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TITLE:

Method for High Speed Stretch Injury of Human Induced Pluripotent Stem Cell-derived Neurons in a 96-Well Format

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KEYWORDS:

Stretch, neurotrauma, neuron, human induced pluripotent stem cell, silicone, cell culture, high content imaging

SUMMARY:

Here we present a method for a human *in vitro* model of stretch injury in a 96-well format on a timescale relevant to impact trauma. This includes methods for fabricating stretchable plates, quantifying the mechanical insult, culturing and injuring cells, imaging, and high content analysis to quantify injury.

ABSTRACT:

Traumatic brain injury (TBI) is a major clinical challenge with high morbidity and mortality. Despite decades of pre-clinical research, no proven therapies for TBI have been developed. This paper presents a novel method for pre-clinical neurotrauma research intended to complement existing pre-clinical models. It introduces human pathophysiology through the use of human induced pluripotent stem cell-derived neurons (hiPSCNs). It achieves loading pulse duration similar to the loading durations of clinical closed head impact injury. It employs a 96-well format that facilitates high throughput experiments and makes efficient use of expensive cells and culture reagents. Silicone membranes are first treated to remove neurotoxic uncured polymer and then bonded to commercial 96-well plate bodies to create stretchable 96-well plates. A custom-built device is used to indent some or all of the well bottoms from beneath, inducing equibiaxial mechanical strain that mechanically injures cells in culture in the wells. The relationship between indentation depth and mechanical strain is determined empirically using high speed videography of well bottoms during indentation. Cells, including hiPSCNs, can be

cultured on these silicone membranes using modified versions of conventional cell culture protocols. Fluorescent microscopic images of cell cultures are acquired and analyzed after injury in a semi-automated fashion to quantify the level of injury in each well. The model presented is optimized for hiPSCNs but could in theory be applied to other cell types.

INTRODUCTION:

TBI is a major cause of mortality and morbidity in the United States, causing around 52,000 deaths and 275,000 hospitalizations every year[1]. More than 30 clinical trials of candidate therapeutics for TBI have been conducted without a single success[2]. This uniform failure suggests that human-specific processes separate human TBI from the pathophysiology observed in commonly used pre-clinical rodent models.

The advent of hiPSCNs has created an opportunity to study neurotrauma in a human *in vitro* model. Drug screening with hiPSCN-based models may deliver results that are more predictive of clinical success than models employing rodent cells. Also, hiPSCNs can be genetically manipulated to isolate and study the effect of individual human genetic variants on pathology[3].

The method described in this manuscript is designed to bring the unique advantages of hiPSCN-based disease modeling to neurotrauma. *In vitro* stretch injury models of neurotrauma are well established[4-6] with primary rodent cells and human neural cancer cell lines. Most of these models generate stretch by pneumatically loading a silicone membrane. This approach is effective in a single well format but has proven difficult to scale up to a multi-well format[7]. As a result, there has never been a high throughput screen for agents to treat stretch injured neurons.

In this model, the membrane stretches due to indentation from underneath with a rigid indenter. This approach has been shown repeatedly to generate clinically relevant pathology *in vitro* in single well systems[8-10]. Our recent work has shown that it easily scales up to a 96-well format while maintaining pulse durations on the order of tens of milliseconds[11], which is the time domain of closed head impact events[12,13].

In summary, the key advantages of this *in vitro* injury model are the 96-well format, the use of hiPSCNs, and the clinically relevant time domain of the insult.

PROTOCOL:

1. Silicone Detoxification

1.1. Cut 254 µm thick, 30.48 cm x 30.48 cm silicone membranes into 7.5 cm x 11 cm rectangles using a razor blade and an acrylic template. 10 rectangular membranes can be made with each sheet. Save the paper that comes with the silicone.

1.2. Place the membranes in a tub of deionized (DI) water with glassware soap. One at a time, scrub the membranes vigorously with gloved fingertips for at least 20 s or until lathered.

1.3. Rinse the membranes under running DI water until the lathered soap is visibly removed. Lay the membranes on the paper (from their packaging) and autoclave on a gravity cycle.

1.4. Soak each silicone membrane in 250 mL of 70% ethanol per membrane on an orbital shaker at 60 rpm for 24 h. Use a container made from a material that does not react with ethanol, such as polypropylene.

