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An Open Source Technology Platform to Manufacture Hydrogels in an Automated and Standardized Fashion

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TITLE:

An Open Source Technology Platform to Manufacture Hydrogels in an Automated and Standardized Fashion

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SUMMARY:

This protocol serves as a comprehensive tutorial for standardized and reproducible mixing of viscous materials with a novel open source automation technology. Detailed instructions are provided on the operation of a newly developed open source workstation, the usage of an open source protocol designer, and the validation and verification to identify reproducible mixtures.

ABSTRACT:

Current mixing steps of viscous materials rely on repetitive and time-consuming tasks which are

performed mainly manually in a low throughput mode. These issues represent drawbacks in workflows that can ultimately result in irreproducibility of research findings. Manual-based workflows are further limiting the advancement and widespread adoption of viscous materials, such as hydrogels used for biomedical applications. These challenges can be overcome by using automated workflows with standardized mixing processes to increase reproducibility. In this study, we present step-by-step instructions to use an open source protocol designer, to operate an open source workstation, and to identify reproducible mixtures. Specifically, the open source protocol designer guides the user through the experimental parameter selection and generates a ready-to-use protocol code to operate the workstation. This workstation is optimized for pipetting of viscous materials to enable automated and highly reliable handling by the integration of temperature docks for thermoresponsive materials, positive displacement pipettes for viscous materials, and an optional tip touch dock to remove excess material from the pipette tip. The validation and verification of mixtures are performed by a fast and inexpensive absorbance measurement of Orange G. This protocol presents results to obtain 80% (v/v) glycerol mixtures, a dilution series for gelatin methacryloyl (GelMA), and double network hydrogels of 5% (w/v) GelMA and 2% (w/v) alginate. A troubleshooting guide is included to support users with protocol adoption. The described workflow can be broadly applied to a number of viscous materials to generate user-defined concentrations in an automated fashion.

INTRODUCTION:

Reproducibility and replicability are of paramount importance in scientific work[1–4]. However, recent evidence has highlighted significant challenges in repeating high-impact biomedical studies in fundamental science as well as translational research[4–7]. Factors contributing to irreproducible results are complex and manifold, such as poor or biased study design[6,8], insufficient statistical power[3,9], missing compliance with reporting standards[7,10,11], pressure to publish[6], or unavailable methods or software code[6,9]. Amongst them, subtle changes in the protocol and human errors in the execution of experiments have been identified as further elements accounting for irreproducibility[4]. For instance, manual pipetting tasks introduce intra- and inter-individual imprecision[12,13] and increase the probability of human errors[14]. While commercial liquid handling robots are able to overcome these drawbacks and have demonstrated increased reliability for liquids[15–17], automated handling of materials with significant viscous properties is still challenging.

Commercial liquid handling robots commonly use air cushion pipettes, also known as air piston or air displacement pipettes. The reagent and the piston are separated by an air cushion which shrinks during dispensing steps and expands during aspirating steps. Using air cushion pipettes, viscous materials ‘flow’ only slowly into and out of the tip, and early withdrawal of the pipette from the reservoir may result in the aspiration of air bubbles. During dispensing tasks, the viscous material leaves a film on the inner tip wall which ‘flows’ only slowly or not at all when being forced by air. To overcome these issues, positive displacement pipettes were introduced commercially to actively extrude the viscous material out of the tip using a solid piston. Although these positive displacement pipettes enable accurate and reliable handling of viscous materials, automated solutions with positive displacement pipettes are still too expensive for academic laboratory settings, and, therefore, most workflows with viscous materials rely solely on manual

pipetting tasks[18].

In general, viscosity is defined as the resistance of a fluid to flow, and viscous materials are further being defined as materials with a greater viscosity of water (0.89 mPa·s s at 25 °C). In the field of biomedical applications, experimental setups often contain multiple materials with a greater viscosity than water, such as dimethyl sulfoxide (DMSO; 1.99 mPa·s at 25 °C), glycerol (208.1 mPa·s at 25 °C for 90% glycerol [v/v]), Triton X-100 (240 mPa·s at 25 °C), and water-swollen polymers, referred to as hydrogels[19,20]. Hydrogels are hydrophilic polymer networks arranged in a physical or/and chemical mode used for various applications, including cell encapsulation, drug delivery, and soft actuators[19–22]. The viscosity of hydrogels depends on the polymer concentration and molecular weight[19]. Routinely used hydrogels for biomedical applications exhibit viscosity values between 1 and 1000 mPa·s, while specific hydrogel systems have been reported with values of up to 6 x 10^7 mPa·s[19,23,24]. However, viscosity measurements of hydrogels are not standardized in terms of measurement protocol and sample preparation, and, therefore, viscosity values between different studies are difficult to compare.

Since commercially available automated solutions specifically designed for hydrogels are either missing or too expensive, current workflows for hydrogel depend on manual handling[18]. To understand the limitations of the current manual-based workflow for pipetting of hydrogels, it is important to comprehend essential handling tasks[18]. For example, once a novel hydrogel material has been synthetized, a desired concentration or a dilution series with varying concentrations is generated to identify reliable synthesis protocols and crosslinking characteristics with subsequent analysis of the mechanical properties[25–28]. In general, a stock solution is prepared or purchased, and subsequently mixed with a diluent and/or other reagents to obtain a mixture. The mixing tasks are mostly not performed directly in a well plate (or any output format), and are rather performed in a separate reaction tube, which is commonly referred to as master mix. During these preparation tasks, various aspirating and dispensing steps are required to transfer the viscous material(s), mix the reagents, and transfer the mixture to an output format (e.g., a 96 well plate). These tasks require a high amount of human labor[18], long experimental hours, and increase the probability of human errors which could potentially manifest as inaccurate results. Moreover, manual handling prevents efficient preparation of high sample numbers to screen various parameter combinations for detailed characterization. The manual processing also impedes the usage of hydrogels for high-throughput screening applications, such as the identification of promising compounds during drug development. The current manual-based preparation steps are not feasible to screen drug libraries consisting of thousands of drugs. For these reasons, automated solutions are required to provide an efficient development process and enable the successful translation of hydrogels for drug screening applications.

To move from manual-based workflows to automated processes, we have optimized a commercial open source pipetting robot for the handling of viscous materials by the integration of temperature docks for thermoresponsive materials, the usage of off-the-shelf positive displacement pipettes using capillary piston tips, and an optional tip touch dock for pipette tip cleaning. This pipetting robot has been further integrated as a pipetting module into a newly developed open source workstation, which consists of ready-to-install and customizable

modules[18,29]. Detailed assembly instructions for the developed workstation including hardware and software files are freely accessible from the GitHub (https://github.com/SebastianEggert/OpenWorkstation) and the Zenodo repository (https://doi.org/10.5281/zenodo.3612757). In addition to the hardware development, an open source protocol design application (accessible via GitHub) has been programmed and released to guide the user through the parameter selection process and generate a ready-to-use protocol code. This code runs on the commercial open source pipetting robot as well as on the developed open source workstation.

Herein, a comprehensive tutorial is provided on the operation of the open source workstation to automate mixing tasks for viscous materials (**Figure 1**). The tutorial-specific protocol steps can be carried out with the developed open source workstation as well as the commercial open source pipetting robot. Supported by an in-house developed open source protocol design application, automated mixing and preparation of required concentrations for glycerol, gelatin methacryloyl (GelMA) and alginate is demonstrated. Glycerol has been selected in this tutorial, since it is well characterized[30,31], it is inexpensive and readily available, and, therefore, it is commonly used as viscous reference material for automated pipetting tasks. As examples for hydrogels used in biomedical applications, GelMA and alginate hydrogel precursor solutions have been applied for automated mixing experiments. GelMA presents one of the most commonly used hydrogels for cell encapsulation studies[32,33], and alginate was selected in this study to demonstrate the ability to manufacture double network hydrogels[34,35]. Using Orange G as a dye, a fast and inexpensive procedure was implemented to validate and verify the mixing results with a spectrophotometer[16].

A commercial open source pipetting robot has been integrated as a pipetting module into the developed open source workstation (**Figure 2a**), and therefore, the name 'pipetting module' is further used to describe the pipetting robot. A detailed description of the installed hardware is beyond the scope of this protocol and is available via the provided repositories which also include step-by-step instructions for the general assembly of the open source platform. The pipetting module can be equipped with two pipettes (single- or 8-channel pipette) which are installed on axis A (right) and axis B (left) (**Figure 2b**). The pipetting module offers a 10-deck capacity according to American National Standards Institute/Society for Laboratory Automation and Screening (ANSI/SLAS) standards, and the following location positions are defined on the deck: A1, A2, B1, B2, C1, C2, D1, D2, E1, E2 (**Figure 2c**). To initiate photo-induced polymerization of hydrogel solutions, a separate crosslinker module is required and has been added to the workstation. The crosslinker module is equipped with LEDs with a wavelength of 400 nm and, therefore, substances that excite at a visible light wavelength can be used with the current systems, such as lithium phenyl-2,4,6 trimethylbenzoylphosphinate (LAP)[36,37]. The intensity (in mW/cm^2) of the LEDs can be addressed by the user in the protocol design application to study the crosslinking behavior[38]. The workstation includes also a storage module to enable increased throughput studies; however, this module is not used within this study and, therefore, not further described. In general, it is recommended to operate the pipetting module in a biological safety cabinet to avoid sample contamination. The main power circuit to operate the pipetting module is a 12 V circuit, which is considered as a low-voltage application in most countries. All electrical

components are based in a dedicated control box preventing users from coming into contact with the source of an electrical hazard.

