

# Traction Force Microscopy in 3-Dimensional Extracellular Matrix Networks

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Cell migration through a three-dimensional (3-D) matrix depends strongly on the ability of cells to generate traction forces. To overcome the steric hindrance of the matrix, cells need to generate sufficiently high traction forces but also need to distribute these forces spatially in a migration-promoting way. This unit describes a protocol to measure spatial maps of cell traction forces in 3-D biopolymer networks such as collagen, fibrin, or Matrigel. Traction forces are computed from the relationship between measured force-induced matrix deformations surrounding the cell and the known mechanical properties of the matrix. The method does not rely on knowledge of the cell surface coordinates and takes nonlinear mechanical properties of the matrix into account. © 2017 by John Wiley & Sons, Inc.

Keywords: biopolymer networks • finite elements • traction force microscopy • unconstrained force reconstruction • 3-D cell traction forces

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## INTRODUCTION

This unit describes a method for measuring cell traction forces in physiologically relevant three-dimensional (3-D) biopolymer networks with highly nonlinear mechanical properties, such as reconstituted collagen gels. Most cells that are able to adhere, spread, and migrate onto a 2-D extracellular matrix (ECM) can also adhere, change shape, and migrate when embedded in a biopolymer network of suitable adhesiveness, stiffness, and network porosity. In contrast to a 2-D matrix, however, a 3-D matrix imposes a steric hindrance against shape changes and movements of the cell. To overcome this steric hindrance, the cell needs to generate forces. Studying these forces is therefore important for a mechanistic understanding of many physiological and pathophysiological cell functions in health and disease that involve cell adhesion, shape changes, and migration, such as tissue formation during embryogenesis, tumor cell invasion in tissue, or the homing of immune cells. Furthermore, quantifying these forces is important for understanding the function of muscle cells and other highly contractile cells, such as fibroblasts or blood platelets.

The concept of traction force microscopy (TFM) was first developed for 2-D cell culture conditions (Butler, Tolic-Norrelykke, Fabry, & Fredberg, 2002; Sabass, Gardel, Waterman, & Schwarz, 2008) whereby cells are grown on natural or synthetic optically transparent hydrogels, such as polyacrylamide-bisacrylamide hydrogels, in which fluorescent beads are embedded. The displacement field of the hydrogel caused by cell



tractions is quantified by tracking the positions of the embedded beads before and after the cell tractions are relaxed with a drug cocktail containing trypsin and/or high concentrations of actin-disrupting drugs such as cytochalasin D. Once the matrix displacements are known, the matrix strain field can be computed. Given the known mechanical properties of the matrix, an inverse algorithm is then used to compute the stress field within the hydrogel (Butler et al., 2002; Franck, Maskarinec, Tirrell, & Ravichandran, 2011; Palacio et al., 2013). Besides knowledge of the matrix mechanical properties, most algorithms are constrained in the sense that they allow the reconstructed forces to appear only at predefined locations, e.g., at focal adhesion sites (Sabass et al., 2008) or at the cell surface (Dembo & Wang, 1999; Legant et al., 2010). The concept of TFM can also be extended to 3-D measurements (Legant et al., 2010).

Previous TFM methods have relied on a linear stress-strain response of the matrix and assume affine matrix deformations, meaning that a uniform macroscopic stress gives rise to a uniform microscopic strain. However, the mechanical response of some biopolymer networks is more complex. For example, collagen gels stiffen under shear stress and collapse with an abnormal apparent Poisson's ratio greater than 1 when stretched (Arevalo, Urbach, & Blair, 2010; Licup et al., 2015; Steinwachs et al., 2016; Vader, Kabla, Weitz, & Mahadevan, 2009). The method described here takes into account the nonlinearity as well as the non-affine behavior of collagen gels or other biopolymer networks. Moreover, the method is unconstrained and therefore solves the inverse problem of computing the tractions from the matrix displacements without knowledge of the cell geometry (although the user can also constrain the reconstructed forces for cases where the precise 3-D coordinates of the cell surface are known).

To quantify the traction force-induced deformations of the biopolymer network, the network fibers are imaged using an upright reflection confocal reflection microscope with a dip-in water-immersion objective. Alternatively, the network deformations can be imaged with confocal or conventional fluorescence microscopy using matrix-embedded fluorescent beads that serve as fiducial markers. One image stack or a series of time-lapse image stacks are recorded before cell traction forces are relaxed with cytochalasin D. A second image stack is recorded 30 min after cytochalasin D addition. This second image stack represents the undeformed, force-free configuration of the matrix. The displacement field of the matrix fibers between the deformed and the undeformed image stack is obtained by 3-D particle image velocimetry. The matrix volume is subdivided into a mesh of small finite elements of tetrahedral shape. For each tetrahedron, the constitutive equation that describes the relationship between the forces at the four nodes of the tetrahedron and the stresses and deformations of the four faces are computed numerically. An iterative method is then used to modify the nodal forces of all finite elements until the measured and calculated matrix displacements match. In addition to the cell forces, this method also allows the user to compute the principal matrix stress as well as the principal matrix stiffness.

The unit comprises four basic protocols. Basic Protocol 1 explains the preparation of type I collagen gels with different collagen concentrations, as well as the cell seeding procedure. Basic Protocol 2 describes the technique for performing confocal reflection microscopy of the collagen fibers for obtaining the displacement field of the collagen gel. Basic Protocol 3 is a guide to the force-reconstruction software. In the following, this software is referred to as SAENO (semi-affine elastic network optimizer). Basic Protocol 4 explains how the nonlinear material parameters of the 3-D hydrogel are measured.

The source code of the algorithm can be downloaded free of charge under the MIT license on the collaborative coding platform GitHub (<https://github.com/Tschaul/SAENO>). Moreover, a compiled version of the software and a tutorial are provided as

Supplementary Software (Steinwachs et al., 2016), together with a sample data set (<http://lpmt.biomed.uni-erlangen.de/3DTractions/SampleData.rar>).

## PREPARATION OF TYPE I COLLAGEN GELS WITH CELLS

This section describes how to embed cells in a collagen type I hydrogel with a final concentration of 2.4 mg/ml. The protocol for producing collagen gels as described here was developed in the 1990s by Jürgen Behrens (University Clinics Erlangen, Germany, unpub. observ.). He found that a combination of rat and bovine collagen produces gels that are stiffer compared to gels polymerized from pure calf skin or rat tail collagen: Young's moduli of mixed gels are between 45 Pa (for 0.3 mg/ml gels) and 550 Pa (for 2.4 mg/ml gels), compared to pure calf skin collagen gels [1 to 13 Pa for concentrations ranging from 0.5 to 1.5 mg/ml (Yang & Kaufman, 2009)] or pure rat-tail collagen gels [5 to 60 Pa for concentrations ranging from 1 to 4 mg/ml (Miron-Mendoza, Seemann, & Grinnell, 2010)].

This protocol can be adapted to prepare collagen hydrogels of different concentrations by diluting the base solution before polymerization. The optimal cell seeding concentration is 15,000 cells per ml of collagen gel, to ensure sufficient free space between neighboring cells.

To prevent the uncontrolled polymerization of the collagen hydrogels, it is crucial to maintain all reagents, pipet tips, and Petri dishes at a temperature of 4°C during preparation.