1.4.1. If more than one membrane is placed in a single bin, or if the membranes stick to the bottom of the bin, separate the membranes from their environment with plastic pipette tips and pipette tip racks.

1.5. Transfer the silicone membranes with gloved hands to 250 mL of DI water per membrane and soak on the orbital shaker for 48 h at 60 rpm.

1.6. Lay the membranes back on the paper and place in a 93 °C glassware oven for 4 h.

1.7. Store the membranes on the paper, in a clean and dry place, covered with another sheet of paper to protect them from dust.

2. Plate Fabrication

2.1. Plasma treat a 96-well plate top with a 200 W plasma cleaner (see **Table of Materials**) for 60 s on high power, placing it bottom-side-up inside the plasma cleaner. Check for a purple glow visible through the window of the chamber, which indicates that an effective plasma has formed. This bonding technique is adapted from Sunkara *et al.*[14]

2.2. Within 60 s, place the plate top in 200 mL of 1.5% (3-Aminopropyl) triethoxysilane (APTES) in DI water for 20 min, bottom-side-down. This solution is unstable: prepare it no more than 60 s before introducing the plate.

Caution: Work with APTES in a fume hood.

2.3. Plasma treat an extracted silicone membrane in the plasma cleaner for 60 s on high power. Time the plasma treatment such that it finishes no more than 5 min before the end of the 20 min period in step 2.2.

2.4. Use forceps to position the plasma treated membrane on top of a 7.5 × 11 cm^2 parchment paper rectangle. Align a 7.5 cm x 11 cm x 0.5 cm aluminum slab on the lower portion of the plate fabrication clamp. Align the silicone membrane and parchment paper on the aluminum slab using forceps.

Note: Computer-aided design (CAD) files for this device are provided in the supplemental materials as a supplementary code file '**Press Die - Generic 3D.STEP**'. The associated bill of

materials is supplied in **Supplementary Table 1: Custom Built Devices – BOM.xlsx**.

2.5. Remove the plate tops from the APTES bath and shake off excess solution. Dip into a 200 mL DI water bath for 5 s and shake off excess water. Dip into a different 200 mL DI water bath for 5 s and shake off excess water. Replace the water in these baths before dipping another plate top.

2.6. Use compressed air to completely dry the plate top.

2.7. Place the plate top in the upper portion of the plate fabrication clamp. Gently close the plate fabrication clamp to press the plate top and silicone together. Clamp for at least 1 h.

2.8. Allow the plate to cure for 24 h at room temperature before use, protected from dust.

3. Stretching a Plate

3.1. Clean the indenters.

Note: See **Supplementary Figure 1**.

3.1.1. Prepare a 60 W bath-style sonicator with DI water at room temperature.

3.1.2. Support the indenter block above the sonicator bath, inverted so that only the tops of the indenters are submerged to a depth of at least 1 mm. Sonicate the indenters for 8 min at 42 kHz.

3.1.3. Blow dry the indenters with compressed air.

3.2. Align the indenter block.

3.2.1. Position a camera with a live feed above the stretching device so that it is looking down on the indenter array. Focus on the tops of the indenters.

Note: The setup described in Section 4, "Characterizing Membrane Stretch," is one possible camera setup for this task.

3.2.2. Secure a plate on the stage of the injury device using the stage clamps (**Figure 1**) and place a dome light over the device.

Note: See the **Table of Materials** for the instrument control software.

3.2.3. Launch the instrument control software by clicking on the software icon on the desktop of the computer that is controlling the injury device. Run the "in_vitro_neurotrauma.lvproj" project by clicking on it in the launch window. From the project window, launch the motion control and position tracker virtual instruments (VIs), which are named 'motion_control.vi' and 'position_tracker.vi', respectively, by double-clicking on them.

3.2.4. Close the cage surrounding the injury device. Press the **arrow** button in the top left corner of the motion control VI to run the VI and click **Near Bottom** to lower the stage to within 2 mm of contacting the indenters. Click the **Stop** button to stop the VI.

Caution: Keep hands clear of the stretcher when manipulating the camera in the cage. The cage should be fitted with a door switch that delivers power to the stretching device only when the cage door is closed.

3.2.5. In the project window, right click on **Axis 1.** Click on **Interactive Test Panel**. In the window that opens, set the step size to 50 µm by entering '500' units in the **Target Position** field.