By following these standardized mixing protocols, researchers are able to achieve reliable mixtures for viscous as well as nonviscous materials in an automated fashion. The open source approach allows users to optimize mixing sequences and share newly developed protocols with the community. Ultimately, this approach will facilitate the screening of multiple parameter combinations to investigate the interdependencies between different factors and, thereby, accelerate the reliable application and development of viscous materials for biomedical applications.

PROTOCOL:

NOTE: The protocol starts with an introduction to (1) the software and (2) the hardware setup to familiarize the user with required installations and the workstation. Following a section on (3) material preparation and (4) the usage of the protocol designer application, (5) the calibration of the pipetting module and (6) the execution of the automated protocol is highlighted in detail. Finally, (7) validation and verification procedures are described, including absorbance reading and data analysis. A general protocol workflow with individual tasks is displayed in **Figure 1**.

1. Software setup

NOTE: This section includes a detailed instruction to install the application programming interface (API) as well as the required protocol designer application and the calibration terminal. The following instructions are written for a Raspberry Pi (RPi) single-board computer; however also Windows 8, 10 and macOS 10.13+ have been successfully used with the API and the applications.

1.1. Set up the computer environment.

NOTE: Be familiar with the basics of Python[39], how to set up and use a Raspberry Pi[40,41], and how to connect to the internet[42]. The following tutorial steps focus on protocol-specific steps and additional information on the usage of a Raspberry Pi is available online[40].

1.1.1. Open a terminal window from the taskbar or application menu.

1.1.2. Update the system's package list:

```
sudo apt-get update
```

1.1.3. Upgrade all the installed packages:

```
sudo apt-get dist-upgrade
```

1.1.4. Restart the Raspberry Pi:

sudo reboot

1.1.5. Check installed Python version:

python3 --version

Make sure that at least Python 3.5 is installed; if not, install the latest version[43].

1.1.6. Install python pip, which publishes Python packages with the Python Package Index[44]:

sudo apt-get install python3-pip

1.1.7. Install dependencies:

pip install numpy
pip install python-resize-image

1.2. Install the application programming interface (API).

NOTE: The API provides a simple Python framework designed to write experimental protocols script and operate the workstation. The following two APIs are required to successfully execute the generated protocol code.

1.2.1. Install workstation API:

pip install openworkstation

1.2.2. Install Opentrons API to operate the pipetting module:

pip install opentrons==2.5.2

1.2.3. Verify, if API is installed successfully:

python3
>>> import openworkstation
>>> import opentrons

NOTE: The size of the API and the protocol design application is 2.2 MB and 1.2 MB, respectively. No issues were experienced during the installation when used with limited disk space (200 MB). However, the disk space requirements depend on the operating system.

1.3. Select a directory for file download (calibration terminal, protocol design application, etc.).

NOTE: Files can be copied and pasted elsewhere afterwards.

1.4. Clone files from GitHub repository:

git clone https://github.com/SebastianEggert/OpenWorkstation

NOTE: The 'git clone' command clones and subsequently saves all files into the directory, which is open in the terminal at this time. Since the repository also includes the hardware files for the assembly, not the entire repository is required to execute the presented protocols. All required files to replicate the experiments are available as **Supplemental File** and in the GitHub repository under "/examples/publication-JoVE".

1.5. Open the downloaded folder. If the entire repository was downloaded, navigate to the 'publication-JoVE' folder via

cd openworkstation/examples/publication-JoVE

NOTE: This folder includes files required for the operation of the workstation and the usage of the protocol designer application and the calibration terminal.

2. Hardware setup

2.1. Place the workstation in a biological safety cabinet to avoid sample contamination.

2.2. Install pipettes on the workstation.

2.2.1. Select the pipette size based on the experimental setup. In general, take a pipette size which volume to be aspirated is on the higher end of the range. If mixing tasks with volumes greater than 1 mL are required for a specific setup (e.g., aspirating/dispensing of 2 mL), choose the M1000E with a maximal aspirating/dispensing volume of 1,000 µL to minimize pipetting steps and save time.

NOTE: A detailed instruction for air-displacement pipettes is available online[45]. The developed pipetting module is able to integrate the following off-the-shelf positive displacement pipettes: M10E (1–10 µL), M25E (3–25 µL), M50E (20–50 µL), M100E (10–100 µL), M250E (50–250 µL), M1000E (100–1,000 µL).

2.2.2. Use an M4 Allen key to loosen and tighten screws.

2.2.3. Attach the two pipette fixation plates (white acrylic plates) to the aluminum rail and tighten M5 screws loosely.

2.2.4. Insert the pipette into the two pipette fixation plates and ensure that the ergonomic tail of the pipette is resting on the opposite side of the acrylic mounting plate.

2.2.5. Tighten the four screws of the two pipette fixation plates firmly.

2.2.6. Slide the two square fastening nuts, which are attached to the acrylic mounting plate, into the extrusion slot of the z-axis and tighten screws.

NOTE: Fasten pipette tightly to avoid any movement during operation.

3. Material preparation

NOTE: The viscous materials (glycerol, GelMA, alginate) are used for the experiments presented in this study, and, therefore, prepared volumes and handling tasks (e.g., add 5 mL of stock solution in 5 mL reaction tubes) are specifically for this experimental setup.

3.1. Gelatin methacryloyl (GelMA)

NOTE: GelMA functionalization, dialysis, and lyophilization are not the scope of this paper, and a step-by-step protocol is available in Loessner et al.[33]. The protocol starts using lyophilized GelMA, which can be prepared in-house or purchased commercially.

3.1.1. Calculate required mass of GelMA (m_{GelMA}) based on the desired final stock concentration (c_{GelMA}) and volume (V_{GelMA}) using the equation:

$$m_{GelMA} = c_{GelMA} \times V_{GelMA}$$

NOTE: V_{GelMA} depends on the experimental setup and it is recommended to prepare 20−30% excess material. The presented protocols start with 5 mL of 20% (w/v) GelMA as a stock solution.

3.1.2. Weigh the required amount of lyophilized GelMA, add it into a 50 mL reaction tube and add the required amount of phosphate buffered saline (PBS).

3.1.3. Mix GelMA either by soaking into the solvent at 4 °C overnight or by heating to 60 °C for 6 h in a water bath.

NOTE: Sterile GelMA solutions can be stored protected from light at 4 °C for at least six months.

3.1.4. Fill 5 mL of GelMA into 5 mL reaction tubes.

3.2. Photoinitiator: Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)

NOTE: Avoid additional exposure to room light, since LAP is light sensitive.

3.2.1. Calculate required mass of LAP (m_{LAP}) based on the desired final stock concentration (c_{LAP}) and required volume (V_{LAP}) using the equation:

$m_{LAP} = c_{LAP} \times V_{LAP}$

NOTE: It is recommended to prepare a 3% (w/v) stock solution.

3.2.2. Weigh the required amount of LAP, add it into a 15 mL reaction tube and add PBS.

3.2.3. Wrap the tube in aluminum foil to prevent photo-induced decomposition.

3.2.4. Dissolve LAP by placing the reaction tube in a water bath at 37 °C for 2 h or until fully dissolved.

3.2.5. Fill 1 mL of LAP stock solution in 5 mL tubes.

3.3. Alginate

3.3.1. Calculate required amount of alginate ($m_{alginate}$) based on the desired final stock concentration ($c_{alginate}$) and volume ($V_{alginate}$) using the equation:

$m_{alginate} = c_{alginate} \times V_{alginate}$

NOTE: $V_{alginate}$ depends on the experimental setup and it is recommended to prepare 20−30% excess material. The presented protocols start with 5 mL of 4% (w/v) alginate as a stock solution.

3.3.2. Weigh the required mass of alginate, add it into a 50 mL reaction tubes, and add PBS.

3.3.3. Place the alginate mix into a water bath at 37 °C for 4 h.

NOTE: The usage of a vortex mixer will accelerate the dissolution process, but also generate air bubbles. Dissolved alginate can be stored at 4 °C for at least six months.

3.3.4. Fill 5 mL of alginate into 5 mL reaction tubes.

3.4. Fill 5 mL of glycerol in 5 mL reaction tubes.

3.5. Orange G solution

3.5.1. Prepare a 10 mg/mL stock solution of Orange G in a 50 mL reaction tube.

NOTE: The volume depends on the number of experiments. Depending on the diluent type, prepare stock solution either in ultrapure water, PBS or a suitable diluent reagent. In the presented experiments, ultrapure water was used for diluting glycerol and PBS for diluting GelMA and alginate. PBS was used as a diluent for GelMA and alginate, and can be either prepared using tablets or purchased off-the-shelf.

3.5.2. Mix for 10 s by vortexing.

3.5.3. Wrap the tube in aluminum foil to prevent photo-induced decomposition.

NOTE: Stock solution can be used after 24 h to ensure proper dissolution of Orange G.

3.5.4. Dilute stock solution to a 1 mg/mL working solution in a 50 mL reaction tube.

3.5.5. Transfer working solution to appropriate flasks/tubes for the experimental setup.

NOTE: For the presented experiments, the working solution was filled into 5 mL tubes. Orange G stock and working solution can be stored at 4 °C and used within three months upon preparation.

