshooting**

97) Post-process the data based on your experimental needs. Typically, we use MATLAB or other software such as OriginLab to further analyze the dissociation times to determine an off rate. It is typical to plot histograms of the data and then fit the results with an exponential decay curve (Figure 5d). For multiple forces, off-rates plotted on a log scale typically result in a linear relationship, which can be used to extrapolate to the zero-force condition and to determine the force sensitivity of particular interactions.

Table 4 – Troubleshooting

Step	Issue	Common causes	Possible fixes
3	3D printed parts don’t come out as advertised	Uncalibrated printer Obstruction Old or bad filament Printer heating issues Printer spec variation	Recalibrate printer according to manufacturer, run test print. Ensure a clean and flat printing surface Use new and high-quality filament verified for your printer Ensure nozzle and bed heating and fans are working Try a different printer or printer setting.
4	3D printed holes don’t print well	Printer or filament variation	Adjust hole sizes in Fusion 360 and try again, or holes can be hand drilled instead.
27	Router not powering on	Bad soldering connection Wrong polarity Electrical short	Check polarity to make sure wires weren’t swapped Resolder connections. Trim excess wires. Dab hot glue if necessary to insulate or hold connections.
32	Can’t screw into the 3D printed holes	Imperfect print Lopsided or obstructed holes	If the holes are not well cleared, an Xacto knife can be used to further clean the hole, or a paper clip or small drill bit can be used to round out the hole by hand. Glue also works for extra security if screws aren’t catching properly.
37	Wires to camera are in the way	Camera orientation	The camera can be reoriented in 90-degree increments by unscrewing the flange, rotating the camera assembly, and reattaching. This can help with wire management. Tape, glue, or small zipties can also be used to hold wires wherever convenient. If the ethernet cable is obstructing the base, the rubber strain relief ribbing near the plug can be trimmed back with diagonal cutters.
38	Camera not powering on	Bad soldering connection Wrong polarity Electrical short	Check polarity to make sure wires weren’t swapped Resolder connections Trim excess wires Dab hot glue if necessary to insulate or hold connections
45	Can’t connect to router setup	Competing connection	Disconnect all other ethernet connections and turn off any wireless connections from the computer. Power cycle the router and the computer and try again.
47	Camera not connecting	IP configuration issues	First, try re-opening NI MAX and/or rebooting your computer which often resolves this issue. If the camera is still not discovered in NI MAX, you can force IP configuration using your camera SDK where the router and the camera will share the same IP and thereby

			establish a virtual Ethernet connection between camera and the computer.
51	Loud or vibrating centrifuge	Poor balancing Physical obstruction of buckets	Rebalance the CFM. Make sure buckets can rotate freely.
84	Error in LabView	Camera connection issue Software issue	Close Labview completely. Check that the camera is connected and visible in NIMAX. Make sure to close NIMAX, and restart LabView and retry the program.
85	No beads visible or low image quality	Focus is off Not enough light No beads in chamber Vacuum grease on objective Dirty optics Bubble in chamber	Unscrew the chamber assembly. Remake the chamber if there is suspicion of not enough beads or is a bubble is present. Inspect and clean the optics. Without the chamber assembly, make sure the images are light enough or adjust exposure time or overhead lighting to improve. Slowly remount the chamber assembly until beads come into focus.
85	Can't secure focus properly	Focal shift during tightening locking ring	This takes a little practice; the focus may shift slightly during tightening and so you have to readjust and account for that slight movement until you lock the focus at the right spot.
89	Shifting images during centrifugation	Optical components and/or lens tubes loose	Retighten all components, paying close attention to objective, sample holder, mirrors, and camera.
89	Beads are stuck	Too many tethers Focussed on wrong surface	Check to ensure you are not focused on the back side of the chamber. Perform serial dilutions of constructs to identify ideal conditions, then prepare fresh chamber.
89	Beads don't form tethers	Beads appear in CFM but immediately dissociate upon inversion/centrifugation.	Perform serial dilutions of constructs to identify ideal conditions, then prepare fresh chamber.
89	My beads abruptly disappeared	Bubble in chamber	Remake chamber and be sure that vacuum grease fully seals the chamber.
89	LED burning out	Bad LED or too much current	Replace LED and ensure coin cell is 3V and not 3.7V. Optionally solder 20 Ω resistor in series to LED to limit forward current.
92	Analysis software can't load files	File or pathname issues	Make sure to use a Windows PC (not Mac).
96	Many beads have error code 100001	Too many dropped pixels Shifting bead positions	Analysis of beads can be done manually if there are many dropped frames, using the bead kymographs that are produced. Bead shifting may be reduced by making sure all physical connections are rigid and don't allow movement.

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