3.2.6. Click the green **go** button at the bottom of the **Interactive Test Panel** window repeatedly until the plate first makes contact with any indenters. Check for contact on the live image displayed by the camera. Note the vertical stage position reported in the top left of the **Interactive Test Panel** window; this is the first contact position.

3.2.7. Lower further (as described in step 3.2.6) until every well has made contact with the indenters. If necessary, move the camera to see the whole plate and move the stage up (by specifying a negative **Target Position**) and down. Note the vertical stage position reported in the top left of the window when all posts are in contact (this is the full contact position).

3.2.8. Note the difference between the first contact and full contact positions. Close the **Interactive Test Panel**. Run the **motion control VI** (see step 3.2.4) and click on **Top** to raise the stage. Stop the motion control VI with the **Stop** button.

3.2.9. Once the stage is at the top of its travel, open the door to deactivate the device. Adjust the set screws on the corners of the indenter block. Loosen the set screw on the corner that made contact first, to lower it and tighten the opposite screw to raise the corner that made contact last.

3.2.10. Repeat the process of lowering the stage until the plate contacts the indenters, inspecting the contact images for tilt, raising the stage, and adjusting (step 3.2.9) the indenter block. When the stage and block are aligned, all indenters will make contact at once.

3.2.11. Open the door to deactivate the device. Insert the tie-down screws into their holes on the indenter block and tighten them. Confirm that the block is level with the tie-down screws in place (steps 3.2.4–3.2.8). Take note of the stage position reported by the **Interactive Test Panel** when the plate makes contact. This will be the zero position for indentation experiments.

3.3. Lubricate the indenters.

3.3.1. If the indenter block has just been set up and has not yet been lubricated, clean a solid rubber pad (7.5 cm x 11 cm x 1.5 mm) and a soft, closed-cell foam rubber pad (7.5 cm x 11 cm x 3 mm) with an ethanol-soaked lab wipe. Allow them to air dry.

3.3.2. Soak a lab wipe in corn oil and spread it on the solid rubber pad to a dull shine.

3.3.3. Place the foam pad onto the solid rubber pad to transfer oil to the foam pad. Place a 7.5 cm x 11 cm x 0.5 cm aluminum slab on top of the foam pad and load it with a ballast weight of approximately 360 g (*e.g.*, 6 conical tubes loaded with 45 mL of water each) to ensure consistent transfer of oil from the solid rubber pad to the foam pad. Allow 10 s for the oil to transfer to the foam rubber pad.

3.3.4. Move the foam rubber pad onto the indenter array. Place the aluminum slab and the ballast weight on top of it to ensure consistent transfer of the oil to the tips of the indenters. Allow 10 s for the oil to transfer to the indenters.

3.3.5. If no plate has been stretched since the indenter block was set up and lubricated for the first time, stretch a test plate before beginning the experiment.

Note: This will prevent any inconsistency between the first stretch and subsequent stretches from confounding the experiment. For subsequent stretches, repeat only steps 3.3.2–3.3.5 before each stretch.

3.4. Stretch a plate.

3.4.1. Lubricate the indenters as described in step 3.3.

3.4.2. Secure the plate on the stage of the injury device with the stage clamps. If sterility is required, adjust the position of the lid to secure the plate without exposing the culture surface to room air.

3.4.3. Lower the stage to the zero-position using the **motion control VI** (see steps 3.2.4–3.2.6); zero-position is determined during step 3.2.

3.4.4. Change the file name in the “file path” field in the **position tracking VI** to a unique file name, then run it with the **arrow** button in the top left corner of that window.

3.4.5. Set the depth and duration of indentation (typically 1–4 mm and 30 ms, respectively, maximum depth 5 mm, minimum duration 15 ms) in the **Injury (mm)** and **Injury Duration (ms)** fields in the **movement control panel VI** by clicking on the fields and typing in the desired values.

3.4.6. Run the **movement control panel VI** and click on **Injure** to indent the plate.

3.4.7. Move the stage up to the top of its travel by clicking on **Top**. Then stop the VI with the **Stop** button and deactivate the injury device by opening the door.

3.4.8. Inspect the displacement history of the stage presented in the **position tracker VI** to confirm that the specified maximum displacement was applied. Right click on the graph and click on **export to Excel**. Click on **File**| **Save as** to save the data.

4. Characterizing Membrane Stretch

4.1. Paint the recess at the top of each indenter white to provide a high contrast background for high speed videography.