3.5.6. Fill 5 mL of the 1 mg/mL Orange G working solution in 5 mL reaction tubes.

4. Generate protocol code with the protocol designer application

NOTE: The specified parameters in steps 4.2−4.7 are the same for all conducted experiments, except for the material's stock concentration and the final output concentration. These parameters are summarized in **Table 1** and, in the following, parameters are used to prepare double network hydrogels with 5% (w/v) GelMA, 2% (w/v) alginate, 0.15% (w/v) LAP, and PBS as a diluent.

4.1. Open the protocol designer application by running 'protocol_designer.py'.

NOTE: The app "protocol_designer.py" guides the user through the parameter selection process and automatically generates the ready-to-use protocol to operate the workstation. Either use the terminal function or a Python integrated development environment (IDE) to open and execute the file.

4.2. Select protocol name (e.g., double-network-hydrogels.py) and define excess amount for input and mixing tubes.

NOTE: The selected percentage of the excess volume depends on the container type (e.g., 5 mL tubes compared to 0.5 mL reagent tubes). Within the presented experimental setting, an excess volume of 10% (w/v) was chosen for the input and 30% (w/v) for the mixing tray.

4.3. Define 'Input Tray Setup' by clicking on **Input Tray**.

4.3.1. Define key parameters: Cell position = C1, Tray model = heating-block-3x4, and Define well ordering = by column.

4.3.2. Define well parameters by clicking on 'Define wells': Column #1 = Gel 1 with a 20% (w/v)

stock concentration and 5000 µL tube volume, Column #2 = Gel 2 with a 4% (w/v) stock concentration and 5000 µL tube volume, Column #3 = Photoinitiator with a 3% (w/v) stock concentration and 5000 µL tube volume, and Column #4 = Diluent 1 with a 5000 µL tube volume.

NOTE: Gel 1 and Gel 2 refers to GelMA and alginate, respectively. Generic names were selected for the hydrogels to foster a universal usage for hydrogel applications.

4.4. Define 'Mixture Tray Setup' by clicking on **Mixture Tray**: Include mixture tray = Yes, Cell position = D1, and Tray model = heating-block-3x4.

4.5. Define 'Output Tray Setup' by clicking on **Output Tray**.

4.5.1. Define key parameters: Cell position = D2, Tray model = 96-flat, and Define well ordering = uniformly.

4.5.2. Define well parameters by clicking on **Define wells**: Volume = 60 µL, Desire concentration of Gel 1 = 5% (w/v), Desire concentration of Gel 2 = 2% (w/v), Desire concentration of Photoinitiator = 0.15% (w/v), and Diluent = Diluent 1.

4.6. Define type and characteristics of first pipette (M1000E) by clicking on **Tips Tray**: Cell position = B2, Tray model = tiprack-1000ul, Type = positive-displacement, Aspirating speed = 800, Dispensing speed = 1200, and Height above well bottom calibration = 0 mm.

4.7. Define trash by clicking on **Trash Tray**: Cell position = A2, and Tray model = trash-box.

4.8. Add a second pipette (M100E) under **Additional Cell 1**: Type = Tips, Cell position = B1, Tray model = tiprack-100ul. Under **Define wells**: Pipette type = positive-displacement, Aspirating speed = 600, and Dispensing speed = 1000.

4.9. Define crosslinker parameters by clicking on **Crosslinking Module**: Light intensity = 2 mW/cm^2, and Exposure time = 30 seconds.

4.10. Click on **Write Protocol** to generate the protocol for the setup.

NOTE: The developed protocol designer app generates automatically a new folder whenever a new protocol is generated. All files which are required for this experiment and to operate the workstation are saved in this folder which is named after the protocol name. The folder can be copied into different directories without causing issues. A selected deck setup can be saved as default setup using the purple **Save Setup** button.

5. Calibration of the pipetting module

NOTE: Containers (e.g., well plates, tip rack, trash) and pipettes (e.g., M1000E) must be calibrated initially. If a container and/or a pipette position are modified/changed, the new position must be

calibrated.

5.1. Open the calibration terminal by running ‘calibrate.py’.

NOTE: The interface “calibrate.py” interface guides the user through the calibration of the deck setup and pipettes.

5.2. Calibrate the pipette.

5.2.1. Press keyboard shortcut **P** to select pipette size.

5.2.2. Press keyboard shortcut **V** to enter the plunger calibration mode.

5.2.3. Select movement increments for plunger movement with the numeric keypad (1–8): ‘1’ for 0.1 mm, ‘2’ for 0.5 mm, ‘3’ for 1 mm, ‘4’ for 5 mm, ‘5’ for 10, ‘6’ for 20 mm, ‘7’ for 40 mm, and ‘8’ for 80 mm.

NOTE: It is recommended to start with small increments (2, 5, and 10 mm) to get familiar with the increment size and movement action of the pipette head.

5.2.4. Calibrate the following plunger positions for a positive displacement pipette: T–Top = rest position; B–Bottom = plunger is pushed until resistance is met; P–Pick-up = plunger is pushed to a position where a piston tip can be attached; E–Eject = plunger is pushed until an attached tip is ejected. Vary plunger positions using the upwards and downwards arrows on the keyboard, and save the final position using **S** on the keyboard.

5.3. Calibrate container position relative to the pipette tip.

5.3.1. Press keyboard shortcut **P** to select pipette type. Make sure that a tip is connected to the selected pipetted.

5.3.2. Press keyboard shortcut **C** to select container type.

5.3.3. Select an appropriate movement increment and move pipette tip to the following positions. For well plates, calibrate to the ‘A1’ well position at the bottom; For tip rack, calibrate to the ‘A1’ position; For trash, choose a position (defined as a point) where the tip can be ejected into the trash.

5.3.4. Press keyboard shortcut **S** to save position.

5.3.5. Repeat steps 5.3.1–5.3.3 for all containers listed under ‘C’ for the selected pipette type.

5.3.6. Repeat 5.3.1–5.3.5 for the second pipette type.

5.3.7. Close the calibration script.

6. Protocol execution with the workstation

NOTE: Protocol files are accessible via the repository and are also available as **Supplemental File**.

6.1. Position trash container, tip racks, input tray, mixing tray, and output on the deck (defined in step 4.3).

6.2. Calibrate pipettes and instruments as defined in section 5.

6.3. If required, switch the temperature dock ON and select the temperature for input and mixing tray.

NOTE: The experiments in this tutorial were conducted without temperature control and at 40 °C for glycerol, and 37 °C for GelMA and alginate pipetting.

6.4. Position tubes with input reagents in the aluminum blocks on the temperature docks according to the selected setup.

6.5. Wait until input reagents have reached the desired temperature.

NOTE: To ensure proper temperature distribution, an incubation time of 30 min is recommended for GelMA and alginate.

6.6. Execute the generated protocol code.

NOTE: The "protocol.py" file summarizes the specific protocol tasks (e.g., aspirating of 100 µL) for the experiment and needs to be executed in order to operate the workstation.

6.6.1. Open an IDE and load the protocol file (e.g., double-network-hydrogels.py).

NOTE: The protocol can be executed either through the terminal function or a Python IDE, such as Thonny[46] or IDLE[47].

6.6.2. Run the protocol file by clicking on **run**.

NOTE: The selected protocol is now executed by the workstation. The accompanying video highlights automated mixing of GelMA and the distribution of 60 µL into a 96 well plate.

7. Validation and verification process

7.1. Remove the well plate from workstation and transport the well plate with the samples to a spectrophotometer.

7.2. Read absorbance with a spectrophotometer at 450 nm. Read each plate 2x to compare results and ensure consistent results.

7.3. Export and save absorbance readings.

7.4. Data analysis.

NOTE: Experimental data can be processed individually or copied and pasted into the provided template to evaluate the mean, standard deviation, and coefficient of variance (CV) value using spreadsheet software.

7.4.1. Open the **Supplemental File** 'supplementary_template-analysis.xlsx", which is also vailable within the GitHub repository under 'openworkstation/examples/publication-JoVE.

7.4.2. Copy absorbance readings into the 'raw data' sheet and ensure that all cell references are correctly defined in all tables.

NOTE: Depending on the sample distribution on a well plate, the following preset evaluation types are available with the template: the 'Uniform' type is used when all samples have the same composition, the 'By rows' type is used when samples in different rows have different compositions, the 'By columns' type is used when samples in different columns have different compositions, and the 'Customized' type is used when the sample positions are user specific.

REPRESENTATIVE RESULTS:

This tutorial presents results for experiments with glycerol (**Figure 3**) and GelMA with LAP and alginate (**Figure 4**).