**NOTE:** All solutions and equipment coming into contact with cells must be sterile.

### Materials

- T-75 (75-cm<sup>2</sup>) flask with cells of interest at 60% to 75% confluence
  - Complete cell culture medium, 37°C (depending the cell line): e.g., low-glucose DMEM (1g/liter D-glucose) with 10% FBS and 1% penicillin/streptomycin
  - 2 mg/ml, rat tail collagen (Collagen R; Matrix Bioscience)
  - 4 mg/ml bovine skin collagen (Collagen G; Matrix Bioscience)
  - 0.27 M NaHCO<sub>3</sub>: Dissolve 2.3 g NaHCO<sub>3</sub> (Merck KGaA) in 100 ml distilled H<sub>2</sub>O (store up to 1 year at 4°C)
  - 10× DMEM medium (Biochrom)
  - 1 M NaOH: Dissolve 4 g NaOH (Sigma) in 100 ml distilled H<sub>2</sub>O (store up to 1 year at 4°C)
  - Dulbecco's phosphate-buffered saline without Ca or Mg (Thermo Fisher Scientific, cat. no. 14190169)
  - 50-ml conical polypropylene centrifuge tubes
  - 35-mm Petri dishes
  - Aluminum or copper plate to support Petri dishes in ice bucket (~15 × 10 cm, see Fig. 10.22.1)
  - 37°C incubator, 95% relative humidity and 5% CO<sub>2</sub>
  - Cell culture tabletop centrifuge
  - Tissue culture phase-contrast microscope
- Additional reagents and equipment for cell culture (*UNIT 1.1*; Phelan & May, 2015)

### Prepare cell suspension

1. Prepare cells according to usual procedures for cell culture (*UNIT 1.1*; Phelan & May, 2015). Trypsinize, count, and disperse cells in complete cell culture medium (all techniques described in Phelan & May, 2015) at a concentration of 10<sup>6</sup> cells per ml (we will need 30 μl per Petri dish). Place a microcentrifuge tube with cells on wet ice.



**Figure 10.22.1** The reagents and the aluminum plate in an ice bucket.

### ***Preparing 3-D collagen gels and cell seeding***

2. Cool all reagents and a 50-ml conical polypropylene centrifuge tube for at least for 5 min on wet ice. Place as many 35-mm Petri dishes as needed onto a cooled aluminum or copper plate (which provides better mechanical stability than simply placing the Petri dish onto the ice, see Fig. 10.22.1).

*IMPORTANT NOTE: The following volumes and measures refer to the amounts needed for two Petri dishes containing 2 ml of collagen with a concentration of 2.4 mg/ml. Step 5 explains how to adjust the solution for other collagen concentrations.*

3. Pipet 1600  $\mu$ l of 2 mg/ml rat tail collagen and 1600  $\mu$ l of 4 mg/ml bovine skin collagen in a 50-ml conical polypropylene centrifuge tube (previously placed on wet ice). Mix the collagen solution slowly and gently without introducing air bubbles.

*It is recommended not to withdraw the tip of the pipet from the solution during the mixing process. Always keep the tube with the collagen solution on ice.*

4. Add 400  $\mu$ l of 0.36 M  $\text{NaHCO}_3$  and 400  $\mu$ l of 10 $\times$  DMEM medium. Gently mix the solution.
5. For final collagen concentrations of less than 2.4 mg/ml, dilute the solution with a mixture of 1 part (v/v) 0.36 M  $\text{NaHCO}_3$ , 1 part 10 $\times$  DMEM, and 8 parts distilled water.
6. Adjust the solution to pH 10 by adding 60  $\mu$ l of 1 M NaOH.

*It is possible that the amount of NaOH needs to be adjusted depending on the stock solution's pH. Hence, it is recommended to use pH test paper or a pH meter to measure the pH of the final solution. If less than 60  $\mu$ l NaOH is needed, make up the difference with distilled  $\text{H}_2\text{O}$ ; if more is needed, prepare a higher NaOH concentration.*

7. Pipet 60  $\mu$ l of a 15,000 cell/ml cell suspension into the collagen solution, and gently mix.

*Addition of NaOH and cell suspension will slightly reduce the collagen concentration of the final mixture (2.31 mg/ml in the case of 60  $\mu$ l NaOH and 60  $\mu$ l cell suspension).*

8. Pipet 2 ml of the cell-collagen solution into a 35-mm Petri dish to obtain a collagen thickness of approximately 2 mm.

*Sufficient thickness of the collagen sample is crucial for the 3-D cell traction force reconstruction, as it is important to find cells at a distance of at least 300  $\mu$ m from the bottom or top surface of the collagen gel.*

9. To initiate polymerization, quickly transfer the Petri dish with the cell-collagen solution to an incubator at 37°C with 95% relative humidity and 5%  $\text{CO}_2$  for 1 hr.

*It is advisable not to open the incubator during the polymerization time, to ensure constant temperature and humidity conditions for the sample. It is also possible to polymerize the collagen gel at different temperatures or with different temperature-time profiles, but this will inevitably alter the pore size and mechanical properties of the polymerized gel. Polymerizing the gels at lower temperatures leads to wider pores and a higher bulk stiffness (Yang & Kaufman, 2009).*

10. Gently pipet 2 ml of 37°C complete cell culture medium onto the polymerized collagen gel.

*It is important to pipet the cell culture medium at 37°C, taking care not to damage the gel surface or detach the gel. For all following steps, it is also crucial to handle the sample gently and carefully to avoid damage to the collagen gel.*

11. Incubate overnight.

*Incubation time depends on the cell type and the time needed to establish cell polarity and migration through 3-D collagen matrix.*

## CONFOCAL REFLECTION MICROSCOPY OF 3-D COLLAGEN GELS

This protocol describes how to configure the confocal microscope hardware and software for the acquisition of the two image stacks around selected cells. The first image stack, subsequently referred to as *stack-1*, corresponds to the deformed configuration of the matrix. The second image stack, subsequently referred to as *stack-2*, is recorded after cell traction forces have been ablated with a drug cocktail. Thus, *stack-2* corresponds to the force-free undeformed state of the matrix.

Voxel dimensions are 0.72  $\mu\text{m}$  horizontally in the  $x$  and  $y$  direction, and also 0.72  $\mu\text{m}$  vertically in the  $z$  direction. The individual fibers of the biopolymer network matrix around the cell are imaged in confocal reflection mode, which collects the back-scattered light from the fibers. No fluorescence staining of the matrix is needed in this imaging mode, and since the back-scattered light intensities are large even for small excitation laser intensities, a fast scanning speed without line or frame averaging can be selected. With typically 512  $z$  stacks and  $512 \times 512$  pixels in the  $x$ - $y$  plane, the acquisition time for one complete image stack is on the order of 4 to 5 min. If more than one cell is analyzed for each Petri dish, or if time-lapse image stacks are recorded, the use of an environmental enclosure or microscope incubation chamber is recommended to keep the cells at 37°C, 5%  $\text{CO}_2$ , and 95% relative humidity.

**NOTE:** For prolonged investigation times, all solutions and equipment coming into contact with cells should be sterile. If an environmental enclosure or microscope incubation chamber is used, it is advisable to turn on the temperature control system the day before the experiment to minimize stage drift.

### Materials

Ethanol

Cells within collagen gel in 35-mm Petri dishes (Basic Protocol 1)

1 mM cytochalasin D: dissolve 50 mg cytochalasin D (Sigma-Aldrich) in 100 ml dimethyl sulfoxide (DMSO; Sigma); store up to 2 years at  $-20^\circ\text{C}$

Complete cell culture medium, 37°C (depending the cell line): e.g., low-glucose DMEM (1g/liter D-glucose) with 10% FBS and 1% penicillin/streptomycin

Upright confocal microscope (Leica TCS SP1)

Lens paper

Argon laser 488 nm or other laser

Microscope environmental chamber or microscope stage incubator

## BASIC PROTOCOL 2

### Extracellular Matrix

## 10.22.5

20× dip-in water-immersion objective with a high numerical aperture (NA)  
Injection needles (optional)

### ***Mount samples for imaging***

1. At a time point 1 hr prior to the measurements and when the temperature inside the microscope incubation chamber has reached a steady state, turn on the CO<sub>2</sub> system as well as the microscope following the manufacturer's instructions.