Caution: Do not paint the rims of the indenters where they make contact with plates.

4.2. 3D print with poly(lactic acid) (PLA), or otherwise fabricate, a cylindrical stamp with which to make a dot in each well. Make the cylinder 5.9 mm in diameter and 12.2 mm high, with a cylindrical protrusion 1.5 mm diameter and 1.0 mm high centered on top.

Note: A 3D printable model '**stamp.STL**' is available as a supplementary file.

4.3. Plasma treat the plate right-side-up to activate the silicone cell culture surface in the wells for 60 s, as mentioned in step 2.1.

4.4. Place the plate on a rubber pad or other soft surface. Prime the small protrusion on the stamp (see step 4.2) with ink from a permanent marker pen. Insert the stamp into the well to be tested and tap to ensure good transfer of ink. Prime the stamp before every well.

4.5. Align the indenter block and lubricate the indenters of the injury device (see steps 3.2–3.3). Clamp the plate onto the stage of the injury device. Position a bright diffuse axial light above the injury device.

4.6. Set up a high-speed camera on a boom stand over the injury device, facing straight downward, with the lens set to the smallest numbered f-stop, and turn it on. Launch the camera software on the computer connected to the camera. In the **frame rate** drop down menu, select **2,000** frames per second, and in the **shutter** drop down menu, select the fastest exposure time which yields high contrast images. Center over the dotted wells.

4.6.1. Position the camera so that the field of view contains 12 wells in a 3 x 4 grid.

Note: This field of view offers an optimal compromise between throughput and resolution for a 1,280 × 1,024 image.

4.7. Lower the plate to the zero-point (see steps 3.2.4–3.2.6). One-click the record button on the camera software so that it reads **trigger in**. Initiate the position tracker VI (step 3.4.4).

4.8. Turn on the bright diffuse axial light. Indent the plate as described in step 3.4.6.

4.9. Turn off the bright diffuse axial light.

4.10. On the camera control computer, find the 30–40 ms window of the recording in which the indentation occurs: drag the beginning and end arrows in the high-speed video play bar in the camera software. Click **Save**, set the name in the **File Name** field, select **TIFF** in the **Format** field, and click on **Save**.

4.10.1. Look through the .TIF-files for images of the least stretched (beginning) and most stretched (peak indentation) states.

4.10.2. To measure the height and width of the dots in both images, use Fiji to open the images of the least and peak stretch side-by-side. Using the default rectangular selection tool, click and drag to draw a box around a dot in the least stretch image. Measure the height and width with **Analyze**| **Measure**.

4.10.3. Repeat for the dot in the same well on the peak stretch image. Repeat for the rest of the wells.

4.11. Calculate the Lagrangian strain in the *x* and *y* directions as follows:

$$E_{xx} = \frac{X_f - X_i}{X_i} + \frac{1}{2}\left(\frac{X_f - X_i}{X_i}\right)^2$$

$$E_{yy} = \frac{Y_f - Y_i}{Y_i} + \frac{1}{2}\left(\frac{Y_f - Y_i}{Y_i}\right)^2$$

Note: Here, E_{xx} is the Lagrangian strain in the *x* direction, E_{yy} is the Lagrangian strain in the *y* direction, *X* is the width of the dot, *Y* is the height of the dot, $_f$ denotes the final image (*i.e.*, the peak indentation image), and $_i$ denotes the initial image (*i.e.*, the pre-indentation image). Average the two values to determine the strain in that well.

5. Plating the Cultured Cells

5.1. Autoclave the bins and ballast weights that will be used for sterilization.

5.2. Plasma treat the silicone-bottomed plates right-side-up for 60 s (see step 2.1). Immediately submerge the plates in sterile bins containing 70% ethanol for 15 min.

5.3. Submerge the plates in sterile phosphate buffered saline (PBS) in separate sterile bins for 30 min.

5.4. Aspirate the PBS from the wells. Only dry one plate at a time to prevent the wells from losing the plasma treatment.

Caution: Silicone bottomed plates provide less tactile feedback than rigid plates when pipetting and are more likely to block the tip of a pipette.

5.5. Add 100 µL of 0.1 mg/mL poly-L-ornithine (PLO) per well to the sterilized plates. Incubate at room temperature for 1 h.

5.6. Rinse the wells twice with 100 µL sterile PBS, leaving the second wash on the wells until the cell suspension is ready.