The generation of an 80% (v/v) glycerol solution was investigated either without temperature control (room temperature, 22 °C) and without tip touch (defined as setup 1), with temperature control (40 °C) and without tip touch (setup 2), or with temperature control (40 °C) and with tip touch (setup 3) (**Figure 3a-i**). These two temperature settings were chosen to evaluate the handling difference, since glycerol's viscosity is decreasing almost by a factor of 3 when heated from 22 °C (139.5 mPa·s) to 40 °C (46.6 mPa·s)[30]. An 85% (v/v) stock solution of glycerol was diluted to a final concentration of 80% and uniformly distributed into a 96 well plate (n = 96 per setup). The experimental time, which includes the dispensing of each material into the mixture tube, the respective mixing tasks, and sample distribution into a 96 well plate, was 30 min 42 s. To identify differences between dilution mixtures, ultrapure water–as the diluent for glycerol–was prepared with 1 mg/mL Orange G. The absorbance readings highlight that the integration of the temperature control and the tip touch significantly impacts the mixtures ($p < 0.0001$). In addition to the performed two-way analysis of variance (ANOVA), the CV values were calculated to evaluate the relative standard deviation. The coefficient of variation describes a standardized indicator to identify the degree of deviation in relation to the mean and is expressed as a percentage. If the sample means are not particularly the point of interest, but the variability

within the measurements, the coefficient of variation provides additional insights to identify reproducible mixtures[48]. Within this experiment with three different setups, the absorbance values showed decreasing CV values from 5.6%, 4.2%, to 2.0% for setup 1, setup 2, and setup 3, respectively, demonstrating the significant influence of the temperature dock and the tip touch function on producing reliable results (**Figure 3a-ii**). Plotting of sample absorbance values for setup 3 (sample number #1 to #96 in a 96 well plate) yields no increasing or decreasing values throughout the experiment and, therefore, indicates no influence of the sample position on the absorbance values (**Figure 3a-iii**). Visualizing the data for each measured well plate with heat maps provides additional insights to identify heterogeneities for a specific row or column, or varying absorbance values throughout the dispensing tasks. The visualized heatmaps for the three setups display decreased heterogeneities across the entire well plates from setup 1 to setup 3 (**Figure 3b**). Finally, the replicability of the conducted mixing was evaluated within eight independent runs (**Figure 3c-i,ii**), where each run took 6 min 57 s. Single mixing runs showed low CV values between 1.1% to 2.6%, which indicate very reliable mixing and dispensing tasks for the individual runs. Absorbance values of all eight runs yielded a CV value of 3.3% and demonstrated the reproducibility of the established mixing protocol.

GelMA dilution series were prepared by diluting a 20% (w/v) stock solution with PBS to 14, 12, 10, 8, 6, 4, 2, and 0% (w/v) and adding LAP to a constant concentration of 0.15% (w/v) (**Figure 4a-i**), which took in total 55 min 12 s. As specified in the experimental protocol script, the hydrogel was crosslinking for 30 s with an intensity of 2.0 mW/cm^2 at 400 nm. To evaluate the differences between the mixtures, PBS—as the diluent for GelMA and alginate—was prepared with 1 mg/mL Orange G. Hence, absorbance difference between samples within one mixture as well as between the serial dilutions are identified with a spectrophotometer. Measured absorbance values of each concentration step are significantly different ($p < 0.0001$) and have very low CV values between 1.2% and 3.4% throughout the concentration steps ($n = 12$ per concentration step). Linear regression demonstrated high fit with an R^2 value of 0.9869 (**Figure 4a-ii**) and a heatmap confirmed the homogenous distribution for each concentration and the difference between the concentrations (**Figure 4a-iii**). Automated mixing of four reagents was conducted for the generation of 5% (w/v) GelMA, 2% alginate (w/v), 0.15% (w/v) LAP, and PBS as diluent without (setup 2) and with (setup 3) touch tip ($n = 96$ for each setup) with the same crosslinking parameters (30 s, 2.0 mW/cm^2, 400 nm). Dispensing of the four materials, mixing, and distributing into a 96 well plate took 32 min 22 s. All experiments with GelMA and alginate were conducted at 37 °C to prevent thermal gelling which prevents pipetting of GelMA. With the tip touch option, the CV value was reduced from 5.2% to 3.4% and, especially, outliers in the lower region were prevented by removing excess material from the tip (**Figure 4b-i**). Although the mean value of 1.927 and 1.944 for setup 2 and setup 3 are very close, the coefficient of variation highlights the decreasing deviation in relation to the mean. Single rows of the 96 well plate can be compared with each other using a heatmap visualization to detect row and/or column differences (**Figure 4b-ii**).

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol workflow with individual tasks. The described workflow is divided into eight

tasks, which are separated into setup, preparation, execution, and analysis. In the beginning, the software (task 1) as well as the hardware (task 2) must be set up. After the preparation of the materials (task 3) and the generation of the protocol script (task 4), the pipetting module is calibrated by defining the pipette and container positions (task 5). Next, the protocol script is executed on the workstation (task 6) and validation and verification (task 7) of mixtures are carried out to evaluate mixtures.

Figure 2: Open source workstation and deck setup of the pipetting module. (**a**) The developed workstation is inspired by an assembly line approach, where samples are being transported through different modules, and consists of the following modules: pipetting, crosslinker, storage, transport, and computational module. (**b**) The deck of the pipetting module is set up depending on the experimental layout (e.g., well plate type, tube volume, etc.). The displayed deck setup was used for the presented experiments and consists of positive displacement pipettes with a range from 10−100 µL (M100E) and 100−1,000 µL (M1000E), the tip racks with capillary pistons (CP) for 100 µL (CP1000) and 1,000 µL (CP1000), a trash container, a mixing tray, and an input tray for the input reagents. (**c**) The available deck positions are defined with the displayed numbers.

Figure 3: Results for automated pipetting of glycerol mixtures. (**a**) The flexible workstation design enables the evaluation of three different setups (**i**) to identify optimal parameters for reproducible results. (**ii**) The addition of a tip touch and heating of the material resulted in a decreased coefficient of variance (CV) values and highly reproducible mixtures for setup 3. Each experiment was conducted with 96 samples. (**iii**) Plotting of single sample values showed no influence on the pipetting sequence. (**b**) Experimental results of each setup were visualized with heat maps to identify the influence on raw/column differences, edges, or master mixture. (**c**) The reproducibility of setup 3 was analyzed within eight independent runs using (**i**) median, standard deviation, CV value, and (**ii**) heatmaps. Data in panels **a-ii** (n = 96) and **b-i** (n = 12) are presented with the means and the single data points. Statistical significance was defined as ****$p < 0.0001$ using two-way analysis of variance (ANOVA).

Figure 4: Results for mixing tasks with hydrogels. (**a**) From a gelatin metacryloyl (GelMA) 20% (w/v) stock solution, a serial dilution of 14, 12, 10, 8, 6, 4, 2, and 0% (w/v) was generated within one experimental run using a 96 well plate (n = 12 per concentration). (**i**) The coefficient of variance (CV) values varied between 1.2% and 3.4% throughout the prepared concentrations, and (**ii**) linear regression showed a high fit with an R^2 value of 0.9869. (**iii**) Homogenous dilutions were confirmed visually with the generated heatmap. (**b**) Double network hydrogels were generated with 5% (w/v) GelMA, 2% (w/v) alginate, and 0.15% (w/v) LAP (**i**) with and without tip touch (n = 96 for each setup) and crosslinked for 30 s with an intensity of 2.0 mW/cm^2 at 400 nm. The integration of the tip touch resulted in decreasing CV values from 5.2% to 3.4%. (**ii,iii**) Heatmaps confirm fewer deviations when using tip touch to remove excess material from tip. Data in panels **a-i** and **b-i** are presented with the means and the single data points. Statistical significance was defined as *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ using one-way analysis of variance (ANOVA).

Figure 5: Summary of the pipette type difference and issues with viscous biomaterials. (**a**) The reagent and the piston are separated by an air cushion that shrinks during dispensing steps and expands during aspirating steps. When aspirating and dispensing viscous materials, the slow 'flow' introduces problems such as air bubbles and irregular pipetting behavior. (**b**) Positive displacement pipettes enable reliable aspirating and dispensing of viscous material by the usage of a piston inside the tip. (**c**) Pipetting of highly viscous materials (e.g., 4% [w/v] alginate) can result in the accumulation of excess material on the tip, which leads to inaccuracy throughout the experiments. (**d**) The implementation of a simple tip touch tray enables the removing of the excess material on the tip and results in accurate aspirating and dispensing volumes. This is realized by using the inner side of the well plate lid placed onto a tip rack container.

Table 1: Parameter overview for the conducted experiments.

Table 2: Troubleshooting table with identified issues, possible reasons as well as solutions to solve the problems.

DISCUSSION:

Pipetting of viscous materials, especially hydrogels for biomedical applications[19–21,33,49], are routine tasks in many research labs to prepare a user-defined concentration or a dilution series with varying concentrations. Although it is repetitive and the execution is rather simple, it is mostly performed manually with low sample throughput[18]. This tutorial is introducing the operation of an open source workstation, which has been specifically designed for viscous materials, to enable automated mixing of viscous materials for reproducible generation of desired concentrations. This workstation is optimized for pipetting of hydrogels to enable automated and highly reliable handling by the integration of temperature docks for thermoresponsive materials, positive displacement pipettes for viscous materials, and an optional tip touch dock to remove excess material from the tip. The pipetting module has been specifically optimized to enable the processing of viscous material in a standardized and automated manner. In comparison to air cushion pipettes (**Figure 5a**), positive displacement pipettes (**Figure 5b**) dispense viscous materials without leaving residual material left in the tip, resulting in accurate aspirating and dispensing volumes. The optional tip touch dock removes excess sample material from the tip (**Figure 5c,d**), which is useful for gluey materials (e.g., 4% [w/v] alginate).