*The sample should not be introduced into the microscope incubator chamber until the correct level of 5% CO<sub>2</sub> and temperature of 37°C is reached.*

2. Turn on the lasers for the measurements.

*For confocal reflection mode, a laser power of 1.5 mW (typically 3% of the maximum argon laser power at 488 nm) is sufficient.*

*Wait at least 30 min before starting the image acquisition to reach a stable laser output.*

3. Sterilize the dip-in objective lens with ethanol using lens paper.
4. Carefully place the sample onto the stage plate.

*Allow the sample to equilibrate to the microscope chamber temperature for 10 to 15 min.*

5. Remove the Petri dish lid, dip in the microscope objective, and focus onto a cell.

*The objective should be immersed in the cell culture medium at all times but must not touch the collagen gel surface, to prevent damage to the collagen gel.*

### ***Set up acquisition and scanning parameters for the reflection mode***

If the scanner settings are modified, the parameters of the algorithm (described in Basic Protocol 3) must be changed accordingly.

6. Adjust the laser power level of the required laser line.

*For reflection images of the collagen fibers, it is sufficient to use 3% (1.5 mW) of the total power of the 488 laser line.*

7. Select the appropriate optical zoom/magnification for an *x-y* pixel size of 720 nm.
8. Select a pinhole diameter of 0.75 Airy units or less.

*The optimum pinhole diameter depends on the magnification factor and the numerical aperture of the objective. It is important to define a proper pinhole size, as it determines the thickness of the optical sections and therefore the *z*-resolution.*

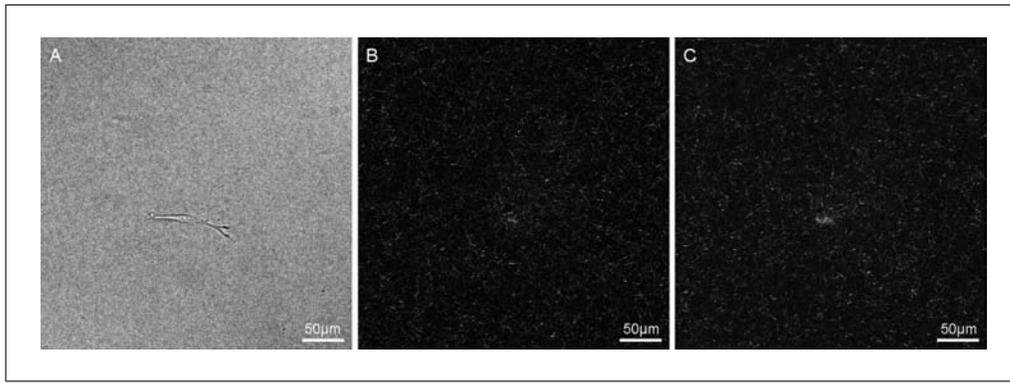
9. Select the *xyz* scan mode and scan frequency to optimize for short scan time and high signal-to-noise ratio.
10. Select an image resolution of 512 × 512 pixels, corresponding to a total imaged area of 370 μm × 370 μm.

### ***Set up the z-stack***

11. Select a stable and reproducible *z*-focus mode (e.g., objective piezo or stepper motor, but not a *z*-Galvo microscope stage).

*Verify the reproducibility of the *z*-focus of your microscope by repeatedly taking *z*-stacks of a calibration slide, e.g., consisting of fluorescent beads embedded in a polyacrylamide hydrogel.*

12. Select a *z*-stack distance (step size) equal to or as close as possible to the *x-y* pixel dimensions of 720 nm, and a total number of ~512 *z*-stacks, so that the total *z*-travel is equal to the *x-y* field of view.



**Figure 10.22.2** (A) Bright-field image of a MDA-MD-231 breast carcinoma cell migrating within the collagen gel. Confocal reflection images of the collagen fibers: (B) before and (C) after cell relaxation.

### **Select the cells to be analyzed**

13. Use bright-field illumination to locate the cells to be analyzed (Fig. 10.22.2A).

*The cell should be at least 300  $\mu\text{m}$  away from the top or bottom gel surface or from neighboring cells.*

*It is advisable to check that the hydrogel in the region of interest around the cell does not show any irregularities.*

14. Store the xyz center coordinates of all cells to be analyzed.

*To avoid very long image acquisition times, it is advisable not to select more than 10 cells per experiment, as the image acquisition takes around 5 min per cell for the configuration defined above.*

### **Acquire the first (pre-relaxation) z-stack**

15. Acquire the first z-stack (*stack-1*) of the collagen fibers for each selected cell.

*This first z-stack corresponds to the deformed state of matrix prior to force relaxation (Fig. 10.22.2B).*

### **Force relaxation**

16. Dilute 10  $\mu\text{l}$  of 1 mM cytochalasin D in 500  $\mu\text{l}$  of complete cell culture medium and add to the cell culture dish (final concentration will be 1.96  $\mu\text{M}$ ).

*It is important to pipet the solution without touching the sample or moving the dish. It is advisable to add the solution using an injection needle to prevent movement of the sample.*

17. Wait 30 min to ensure that the cell is completely relaxed.

### **Acquire the second (force-free) z-stack**

18. Scan the sample in live mode to check that the cells are in the same positions.

*If the sample was moved by more than approximately 50  $\mu\text{m}$ , the measurement cannot be used.*

19. Acquire the second z-stack (*stack-2*) of the collagen gel for each selected cell (Fig. 10.22.2C).

20. Export the two z-stacks (*stack-1* and *stack-2*) in uncompressed TIF format.

*Each image needs to be saved with a filename that includes the cell or position number, the z-plane, and the selected laser channel, e.g., Pos001\_z001\_ch00.tif. The image files of the stacks should match a common wildcard-based file filter (e.g., \*\_ch00.tif)*

in which the \* character is a wildcard that stands for any possible string. This file filter will be used later (in Basic Protocol 3) by the force-reconstruction software to recognize the confocal reflection images. Images from different time points should be stored in separate folders, e.g., `stack_alive` and `stack_relaxed`, or they can be simply named `stack_1` and `stack_2`.

### **UNCONSTRAINED 3-D FORCE RECONSTRUCTION**

This protocol describes how to run the unconstrained 3-D force reconstruction algorithm using the SAENO software. The input data for the algorithm are the two image stacks (*stack-1* and *stack-2*) as well as four parameters that describe the material properties of the collagen network. These four parameters are defined in a configuration file, as described below in more detail (see also Table 10.22.1 and Basic Protocol 4).

The software first calculates the displacement field of the collagen network between *stack-1* and *stack-2*. To do so, both image stacks are aligned to remove stage drift and z-focus drift. The alignment algorithm is based on rigid registration. Once the images are aligned, the program computes the matrix displacement field with particle image velocimetry. Next, the software performs an unconstrained force reconstruction algorithm to calculate the cell traction forces.

#### **Materials**

A compiled version of the SAENO software (<https://github.com/Tschaul/SAENO>)  
Matlab software V.R2012b or newer (older versions of Matlab may not work with SAENO)  
PC with Windows operating system

#### **Select the path and data files to be analyzed**

Supplementary information on the method, as well as a tutorial and source data files, are available in the online version of Steinwachs et al. (2016).