5.7. Remove the vial of hiPSCNs from liquid nitrogen storage using safety gloves and place on dry ice. Quickly transport the vial to a 37 °C water bath and thaw for exactly 3 min. Do not swirl the vial.

5.8. In a sterile hood, gently transfer the contents of the vial to a 50-mL conical tube using a 1 mL serological pipette.

5.9. Rinse the empty cryogenic vial with 1 mL of room temperature complete maintenance medium (media base + supplement).

5.10. Transfer the 1 mL of media into the 50-mL tube drop-wise at about one drop per second. Gently swirl the tube while adding. Add 8 mL of room temperature complete maintenance medium to the 50-mL tube at about 2 drops/s.

5.11. Cap the tube and invert 2–3 times. Count the cells with a hemocytometer. Compute the volume of the additional media required to dilute the cell suspension to 225,000 cells/mL.

5.12. Gently pipette the amount of media (calculated above) into the tube of cell suspension using a 25 mL serological pipette.

5.13. Add 10 µL of 1 mg/mL laminin stock per 1 mL of cell suspension with a 1,000 µL micropipette to achieve 10 µg/mL of laminin in the cell suspension. Aspirate up and down once with the tip used for laminin, then cap the tube and invert the tube once.

5.14. Aspirate the PBS from the plates, one plate at a time. Use a multichannel pipette to add 100 µL of the hiPSCN cell suspension to each well. The wells have a culture area of 0.33 cm^2, so the cell density per area is 67,500 cells/cm^2.

5.15. Rest the plates at room temperature for 15 min after seeding to promote attachment. To avoid fan vibrations of sterile culture hood, place the covered plates on the lab bench. Incubate the cultures at 37 °C with 5% CO_2.

5.16. Perform a full media change at 24 h, refilling the wells with 200 µL of complete maintenance media. Perform a half media change of 100 µL/well every 2–3 days.

6. Injuring Cultures

6.1. Align the indenter block and find the zero position as described in step 3.2. Lubricate the indenters as described in step 3.3.

6.2. Set the filename for the displacement history in the **position tracker VI** (step 3.4.4). Set the injury parameters in the **motion control VI** (step 3.4.5).

6.3. Take the plate to be injured out of the incubator and clamp it onto the stage. Adjust the position of the lid to secure the plate without exposing the cultures to room air.

6.4. Lower the plate to the zero-point (steps 3.2.4–3.2.6) using the **motion control VI**. Start the **position tracker VI** (step 3.4.4).

6.5. Use the movement control VI to indent the plate (as described in step 3.4.6). For sham stretches, skip only this step.

6.6. Return the stage to the top of its range of motion (see step 3.4.7). Return the plate to the incubator.

6.7. Inspect and save the displacement trace (as in step 3.4.8).

7. Microscopy

7.1. Prepare a 10x staining solution with 2 µg/mL Hoechst 33342 and 5 µg/mL Calcein AM in maintenance media.

7.2. Stain each well with a 20 µL spike of 10x staining solution.

7.3. Incubate for 15 min with stain at 37 °C.

7.4. Acquire wide field fluorescent images. Use conventional FITC and DAPI filter sets to image the Calcein AM and Hoechst 33342 signals, respectively. If the multi-well imaging sequence will take more than 10 min, enclose the plate in a stage top incubator to maintain the health of the cultures.

Note: A 10x, 0.30 NA lens provides sufficient detail for determination of cell viability and morphology. Adjust gains to ensure good visualization of the neurites, even if this causes some saturation of the much brighter soma.

7.5. Segment live cell images in the green Calcein AM channel to identify soma and neurites. Use nuclear images in the blue Hoechst 33342 channel to assist with the identification of the soma.

REPRESENTATIVE RESULTS:

The stretcher device is capable of moving the stage repeatably with pulse durations as short as 10–15 ms depending on the amplitude of the pulse (**Figure 2A**). The pulse amplitudes are highly repeatable, but the pulse duration varies by approximately 1 ms between repetitions. The actual pulse amplitude diverges from the prescribed pulse amplitude when a large number of wells are loaded, and the prescribed amplitude is high (see **Figure 2B**). As the amplitude of stage displacement is increased beyond 3 mm, the actual displacement amplitude increasingly falls short of the prescribed displacement amplitude (see **Figure 2B**). Careful alignment of the post block eliminates any trend in the membrane strain across rows or columns (**Figure 2C**). At the 3.5 mm prescribed stage displacement amplitude (3.3 mm actual displacement amplitude) with 52 wells indented, the mean Lagrangian strain across all well locations was 0.451 (standard deviation of means for all locations = 0.051, mean of standard deviations for all locations = 0.065, n = 5 measurements per well). These results are presented here for completeness although some of them have already been reported[11].