The protocol designer application has been specifically programmed for hydrogels and allows the dilution of up to four reagents with different concentrations and up to two diluents. The risk of errors in the calculation of final dilutions is prevented in this application, as users only choose the desired concentration or the serial dilution steps. Required aspirating and dispensing volumes are calculated automatically, saved in a separate documentation text file, and then filled into the protocol script. This protocol design application gives the user full control of all experimental parameters (e.g., pipetting speed) and ensures internal documentation of the important parameters. The protocol design app takes the filling level of the reservoir (e.g., well) into account and varies the aspirating/dispensing height to prevent unnecessary dipping into the viscous materials. This integrated feature avoids material accumulation on the outer wall of the tip, and,

thereby, ensures reliable aspirating and dispensing tasks throughout the protocol. Although the protocol designer application has been developed for hydrogel dilution steps, it can be also used for dilution of nonviscous liquids, such as Orange G dyes. The protocol designer application, which is accessible via the repository under '/examples/publication-JoVE', is the version which is explained in the protocol section and highlighted in the video. This version will not be updated. However, an updated version of the protocol designer application is available via the main repository page. The calibration terminal was initially developed by Sanderson[50] and has been optimized for the calibration of positive displacement pipettes.

As described in the protocol section 4, pipettes as well as containers must be calibrated initially. This calibration process is crucial to define and save the positions which are then used to calculate the movement increments. Therefore, successful protocol execution relies on well-defined calibration positions, as wrong calibration points could result in crashing of the tip into a container. Since the plunger positions of the pipettes must be calibrated manually, pipetting accuracy and precision depend greatly on the performed calibration. These calibration procedures depend highly on the user experience with the pipetting module, and, therefore, training with experienced staff is recommended at the beginning to ensure proper calibration procedures. In addition to the manual calibration on the pipetting module, the pipette itself must be calibrated to ensure accurate pipetting. It is recommended to calibrate the pipettes at least every 12 months to meet to acceptance criteria as specified in ISO 8655. To evaluate the pipette calibration internally, validation and verification are available as described by Stangegaard et al.[16].

For the generation of a reliable data set, it is crucial to start with reagents of high quality. This is especially important for hydrogel processing tasks, as batch-to-batch variations might impact the generated results within this protocol. In addition to batch-to-batch variations, subtle changes in the preparation of small volumes may also contribute to property differences. To prevent this, preparation of larger volumes is recommended, which can be used for the entire experiments.

The validation and verification procedures rely on the usage of a dye to identify reliable mixtures. The presented protocol describes the application of Orange G, but the general protocol and analysis workflow can be also adapted to fluorescent dyes[51,52]. The usage of Orange G reduces the technical requirements of the spectrophotometer and eliminates precautions taken to prevent bleaching of the fluorescent dyes after exposure to light. Issues in the dissolving behavior or cluster formation of the dye have not been observed with the presented materials during experiments but might appear with other materials. Potential cluster formation and, therefore, the interaction between dye and material could be easily detected with a microscope.

The procedures and techniques presented in this tutorial add automation capability to current workflows for viscous materials to achieve highly reliable tasks with minimal human labor. The provided troubleshooting table (**Table 2**) includes identified issues and presents possible reasons as well as solutions to solve the problems. The presented workstation has been successfully applied to natural (gelatin, gellan gum, matrigel) and synthetic (e.g., poly(ethylene glycol) [PEG], Pluronic F127, Lutrol F127) polymeric materials for automated pipetting tasks. In particular, the

combination of an open source workstation and an open source protocol design application designed for viscous materials will be very useful for researchers working in the fields of biomedical engineering, material science, and microbiology.

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DISCLOSURES:

CM and DWH are founders and shareholders of Gelomics Pty Ltd. CM is also the Director of Gelomics Pty Ltd. The authors declare no conflict of interests relevant to the subject of this article. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the article apart from those disclosed.

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figure1

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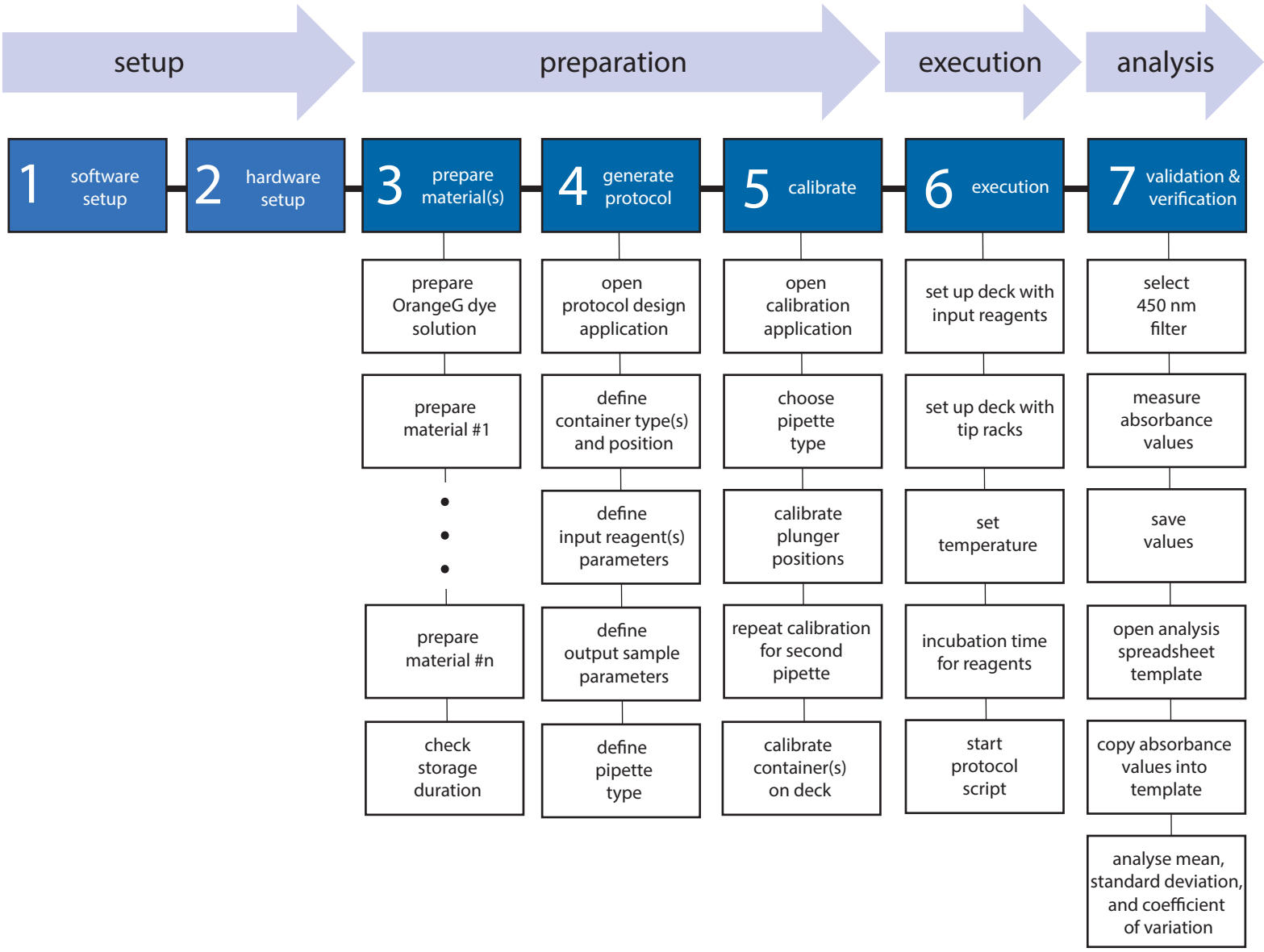

figure2

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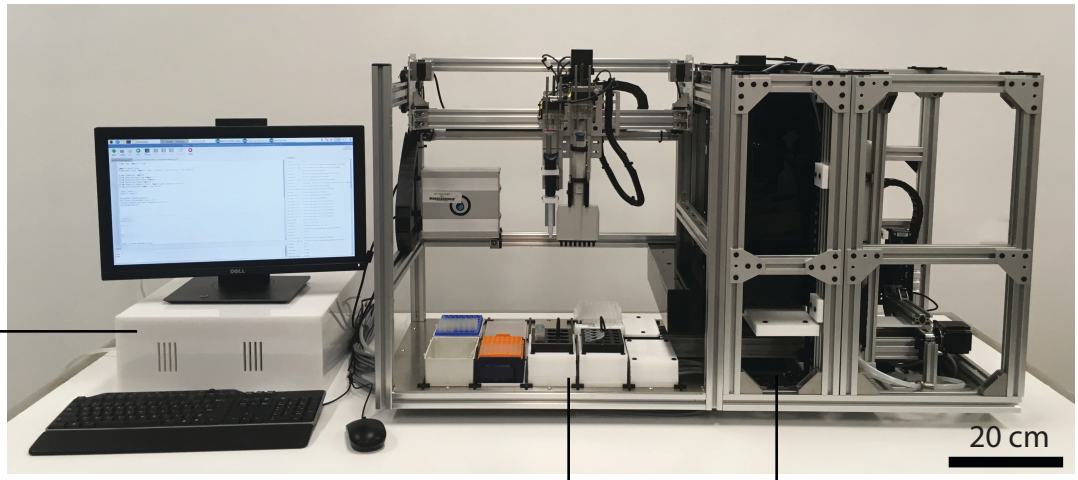
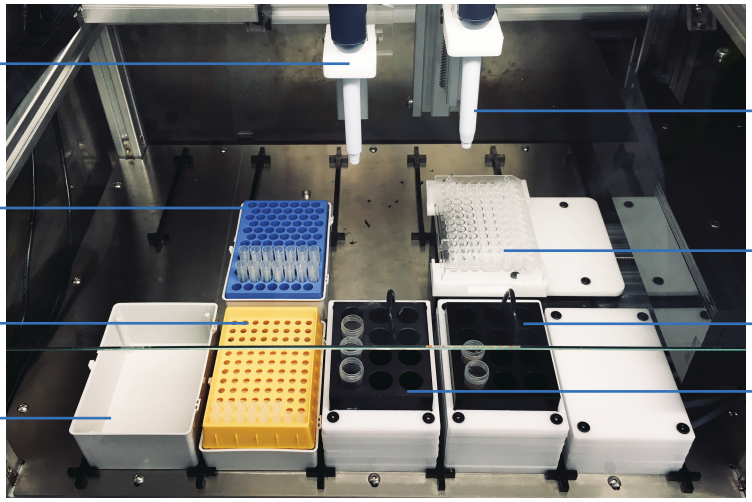
a