1. Open Matlab.
2. Add the path where SAENO is saved (see Fig. 10.22.3). To do this, in the HOME tab (a), click on Set Path (b). Then, click Add with Subfolders (c) and select the SAENO\_matlab directory where the compiled files are located (d).
3. Inside a parent data folder, place the two image stacks (*stack\_1* and *stack\_2*) of each cell into different folders (e.g., `cell1007_alive` and `cell1007_relaxed`). The two folder names belonging to the same cell must be the same except for an arbitrary suffix, e.g., `_alive` or `_relaxed`. Make sure that there is no file in these two folders other than the image files belonging to the same image stack, and that the folder names and the image file names do not contain white spaces; otherwise the program will not recognize them properly. The parent data folder structure should look as shown in Figure 10.22.4.

In the case of time-lapse measurement (multiple stacks per cell), the parent data folder structure should look as shown in Figure 10.22.5.

#### **Specify the material parameters**

4. Specify the four parameters that describe the material properties of the matrix in the `config.txt` file. A default `config.txt` file can be found in the SAENO\_matlab directory that can also be edited, but local copies of this `config.txt` file can be copied and stored at arbitrary locations. Open the file with a text editor (e.g., Notepad++) and modify the values for  $K_0$ ,  $d_0$ ,  $L_s$ , and  $d_s$  (lines 6

**Table 10.22.1** Table of parameters

Name	Value in the example config file	Description/ meaning
MODE	Regularization	Do a unconstrained force reconstruction
BEAMS	300	Number of fibers per tetrahedron
K_0	2000	Bulk modulus of the material in Pascal
D_0	0.00032	Buckling coefficient of the fibers
L_S	0.0075	Onset of strain stiffening of the fibers
D_S	0.0333	Strain stiffening coefficient of the fibers
DRIFTCORRECTION	1	Set to 1 to perform a drift correction. Set to 0 otherwise.
DRIFT_STEP	2.0e-06	Initial step width for the drift correction algorithm (in units of meters). Larger values speed up the drift correction but increase the risk that drifted stacks cannot be aligned.
DRIFT_RANGE	30.3e-06	Maximum search range (in units of meter) over which the drift correction is performed
ALIGNSTACKS	1	Set to 1 to perform a z-dependent drift correction (stack alignment). Set to 0 otherwise
FIBERPATTERNMATCHING	1	Set to 1 to run the module <code>extractDeformations</code> . Set to 0 to run the module <code>loadDeformations</code> .
SUBPIXEL	0.0005	Convergence criterion for the uncertainty of the fiber pattern matching, in units of voxels
VB_MINMATCH	0.7	Only sections of the fiber matrix are considered for force reconstructions when the image cross-correlation of matching fibers is larger than the value specified. Higher values exclude poorly matched sections.
VB_SX	12	Size of cross-correlated sections in x direction, in units of voxels.
VB_SY	12	Size of cross-correlated sections in y direction, in units of voxels.
VB_SZ	12	Size of cross-correlated sections in z direction, in units of voxels.
BOXMESH	1	Set to 1 to build a mesh. Set to 0 otherwise.
BM_GRAIN	7.5e-06	Grid constant of the regular cubic finite element mesh, in units of meters
BM_N	54	Number of nodes in every dimension (x, y, and z)

*continued***Extracellular Matrix****10.22.9**

**Table 10.22.1** Table of parameters, *continued*

Name	Value in the example config file	Description/ meaning
BM_MULOUT	1.2	Stretch factor. The outer (beyond a length specified under BM_RIN) are nodes stretched away from the center by this factor. The size of the volume over which forces are reconstructed is $(BM\_GRAIN \times BM\_N \times BM\_MULOUT)^3$
BM_RIN	160e-06	Only nodes that are farther away from the center than this value are stretched (in units of meters)
ALPHA	3.0e-09	Regularization parameter in units of square Newtons per square meter
REG_ITERATIONS	50	Maximal number of iterations
REG_CONV_CRIT	0.01	The algorithm stops if the relative standard deviation of the residuum in the last 6 iterations falls below this value
REG_SOLVER_STEP	0.33	Step width parameter of the regularization. A higher value leads to a faster but less robust convergence of the algorithm. For a more linear material (like Matrigel), you can increase this parameter up to a value of 1.0.
FR_RMAX	100e-06	Only forces that lie within this range (in units of meters) from the force epicenter are considered for the analysis of the cellular forces

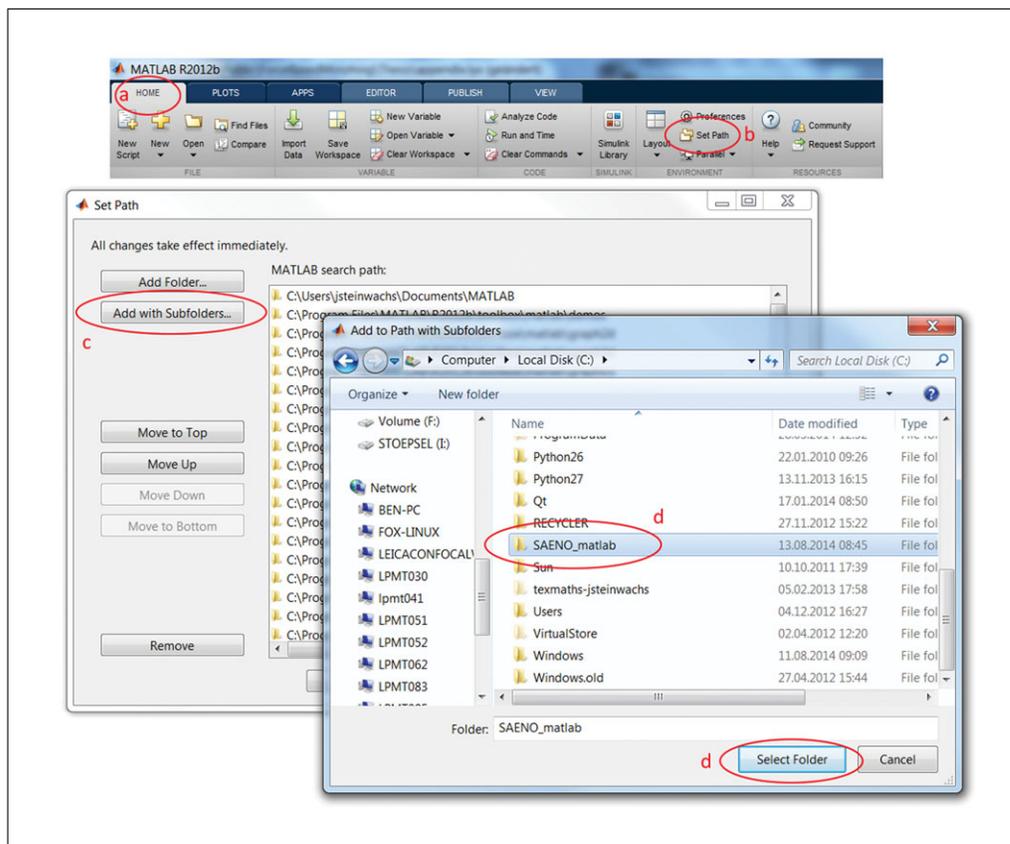
to 9). How these parameters can be obtained is described in Basic Protocol 4. The other parameters in the `config.txt` file should only be changed by expert users.