An optimal, uninjured culture will have few if any clumps of more than 5 cells. Neurites will be individual, long, slender, and curved with little or no sign of tension or beading (**Figure 3A**). Under ideal conditions, the viability of the cultures should closely approach the viability specified in the manufacturer's data sheet (typically 60–70%) and cultures on silicone should resemble those maintained on conventional rigid culture substrates (**Figure 3B**). Neurites may or may not be visible on a low power bright field microscope. Laminin concentration and cell density both influence cultures at baseline and after injury. Increasing the cell density increased the number and size of clumps that formed in culture. Increasing the laminin concentration often counteracted this effect (**Figure 3A**). However, increasing the laminin concentration too much blunted the sensitivity of the cultures to injury (**Figure 4**). The optimal laminin concentration for uninjured cultures was 50 μg/mL of laminin (**Figure 3**), but the optimal separation between the sham and stretch injured populations was obtained at 10 μg/mL of laminin (**Figure 4**). Higher laminin concentrations reduced the sensitivity of the cultures to injury at short time points (**Figure 4**), but also improved baseline cell viability at longer time points (*e.g.*, 7 days). In summary, it is worthwhile to optimize the laminin concentration for each experimental scenario.

Membrane strain, post-injury imaging time point, laminin concentration, and cell density all exerted a highly statistically significant main effect on the neurite length per cell (ANOVA, $p < 0.001$). The effect of membrane strain on neurite length per cell had highly statistically significant interactions with post-injury imaging time point and laminin concentration (ANOVA, $p < 0.001$) and a statistically significant interaction with cell density (ANOVA, $p < 0.05$). Similarly, membrane strain, post-injury imaging time point, cell density, and laminin concentration all exerted a highly statistically significant main effect on cell viability (ANOVA, $p < 0.001$). The effect of the membrane strain on cell viability had a highly statistically significant interaction with the post-injury imaging time point (ANOVA, $p < 0.001$) and a statistically significant interaction with the cell density (ANOVA, $p < 0.05$). These results prove that timing, cell density, and laminin

concentration exert an important influence on the relationship between the applied insult and experimental outcomes, so each should be optimized carefully.

Low cell viability and beaded neurites, along with stunted neurite growth, indicate toxic culture conditions that can arise from improperly prepared silicone. Skipping or shortening the water soak or the oven dry can leave absorbed ethanol or water in the membrane, respectively, which can diffuse into the media and stress the cells. Well-injured cultures will have reduced cell viability, shortened or missing neurites, beaded neurites, and neurites that look taut or tensioned. Injury may induce clumping in cell cultures that were well-dispersed pre-injury. Large clumps can confound the morphological analysis. For morphological analysis, the injury level should be tuned such that noticeable changes occur, but cells are still present with some neurites.

FIGURE AND TABLE LEGENDS:

Figure 1: A labeled schematic of the injury device. (**A**) Top view, (**B**) isometric view, (**C**) front view, (**D**) right side view. The scale bar applies to the orthographic views (A, B and D).

Figure 2: Kinematics of the mechanical insult. (**A**) The stage displacement histories over 5 pulses at a range of prescribed amplitudes (prescribed amplitudes are listed in the legend) when no wells are loaded. (**B**) The stage displacement histories over 10 pulses at a range of prescribed amplitudes (prescribed amplitudes are listed in the legend) when 52 wells are loaded. (**C**) The average strain in each well with stage displacement amplitude of 3.3 mm (n = 5 measurements per well, average standard error per well = 0.029). Note that C4–F4 and C9–F9 are unstretched control wells. This figure has been modified from Sherman *et al.*[11]