open source workstation



b

pipetting module: deck setup



c

pipetting module: definition of positions

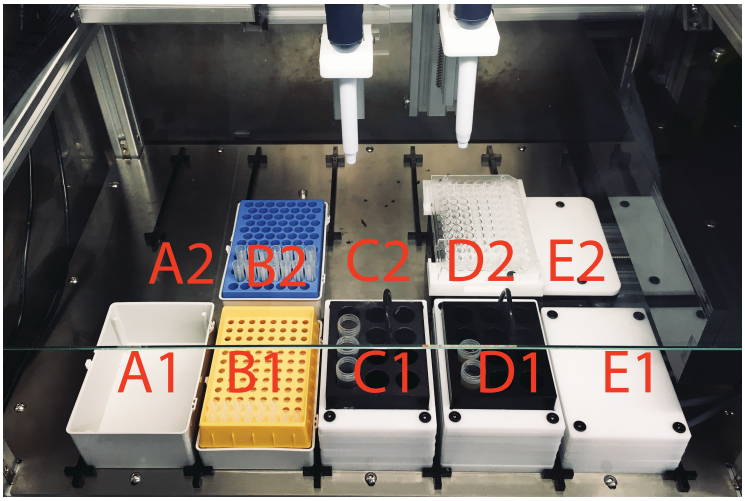


figure3

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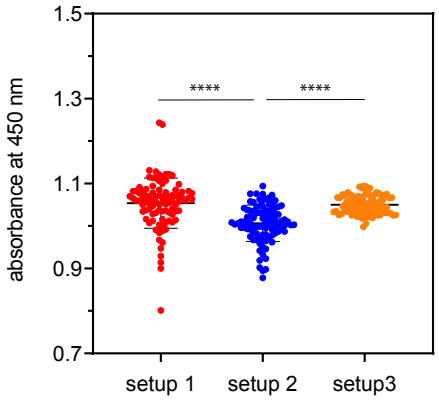
a

setup optimization for 85% glycerol

i

	setup 1	setup 2	setup 3
temperature	22 °C (± 0.3 °C)	40 °C (± 0.2 °C)	40 °C (± 0.2 °C)
tip touch	no	no	yes
coefficient of variation (CV)	5.6 %	4.2 %	2.0 %

ii



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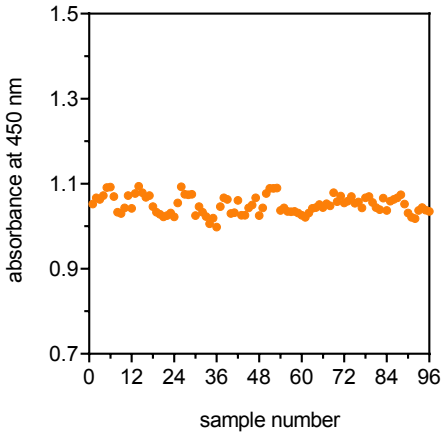
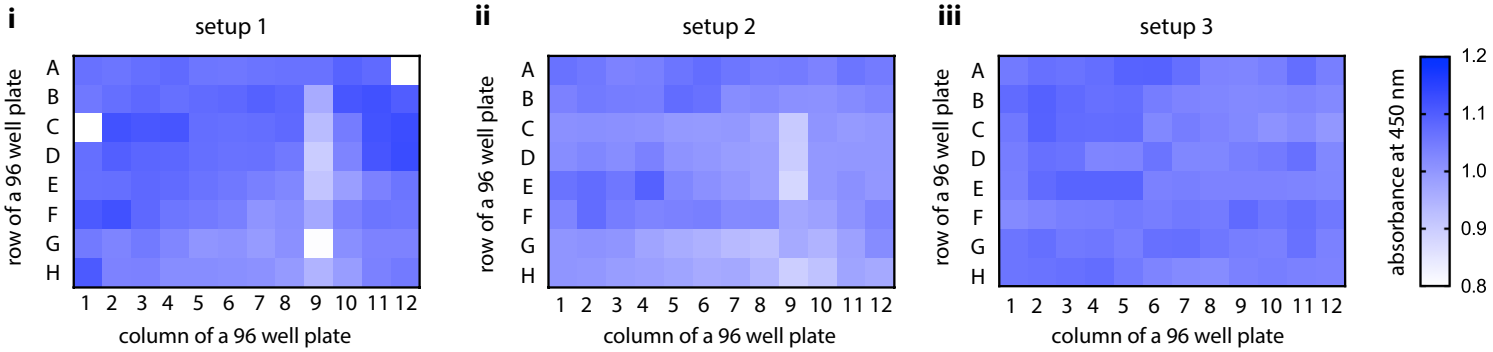


b

absorbance value distribution on a 96 well plate

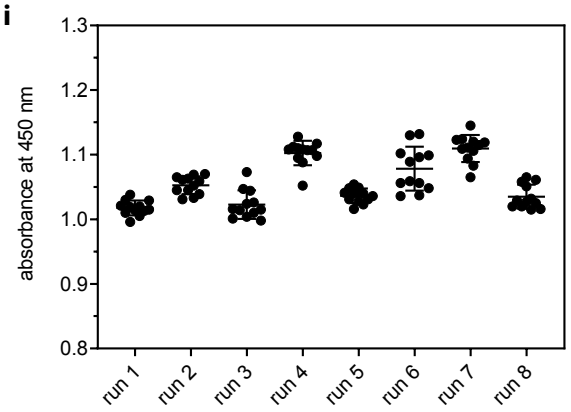
i ii iii



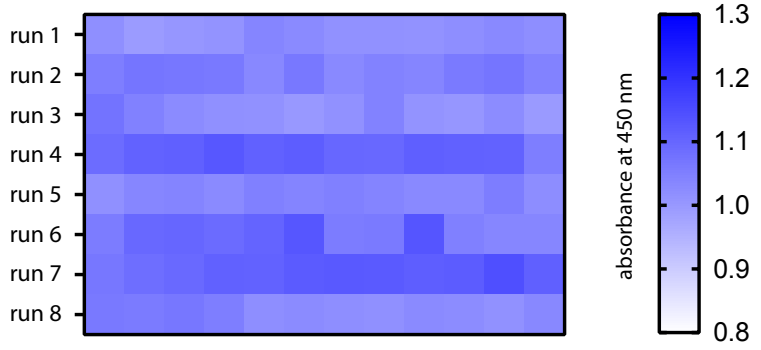
c

reproducible mixing in 8 independent repeats with setup 3

i

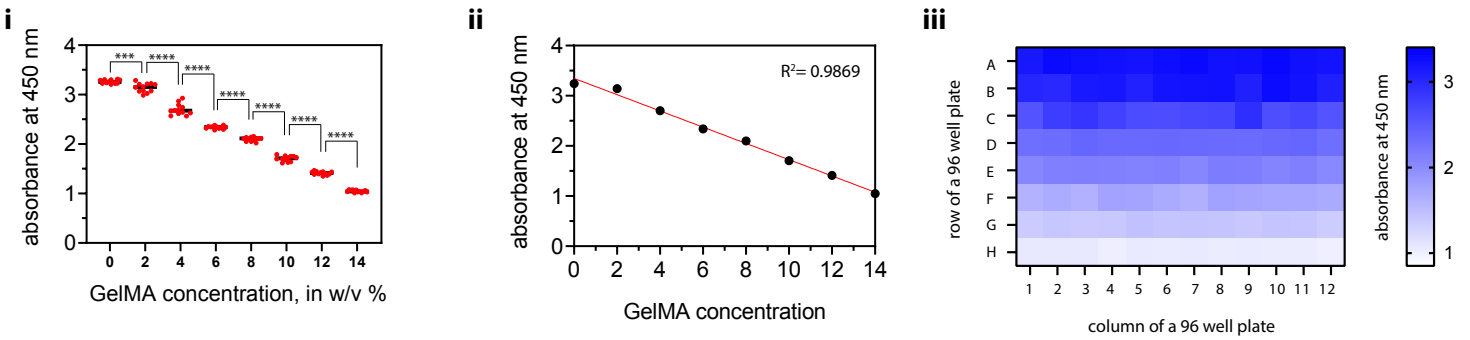


ii



a

Dilution series of gelatin methacryloyl (GelMA)