*For more information on how to adjust the different parameters in the `config.txt` file, see Table 10.22.1 as well as the GitHub documentation (<https://github.com/Tschaul/SAENO/wiki/Introduction>).*

#### **Run** SAENO\_setup

- Run the program `SAENO_setup` in the Matlab command window, as shown in Fig. 10.22.6A. This program opens the `SAENO_setup` window and allows the user to define the different parameters of the calculation.
- The first line shows by default the path of the `SAENO.exe` program (see Fig. 10.22.6B). If no path or an incorrect path is displayed, select the correct path where the `SAENO.exe` program is located.
- On the second line, specify the number of processes to run at the same time (see Fig. 10.22.6B). For instance, for a PC with four CPU cores, this value should be set to 4. This means that the traction force calculation of four cells will be performed in parallel.

*If the computer has more than four CPU cores, this number may be increased to run more processes at the same time, but consider that at least 1 GB of free RAM is needed for each process.*



**Figure 10.22.3** Adding the SAENO\_matlab directory to the Matlab path.

```

/Experiment_1 (parent data folder)
  /cell1001_alive
    Pos001_z001_ch00.tif
    Pos001_z002_ch00.tif
    Pos001_z003_ch00.tif
    ...
  /cell1001_relaxed
    Pos001_z001_ch00.tif
    Pos001_z002_ch00.tif
    Pos001_z003_ch00.tif
    ...
  /cell1002_alive
    Pos002_z001_ch00.tif
    Pos002_z002_ch00.tif
    Pos002_z003_ch00.tif
    ...
  /cell1002_relaxed
    Pos002_z001_ch00.tif
    Pos002_z002_ch00.tif
    Pos002_z003_ch00.tif
    ...
/etc.

```

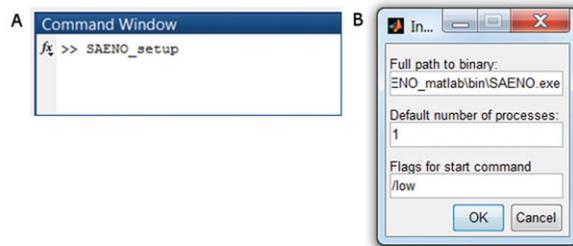
**Figure 10.22.4** Structure of parent data folder.

```

/Experiment_1 (parent data folder)
  /cell1001_alive
    /t000
      Pos001_z001_ch00.tif
      Pos001_z002_ch00.tif
      Pos001_z003_ch00.tif
      ...
    /t001
      Pos002_z001_ch00.tif
      Pos002_z002_ch00.tif
      Pos002_z003_ch00.tif
      ...
    /t002
      Pos003_z001_ch00.tif
      Pos003_z002_ch00.tif
      Pos003_z003_ch00.tif
      ...
  ...
  /cell1001_relaxed
    Pos004_z001_ch00.tif
    Pos004_z002_ch00.tif
    Pos004_z003_ch00.tif
  ...
/etc.

```

**Figure 10.22.5** Structure of parent data folder in case of time-lapse measurements.



**Figure 10.22.6** Running SAENO\_setup in Matlab command window (A) to open the setup window (B).

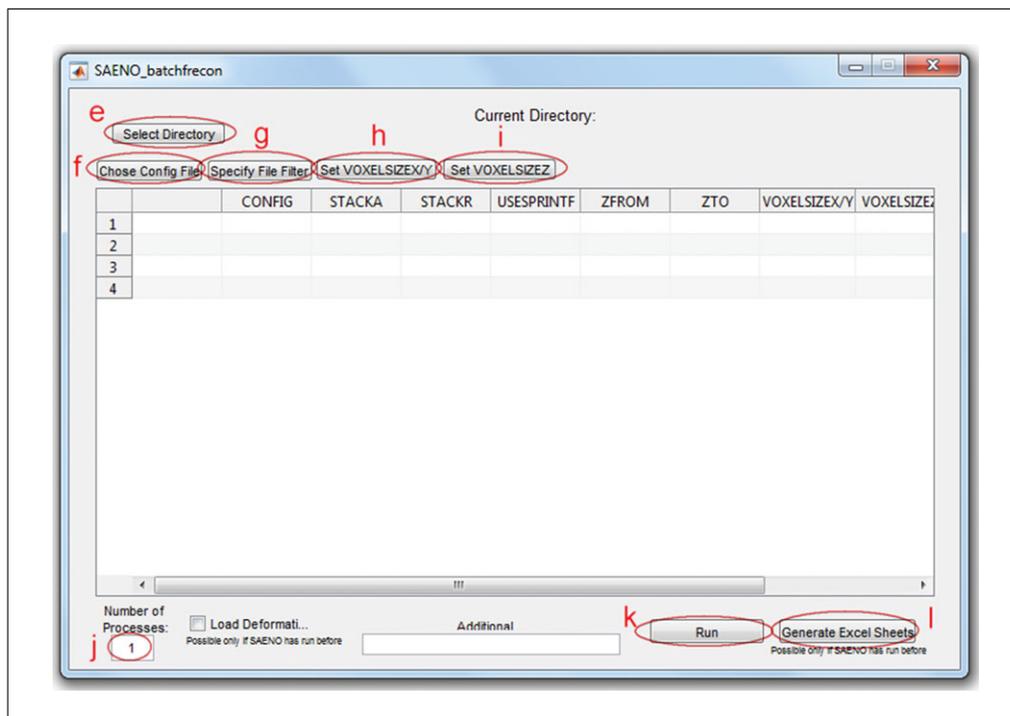


**Figure 10.22.7** Running SAENO\_batchfrecon in the Matlab command window.

8. On the third line, specify the priority of the traction forces reconstruction program (see Fig. 10.22.6B). If the '/low' option is selected, the reconstruction runs in the background so that other processes of the computer are not markedly slowed down. If the '/high' option is selected, the reconstruction has priority over other applications, which in turn will be markedly slowed down.

**Run** SAENO\_batchfrecon

9. Run the program SAENO\_batchfrecon in the Matlab command window, as shown in Fig. 10.22.7. This program opens the SAENO\_batchfrecon window



**Figure 10.22.8** The batch processing window.

(see Fig. 10.22.8) and allows for parallel batch processing of multiple cells or time-lapse image stacks of the same cell. The number of cells or time points that can be simultaneously analyzed corresponds to the number of processes selected in step 7.

10. Click on Select Directory ('e' in Fig. 10.22.8) to select the parent directory defined in step 3 that contains the data files. All subfolders in the parent directory will be processed.

*Check in the processes box ('j') that the number of processes coincide with the number of processes selected in the setup window (Fig. 10.22.6B). It is possible now to re-adjust the number of processes that will be launched in parallel. For the best performance, this number should match the number of cores of your CPU, as explained in step 7. If the parent directory contains more cells than the number of processes selected, SAENO will process the remaining cells once it has finished computing the first ones.*

11. Click on Choose Config File ('f' in Fig. 10.22.8) and select the `config.txt` file in which the calculation parameters are defined.
12. Click on Specify File Filter ('g' in Fig. 10.22.8) to specify the laser channel and file format of the individual files to be analyzed (e.g., `*_ch00.tif`; see Fig. 10.22.8). Do not forget to type `*_ch00.tif` in this example, where the character `*` is a so-called wildcard that stands for any string of characters.
13. Click on Set VOXELSIZE X/Y ('h' in Fig. 10.22.8) and Set VOXELSIZE Z ('i' in Fig. 10.22.8) to set the voxel size in the *x*, *y*, and *z* directions. This value must be in units of meters, i.e., for a size of  $0.72 \mu\text{m}$ , type `0.72e-06`.
14. Click on Run ('k' in Fig. 10.22.8) to start the batch processing (see Fig. 10.22.8). A number of command windows will open up automatically, depending to the number of parallel processes specified in step 7. No further action from the user is needed at this stage.

*Do not close the command windows until all cells have been analyzed.*

```
Command Window
fx >> SAENO_3dplot
```

Figure 10.22.9 Running SAENO\_3dplot in the Matlab command window.

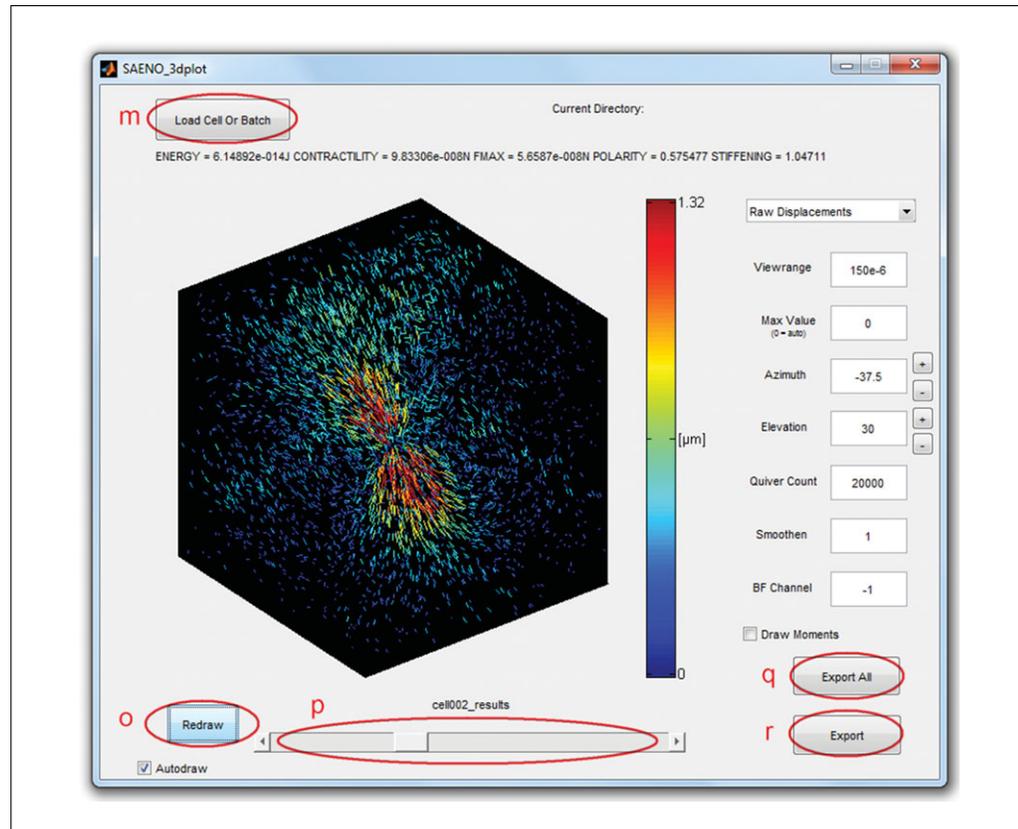


Figure 10.22.10 The 3-D plot window.

15. After all command windows have completed their tasks, you can click on Generate Excel Sheets ('1' in Fig. 10.22.8) to collect all of the information (e.g., total contractility, force polarity, matrix stiffening, etc.) in a `resultsall.xls` file in your parent data directory. If the data set contains only one cell, data collection from multiple cells makes no sense and does not work. In this case, you can find the same information in the `results.txt` file in the `results` directory of the cell, which was automatically created during the calculation.

### ***Generating 3-D plots of the cell deformation and force field***

16. From the Matlab command window, start the program SAENO\_3dplot, as shown in Fig. 10.22.9. This program opens the SAENO\_3dplot window (see Fig. 10.22.10) and plots the deformation and force field that was computed with the SAENO software.
17. Click on Load Cell or Batch ('m' in Fig. 10.22.10) to load the data from one cell or multiple cells for plotting (see Fig. 10.22.10). To that end, select the parent directory where the data and the results are located. The loading process can take a while depending on the number of cells to load. It is advisable to select the autodraw option in order to automatically redraw all results after parameter adjustments. If the autodraw option has not been selected, remember to click the Redraw button ('o' in Fig. 10.22.10) to refresh the results.

18. In the right panel of the plot window, it is possible to change the plotting options. Wait until all data are loaded before changing the plotting options. Click on the drop-down menu to select the data to plot (see Fig. 10.22.10). The data available for plotting are: the measured displacements, the fitted displacements, the traction forces, and the displacement noise. Plotting will take some time as a considerable amount of data needs to be loaded.

*The raw displacements are those calculated with the fiber pattern-matching algorithm. The fitted displacements and the traction forces are computed by the force-reconstruction algorithm. The displacement noise is the difference between the fitted and the raw displacements.*

19. The Viewrange option modifies the size of the volume to be displayed (see Fig. 10.22.10). This parameter is specified in units of meters. The edge length of the displayed cubic volume is two times the Viewrange parameter.
20. The Max Value (see Fig. 10.22.10) controls the upper limit of the color bar. This parameter is specified in units of meters for the displacements and the noise, and in units of squared Newtons per cubic meter in the case of the force density. If a value of zero is entered, the maximum value of the scale will be adjusted automatically.
21. The Azimuth and Elevation options modify the horizontal and vertical viewing angle (see Fig. 10.22.10).
22. The Quiver Count option sets the number of generated arrows (see Fig. 10.22.10).
23. The Smoothen option controls the smoothing of the data (see Fig. 10.22.10). A value of 0 means that no smoothing is applied. For a value of 1, the data points are averaged over a cubic volume with the size of  $3 \times 3 \times 3$  times the grid constant (which is  $7.5 \mu\text{m}$  by default; see Table 10.22.1).
24. The Draw Moments option plots the axis of maximum contractility (see Fig. 10.22.10). The axis is shown as a white line ( $50 \mu\text{m}$  length) in the plot.

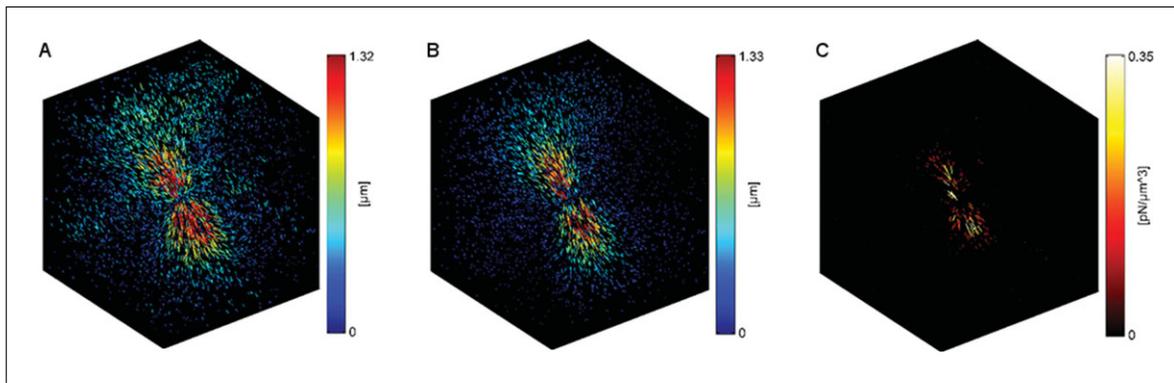
### **Export the generated 3-D plots**

25. Before exporting the plots, switch between different cells and timepoints to check that the selected plot options work for all cells and timepoints. To switch between cells, click and move the slider ('p' in Fig. 10.22.10) located at the bottom part of the plot window.