b

Generation of a 5% GelMA, 2% alginate, and 0.15% LAP mixture

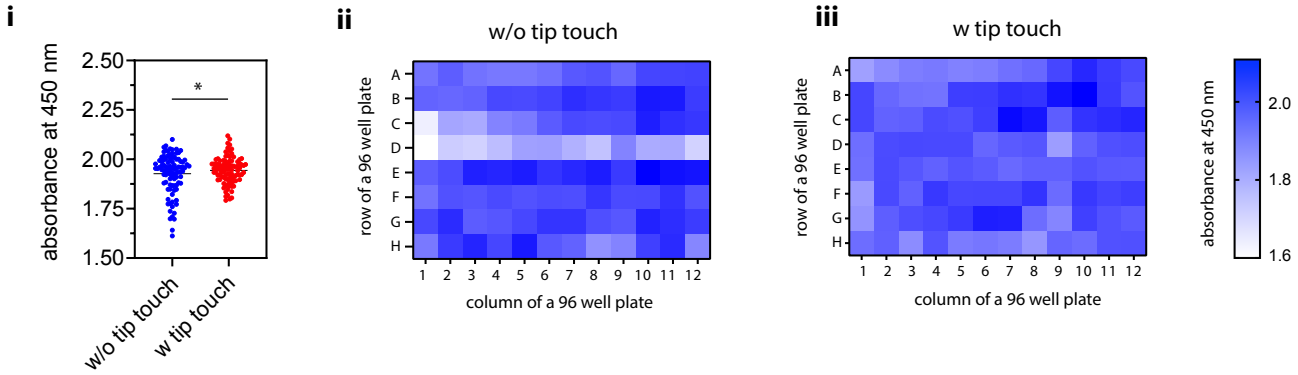


Figure5

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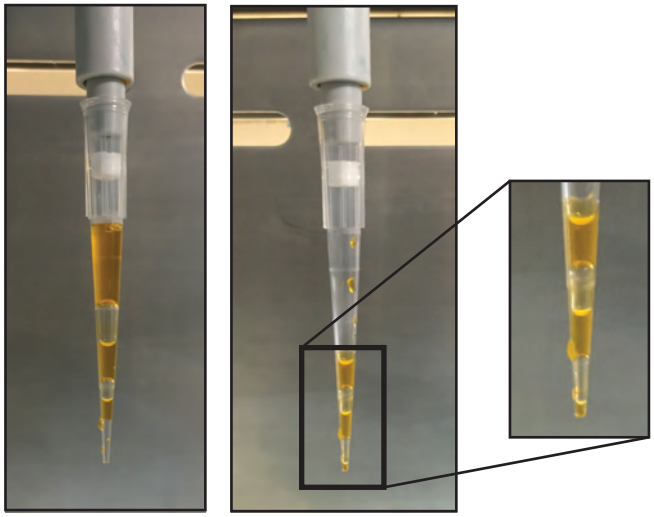
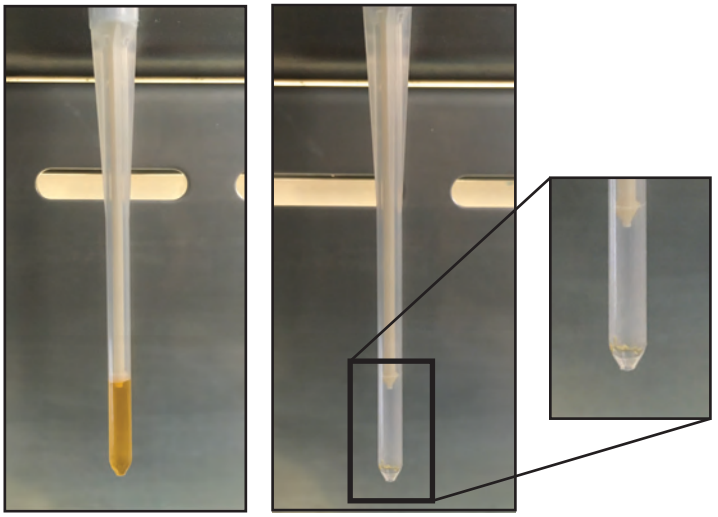
a air cushion pipette

aspirated dispensed

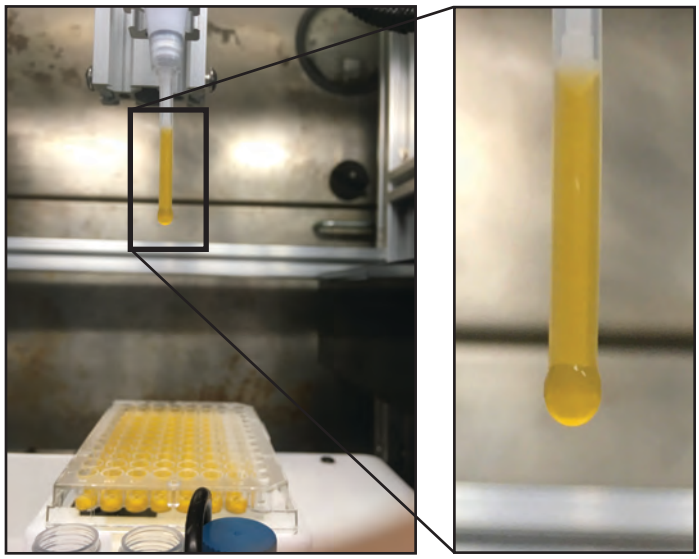


b positive displacement pipette

aspirated dispensed



c excess material on tip



d tip touch to remove excess material

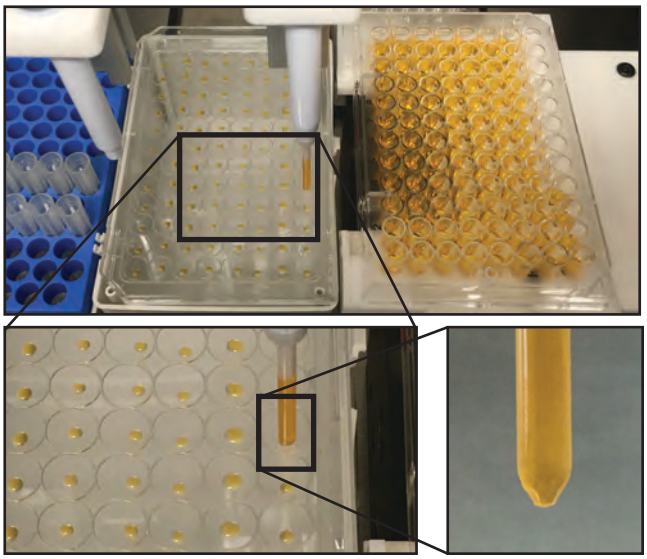


Table1

Material #1 (stock concentration)	**Final concentration of material #1**	**Material #2 (stock concentration)**
Glycerol (85% [w/v])	80% (w/v)	
GelMA (20% [w/v])	0% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	2% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	4% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	6% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	8% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	10% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	12% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	14% (w/v)	LAP (3% [w/v])
GelMA (20% [w/v])	5% (w/v)	Alginate (4% [w/v])

Final concentration of material #2	Materials #3 (stock concentration)	Final concentration of material #3
0.15% (w/v)		
0.15% (w/v)		
0.15% (w/v)		
0.15% (w/v)		
0.15% (w/v)		
0.15% (w/v)		
0.15% (w/v)		
0.15% (w/v)		
2% (w/v)	LAP (3% [w/v])	0.15% (w/v)

Diluent (Orange G working solution)	**Final Orange G concentration in mixture**	**Displayed in figure**
water (1 mg/mL Orange G)	0.059 mg/mL	Figure 3a−c
PBS (1 mg/mL Orange G)	1 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.85 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.75 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.65 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.55 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.45 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.35 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.25 mg/mL	Figure 4a
PBS (1 mg/mL Orange G)	0.2 mg/mL	Figure 4b

Protocol step	Problem	Possible reason
1.1	Software cannot be installed or updated	Running out of disk space on SD card
1.2	API cannot be installed	Users ability for installation is restricted (no root user permission)
3.1	Troubles with GelMA	Functionalization, dialysis or lyophilization
5.1 and 6.2	Workstation is not reacting to commands	Connection issues
5.1 and 6.2	Workstation is not reacting to commands	Connection issues
5.1 and 6.2	Cannot open file	Wrong directory
6.6.2	Tip is not properly attached or falls during movement	Calibration issue
6.6.2	Tip is not properly attached or falls during movement	Attachment issue
6.6.2	Tip is aspirating above material	Calibration issue
6.6.2	Tip is aspirating above material	Calibration issue
6.6.2	Material is dipping during movement	Too much excess material on tip
6.6.2	Material is solid or too viscous for pipetting	Thermoresponsive behaviour of material

https://support.opentrons.com/en/articles/2687601-c-having-trouble-cor

Solution
Check disk space on SD card. If required, remove unnecessary items, empty bin, or use an appropriately sized SD card
Use ‘sudo’ command in front of specified commands to gain admin rights. In Linux, this kind of access is known as the superuser.
Detailed step-by-step protocol including troubleshooting list available in Loessner et al.[33].
Turn of everything and shut down the computer. Switch off the power supply for 10 s. Power computer and workstation back on.
Check if the computer is recognizing the USB connection and the USB port is defined correctly. Make sure that firewall is not preventing the connection process (see the link below Table 2).
Check director (folder path) to ensure that the right path is being used. If a file (e.g., interface.py) cannot be found, it is likely that the wrong path is being used.
Repeat calibration steps for the pipette and make sure that the capillary piston is connected properly with the pipette.
Pipette is not properly connected to the pipette axis and moves during the movement steps. Tighten screws firmly to prevent this.
Repeat calibration of this tray type to define height properly.
Check volume in tube and make sure that the volume equals the volume defined in the protocol designer application.
Add tip touch dock option; optionally, also the time for tip touch can be increased.
Check thermoresponsive material characterization and adjust heating/cooling temperature of temperature dock accordingly.