*Switching between cells will take some time, as the new data need to be loaded.*

26. After selecting another cell to plot, click on Redraw ('o' in Fig. 10.22.10) to refresh the results.
27. To export plots of the same type, e.g., the `Raw_displacements`, for all cells in the parent directory, click on Export All ('q' in Fig. 10.22.10). To export only the current plot, click on Export ('r' in Fig. 10.22.10).
28. After clicking Export or Export All, a window is opened for selecting the directory and specifying a filename for the plot(s). It is advisable to specify a name that contains the type of data to export and the file extension (e.g., `Raw_displacements.png`). The names of the generated files will automatically contain the cell and the time point plus the name defined by the user (e.g., `Raw_displacements_cell1001_t000.png`). An example of the results obtained with SAENO\_3dplot is shown in Fig. 10.22.11.

*It is necessary for the user to specify the file extensions, e.g., .png, .jpg, or .tif, and the plot(s) will be automatically saved in the corresponding image format. If no file extension is specified, the plot(s) will be saved in the non-image .rpt report format.*



**Figure 10.22.11** (A) Measured displacements, (B) calculated displacements, and (C) traction forces in a collagen matrix around an invasive MDA-MB-231 breast carcinoma cell. Colors and line length indicate the magnitude of the matrix displacements and the traction forces.

**BASIC  
PROTOCOL 4**

**MACRORHEOLOGY OF COLLAGEN TYPE I HYDROGELS**

To calculate the traction forces from the matrix displacements, it is necessary to know the mechanical properties of the matrix. Note that visco-elastic or plastic matrix properties are neglected in the traction reconstruction method described here. Therefore, it is assumed that the matrix is perfectly elastic and that the matrix configuration, after cell forces have been pharmacologically relaxed, is the same as the matrix configuration before the cell has moved to its current position and has applied any forces to the matrix. Even so, the mechanics of fibrous biopolymer networks such as collagen gels cannot be described by a single elastic modulus. Rather, collagen gels and some other biopolymer networks stiffen exponentially when strained beyond a linear range, and soften exponentially under compression. This behavior can be described with four parameters: a linear elastic stiffness  $K_0$ , a strain range  $L_S$  at which the matrix starts to stiffen, a strain scale  $D_S$  describing the exponential stiffening under stretch, and a strain scale  $D_0$  describing the exponential softening under compression. To measure these four parameters, two different macrorheological experiments need to be performed. In the first experiment, the stress versus strain relationship of the matrix for simple shear deformation is measured in a cone-plate rheometer. In a second experiment, the vertical contraction (collapse) of the matrix under horizontal uniaxial stretch is measured. For more information about these experiments, see supplementary notes in Steinwachs et al. (2016).

To estimate the four material parameters  $K_0$ ,  $d_0$ ,  $L_S$ , and  $d_s$ , the data from the macrorheological measurements (stress versus strain and vertical contraction versus horizontal stretch) are compared to the calculated response based on an initial guess of the four parameters. These four parameters are then iteratively (manually) changed until the mismatch between the experimentally obtained data and the values predicted by the model is minimized. Below we describe how the model predictions are calculated and compared with data from two different rheological experiments that must be performed by the user. It is beyond the scope of this protocol to describe these rheological experiments in detail.

The source code algorithm can be downloaded free of charge under the MIT license on the collaborative coding platform GitHub (<https://github.com/Tschaul/SAENO/tree/master/macrorheology>). Moreover, a Matlab program and a sample data set of two collagen rheology experiments are provided.

**Materials**

- A compiled version of the analysis software CurledBeamsFit
- The Matlab program PlotFitandData
- Matlab software V.R2012b or newer

An open-source GCC compiler for Windows, for example MinGW32  
Computer with Windows operating system

### ***Download and install the software***

This protocol works properly as long as the downloaded folder names are not modified. If the folder or file names need to be changed, they must not contain white spaces.

1. Download the `Macrorheology` folder from <https://github.com/Tschaal/SAENO/tree/master/macrorheology> onto your computer's hard drive (C: drive).

*The Macrorheology folder also can be saved at a different location, but ensure that the path does not contain white spaces.*

2. Open a command window at the location of your `Macrorheology` folder (e.g., `C:\Macrorheology`). A convenient way to do so is to navigate with your file explorer to the C: drive, press down and hold the Shift key, right click the folder `C:\Macrorheology`, and then click "open command window here." After that, a new command window will appear, as shown in Fig. 10.22.12.
3. To view the contents of the `Macrorheology` folder, type `dir` and press Enter. A list of files and folders will be displayed. Check that the `CurledBeamsFit` file appears in the list. If it is not listed, repeat step 1.

### ***Simulation of the matrix responses to shear deformations and uniaxial stretch***

Make sure that a GCC compiler for Windows has been installed previously on your computer. If not, install an open-source GCC compiler for Windows, for example, MinGW32.

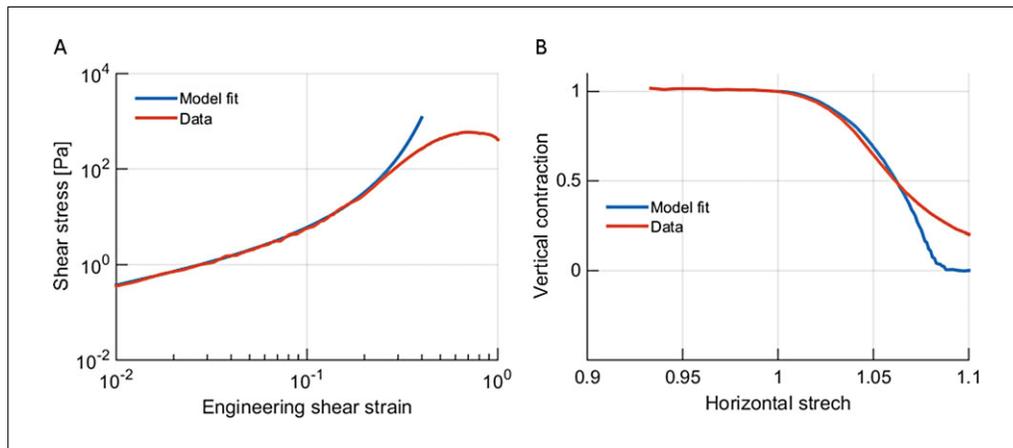
4. Run the `CurledBeamsFit.exe` program as shown in Fig. 10.22.13. This program estimates the stress-strain relationship of the matrix for a pure shear deformation, and the vertical contraction in response to a uniaxial stretch, given the four matrix mechanical parameters  $K_0$ ,  $d_0$ ,  $L_s$ , and  $d_s$ . These four parameters are passed to the `CurledBeamsFit.exe` program from the command line, as shown in Fig. 10.22.13. It is necessary to separate these four parameters by whitespaces, and to pass them in the following order: linear stiffness  $K_0$ , buckling coefficient  $d_0$ , linear strain range  $L_s$ , and strain stiffening coefficient  $d_s$ .
5. After all calculations are completed (typically after 2 to 3 min), two different files are generated in the `Macrorheology` folder. The computational predictions corresponding to the shear-rheometer experiment are contained in the `rheosim.dat` file, and those corresponding to the stretcher experiment are contained in the `stretchersim.dat` file.



**Figure 10.22.12** New command window at `C:\Macrorheology`.



**Figure 10.22.13** Run `CurledBeamsFit` in the command window.



**Figure 10.22.14** (A) Material stress as a function of the engineering shear strain predicted by the semi-affine model (blue), and the corresponding data measured with a cone-plate rheometer (red). (B) Vertical contraction as a function of the horizontal stretch predicted by the semi-affine model (blue), and the corresponding data measured in a uniaxial stretch experiment (red). The experimental data correspond to the response of a 1.2 mg/ml collagen gel (provided as an example in the downloaded files), and the simulated responses, are obtained with the material parameters shown in Figure 10.22.13.

### *Comparing the model predictions with experimental data /fitting the material model*

The two files generated by the `CurledBeamsFit.exe` program containing the estimated shear and uniaxial stretch responses are now compared with the experimental data stored in two separate files located in the sub-folder `C:\Macrorheology\data_exp\`. The experimental data file `rheodata.dat` contains the data from the shear-rheometer experiments, and the file `stretcherdata.dat` contains the data from the uniaxial stretcher experiments. The simulated and experimentally measured data are now compared by plotting stress versus strain and vertical compaction versus horizontal stretch responses using the Matlab program `PlotFitandData.m`. The user may manually optimize the four material parameters until the simulated and experimentally measured data coincide reasonably well.

6. Open Matlab version 2012b or later. Earlier versions of Matlab may not work properly.
7. Select the path where the program `PlotFitandData` is saved (e.g., `C:\Macrorheology\matlab`). To do this, in the HOME tab ('a' in Fig. 10.22.3), click on Set Path ('b' in Fig. 10.22.3). Then, click on Add with Subfolders ('c' in Fig. 10.22.3) and select the directory where the files are located. The directory where the compiled files are located is called `Macrorheology`.
8. Place the experimental data results in two different files in the folder named `data_exp`, which is a subfolder of the `Macrorheology` folder. The experimental data that correspond to the shear-rheometer experiment must be contained in a file named `rheodata.dat` and the results that correspond to the stretcher experiment must be contained in a file named `stretcherdata.dat`.

*The `rheodata.dat` file contains two columns of ASCII-formatted space-separated text. The first column contains the stress in Pa, and the second column the corresponding engineering (or Cauchy) strain.*

*The `stretcherdata.dat` file also contains two columns of ASCII-formatted space-separated text. The first column contains the vertical contraction (height of the collagen layer during stretch, divided by the height at zero stretch), and the second column contains the horizontal stretch (length of the collagen layer during stretch, divided by the unstretched length).*

9. Run the program `PlotFitandData` in the Matlab command window, as explained in step 5 of Basic Protocol 3. The program plots the simulated data (blue lines) and the experimental data (red lines). An example is shown in Figure 10.22.14.
10. If the experimental curves are not properly matched by the simulated data, repeat steps 4 to 9 with different material parameters, until both curves match.

*The most important parameter is the linear stiffness  $K_0$ . For a collagen type I hydrogel, a suitable initial guess for  $K_0$  is around 5000 Pa for a collagen concentration of 2.4 mg/ml and around 1000 Pa for a collagen concentration of 1.2 mg/ml. For more information about how the other parameters influence the macrorheological behavior, see supplementary notes in Steinwachs et al. (2016).*

## COMMENTARY

### Background Information

#### *Adjusting the configuration file*

The configuration file (in the `SAENO_matlab` folder downloaded from the GitHub platform) for fine-tuning the force reconstruction algorithm is summarized in Table 10.22.1.

The material parameters  $K_0$ ,  $d_0$ ,  $L_s$  and  $d_s$  given in the example below are for a collagen gel of 1.2 mg/ml and need to be adjusted by the user depending on the 3-D matrix material properties. These material properties need to be measured as outlined in Basic Protocol 4. More information about optimizing the other parameters can be found in the documentation on the GitHub platform (<https://github.com/Tschaul/SAENO>).

### Critical Parameters and Troubleshooting

The most critical parameter in Basic Protocol 1 is the polymerization temperature. When gels are polymerized at lower temperatures, the pore size and bulk stiffness both increase (Yang & Kaufman, 2009). Similarly important is the exact temperature profile with which the ice-cold unpolymerized collagen mixture is warmed to initiate the polymerization process. This temperature profile depends on the volume and shape of the culture dish, but also on how quickly the dish is transferred, e.g., to the cell culture incubator. A consistent, smooth, and well-rehearsed handling of all steps involved is therefore important.

Another important factor is the consistency of the collagen source from the commercial supplier. It is advisable to test the pore size and branching structure of the collagen gel for each new collagen batch using confocal reflection microscopy and visually compare the images with those obtained from previous batches. If deviations are clearly visible and persist, the problem is often attributable to the new col-

lagen batch. Most commercial suppliers ship small test samples on request.

The most critical parameter in Basic Protocol 2 is the quality of the collagen images and the absence of mechanical drift and noise. Drift and noise can be evaluated with a self-prepared calibration slide containing fluorescent beads embedded in a 3-D stiff hydrogel matrix such as polyacrylamide, or simply a glutaraldehyde-fixed collagen gel. The displacement field from two subsequently recorded image stacks of the calibration slide should be below 200 nm (rms). Larger displacements are a sign either of excessive mechanical noise (place the microscope on a vibration isolation table), photon shot noise (increase the laser intensity), mechanical stage drift (ensure better temperature control or temperature equilibration of the microscope), or poor mechanical reproducibility of the z-focus (this may be an intrinsic problem of the microscope). If confocal reflection images are obtained not with a water-immersion objective but with a water objective through a glass slide, Newton rings can severely degrade the image quality. Newton rings can be reduced or avoided by choosing different laser wavelengths and by staying more than 300  $\mu\text{m}$  away from the bottom or the top of the collagen gel surface. If the problem persists, it is better to mix 1  $\mu\text{m}$  carboxylated fluorescent beads with the collagen matrix prior to polymerization and determine the displacement field either with confocal fluorescence microscopy or simply with a standard motorized epifluorescence microscope.

### Time Considerations

*Basic Protocol 1:* It takes around 30 min to prepare collagen gels and to seed cells, and 1 hr for cell-collagen solution polymerization. After that, incubation overnight is required unless the process of cell spreading in the collagen gel is of interest. The incubation time needed

for the cells to establish cell polarity and to start migration depends on the cell line being studied.

**Basic Protocol 2:** The time needed for the acquisition process depends on the number of cells selected for imaging. With the settings described above, it takes around 5 min to record an image stack for one cell. The time required to relax the cells with the cytochalasin D in the 3-D collagen gels is approximately 30 min, corresponding to the time necessary to wait between the two stacks (*stack-1* and *stack-2*).

**Basic Protocol 3:** The computation time of the algorithm strongly depends on the grid constant of the finite element mesh defined by the user in the configuration file. For the default grid constant of 7.5  $\mu\text{m}$ , the total computation time for four cells (or four time points of the same cell) on an Intel i5 processor with four cores is  $\sim 1$  hr.

**Basic Protocol 4:** The time for computing the stress-strain relationship depends on the dimensions of the material model and the grid constant, but is typically around several minutes.

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### Internet Resources

<https://github.com/Tschaul/SAENO>

The source code of the algorithm SAENO can be downloaded free of charge from the collaborative coding platform GitHub at the above URL. A compiled version of the software and a tutorial are provided as Supplementary Software, together with a sample data set (<http://lpmt.biomed.uni-erlangen.de/3DTractions/SampleData.rar>).

<https://github.com/Tschaul/Macrorheology>

The macrorheology software and a sample data set of two collagen rheology experiments can be downloaded from the GitHub platform at the above URL.