nnecting-try-this-basic-troubleshooting

Name	Company	Catalog Number	
Material			
15 reaction tubes	Fisher Scientific, Inc. (USA)	14-959-53A	
5 mL tubes	Pacific Laboratory Products Australia Pty. Ltd. (Australia)	SCT-5ML	
50 mL reaction tubes	Fisher Scientific, Inc. (USA)	14-432-22	
70% w/w Ethanol	LabChem, Inc. (USA)	aja726-5Lpl	
96-well plate	Thermo Fisher Scientific, Inc. (USA)		
Alginate	NovaMatrix		4200001
Demineralized or ultrapure (MilliQ) water			
Gelatin methacryloyl (GelMA)	Synthetized in-house		
Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)	Sigma-Aldrich, Inc. (USA)		900889
M4 and M5 Allen key	OpenBuilds, inc. (USA)	179, 190	
OrangeG	Fisher Scientific (USA)	O267-25	
Phosphate-buffered saline (PBS)	Thermo Fisher Scientific, Inc. (USA)	14190-144	
Equipment			
Aluminium blocks for temperature dock	Ratek Instruments Pty. Ltd. (Australia)	SB16	
Analytical balance	Sartorius AG (Germany)	ED224S	
Open source liquid handling robot: commercial product	Opentrons Laboratories, Inc. (USA)	OT-One S Pro	
Open source liquid handling robot: open	Assembled in-house following an open source		

source hardware	approach	
Positive displacement pipette: MicromanE	Gilson, Inc. (USA)	FD10006
Spectrophotometer	BMG LABTECH GmbH (Germany)	CLARIOstar
Tips: capillary pistons	Gilson, Inc. (USA)	F148180

Comments/Description

size depends on experimentl protocol; also Eppies (0.5, 1, 1.5 mL) or Falcon tubes (15, 50mL) can be used; product is manufactured by Axygen, Inc.

detailed protocol (incl materials and references) is available in Loessner et al. (2016), Nature Protocols

also available in every hardware store

alternativly: PBS tablets: 18912014 (Thermo Fisher Scientific)

blocks for different tube sizes are available

hardware and software files are freely accessible on GitHub and Zenodo (links provided); building

on GitHub and Zenodo (links provided), building instructions are provided

depends on required size

depends on required size

Link

https://www.pacificlab.com.au/shop/tubes-plastic/sct-5ml-tubewith-screwcap-blue-unassembled-5ml-self-standing/1/name

https://www.thermofisher.com/order/catalog/product/168055
https://www.novamatrix.biz/store/pronova-up-lvg/

https://www.nature.com/articles/nprot.2016.037

https://openbuildspartstore.com/allen-wrench/
https://www.fishersci.com/shop/products/orange-g-certified-biological-stain-fisher-chemical/O26725

http://www.ratek.com.au/products/SB16-Block-with-12x16mm-holes.html

https://shop.opentrons.com/products/ot-one-pro

https://github.com/SebastianEggert/OpenWorkstation

https://zenodo.org/record/3612757#.XipEjBV7F24

https://www.gilson.com/default/shop-products/pipettes/positive-displacement.html

https://www.gilson.com/default/shop-products/pipette-tips.html?technique_en_ww_lk=191

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Xiaoyan Cao, Ph.D.
Review Editor
Journal of Visualized Experiments

1 Alewife Center, Suite 200

Cambridge, MA 02140, USA

March 3rd, 2020

Dear Dr. Cao,

we thank you for providing feedback on the manuscript.

The comments added to the manuscript are addressed in the revised version and general comments are added below. All changes have been tracked within the revised manuscript.

Sincerely yours

Dietmar W. Hutmacher PhD, MBA

Editorial comments:

Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

The attached version was used to incorporate the request changes.

In the JoVE Protocol format, “NOTE” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Please consider moving some of the notes about the protocol to the introduction/discussion section as appropriate. See specific comments marked in the attached manuscript.

The marked “NOTE” sections which included a general description were moved to the introduction and discussion section. Current “NOTE” sections are essential to replicate the presented protocol and provide detailed information or recommendations.

Please address specific comments marked in the attached manuscript.

Your comments are addressed in the revised manuscript.

After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the

highlighting. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Key aspects of the protocol were highlighted in yellow, while “NOTE” sections were excluded. Your additional considerations were taken into account.

Date:	January 20, 2020
Username:	Sebastian Eggert
Path:	C:\desktop\glycerol
Description:	Dilution of glycerol (stock solution with 85% (v/v) to 80% (vv)
Dye:	OrangeG
Well-plate format:	96-well plate

Absorbance values are displayed as OD: see next table for war data

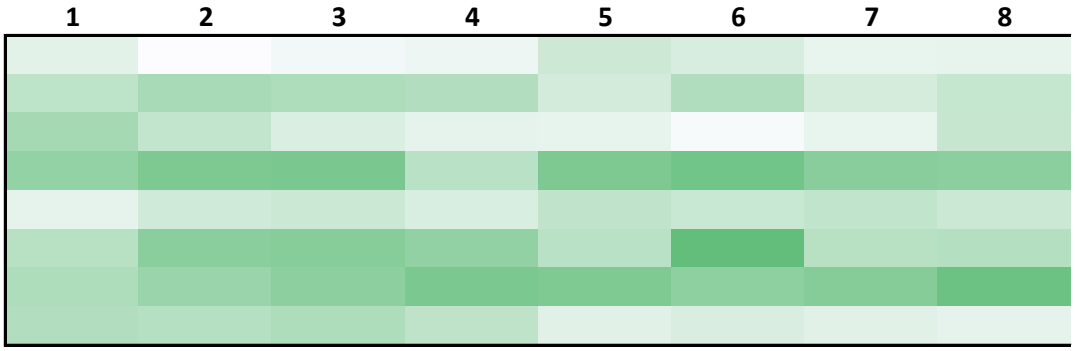
insert raw data from absorbance readings here

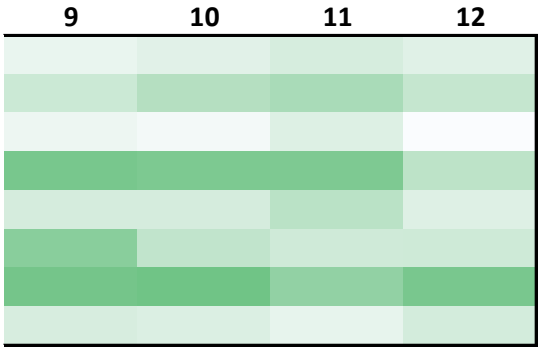
	1	2	3	4	5	6	7	8	9
A	1.052	1.067	1.063	1.072	1.091	1.092	1.07	1.033	1.03
B	1.077	1.094	1.079	1.068	1.072	1.046	1.033	1.028	1.022
C	1.055	1.093	1.075	1.074	1.075	1.025	1.046	1.033	1.023
D	1.046	1.067	1.063	1.03	1.032	1.061	1.026	1.026	1.043
E	1.043	1.077	1.089	1.089	1.09	1.037	1.043	1.035	1.034
F	1.021	1.031	1.042	1.044	1.051	1.044	1.053	1.048	1.079
G	1.059	1.07	1.054	1.057	1.043	1.067	1.07	1.056	1.044
H	1.059	1.063	1.067	1.074	1.052	1.031	1.021	1.018	1.037

10	11	12
1.043	1.072	1.042
1.025	1.031	1.022
1.006	1.019	0.998
1.05	1.067	1.025
1.035	1.031	1.026
1.058	1.071	1.055
1.039	1.066	1.037
1.044	1.038	1.035

	average	Std	CV
uniform	1.050094	0.021397	**2.0%**
by rows			
by rows: A	1.060583	0.01986	1.9%
by rows: B	1.04975	0.025259	2.4%
by rows: C	1.0435	0.029514	2.8%
by rows: D	1.044667	0.016054	1.5%
by rows: E	1.052417	0.024513	2.3%
by rows: F	1.04975	0.015062	1.4%
by rows: G	1.055167	0.011481	1.1%
by rows: H	1.044917	0.017342	1.7%
sum	1.050094	0.021397	**2.0%**
by columns			
by column 1	1.0515	0.015033	1.4%
by column 2	1.07025	0.018512	1.7%
by column 3	1.0665	0.013766	1.3%
by column 4	1.0635	0.017706	1.7%
by column 5	1.06325	0.020505	1.9%
by column 6	1.050375	0.020554	2.0%
by column 7	1.04525	0.017319	1.7%
by column 8	1.034625	0.011379	1.1%
by column 9	1.039	0.016941	1.6%
by column 10	1.0375	0.015025	1.4%
by column 11	1.049375	0.020291	1.9%
by column 12	1.03	0.0157	1.5%
sum	1.050094	0.021397	**2.0%**

individual




9
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12

Supplemental_coding_files

Click here to access/download

Supplemental Coding Files

supplementary_protocol-code.zip