Figure 3: Optimization of culture conditions on silicone. (**A**) The effect of varying cell density and laminin concentration on hiPSCN cultures on silicone. Clumping increases with increasing cell density and decreasing laminin concentration. Cell density and laminin concentration must be optimized to achieve mono-dispersed cultures. Mono-dispersed cultures are less vulnerable to artifacts during quantification. Note that the dynamic range has been adjusted to optimize visualization of the neurites. As a consequence, the much brighter soma are saturated. This presentation is preferred to the alternative of optimizing the dynamic range with respect to the soma, which renders the much dimmer neurites almost invisible. The condition highlighted by the red square was deemed to be optimal for *in vitro* stretch injury experiments. (**B**) Under optimal conditions, cultures on silicone membranes appear similar to cultures on conventional rigid substrates. The left panel shows hiPSCNs cultured at 33,750 cells/cm^2 with 3.3 µg/mL of laminin on a conventional, rigid 96-well plate (the cell culture substrate is a tissue culture treated cyclic olefin co-polymer). The right panel reproduces the panel outlined in red from (A). Scale bars = 100 µm.

Figure 4: The injury phenotype and its dependence on laminin concentration. (**A**) Healthy culture, using 10 µg/mL laminin and 67,500 cells/cm^2. Neurites are long with no beads. There are few dead nuclei, and few clumps. (**B**) Culture using the same culture conditions, injured with 57% peak strain and imaged after 4 h. Neurites are shortened or missing, and some have beads

(indicated by arrows). There are fewer Calcein AM-positive cells and more Calcein AM-negative (*i.e.*, dead) nuclei. Injury has increased clumping among the surviving cells. (**C**) 4 h after injury, neurite length per cell declines with increasing strain in a manner that depends on the laminin concentration. (**D**) 4 h after injury, cell viability declines with increasing strain in a manner that depends on the laminin concentration. (**E**) 24 h after injury, neurite length per cell declines with increasing strain in a manner that depends on the laminin concentration. (**F**) 24 h after injury, cell viability declines with increasing strain in a manner that depends on the laminin concentration. (n = 4 per bar, error bars are ± 1 standard deviation, scale bars = 100 μm). Strain values are deduced from stage displacement using data from a prior publication by Sherman *et al.*[11]

Supplementary Figure 1: Technical drawing of the indenter.

Supplementary Table 1: Custom Built Devices.

Supplementary Table 2: 96 Well Plate-loader Pinout Wiring Diagram.

Supplementary Code File 1: Computer-aided design drawings of the injury device.

Supplementary Code File 2: Computer-aided design drawings of the plate fabrication clamp.

Supplementary Code File 3: 3D representation of the stamp geometry, suitable for use with a 3D printer.

Supplementary Code File 4: SubVI for MuStLiMo_si_initialize.vi, which is a SubVI for motion_control.vi. Converts entries in dialog boxes into parameters for motion.

Supplementary Code File 5: SubVI for Multiple Straight Line Moves_simplified.vi, which is a SubVI for motion_control.vi. Converts entries in dialog boxes into parameters for motion.

Supplementary Code File 6: SubVI for position_tracker.vi. Counter tracks displacement input from linear encoder.

Supplementary Code File 7: Base LabVIEW Project.

Supplementary Code File 8: Top level VI that moves the device.

Supplementary Code File 9: SubVI for motion_control.vi. Executes the rapid displacement that stretches the plate.

Supplementary Code File 10: SubVI for motion_control.vi. Executes the slow displacement that moves the stage.

Supplementary Code File 11: SubVI for motion_control.vi. Plots a (usually undersampled) displacement history in the motion_control.vi control panel.

Supplementary Code File 12: Top level VI that records the displacement history.

Supplementary Code File 13: Contains Variable2, which communicates between motion_control.vi and position_tracker.vi.

Supplementary Code File 14: Schematic for printed circuit board.

Supplementary Code File 15: Layout for printed circuit board.

DISCUSSION:

The key to obtaining a consistent, biofidelic phenotype in this model is applying a consistent biofidelic mechanical insult. This model can generate pulse durations as short as 10–15 ms, which are similar to the pulse durations for human head impacts according to cadaveric experiments[12,13]. The consistency of this insult depends on the alignment of the plate with the indenter block and consistent lubrication of the indenters. When the indenter block is well aligned, there is no trend in the applied strain across rows or columns (**Figure 2C**). A thin layer of lubricant typically creates less friction than a thick layer, and viscous greases are not recommended because they foul the silicone and obstruct the passage of light during microscopy. The actual stage displacement amplitude can fall substantially short of the prescribed displacement amplitude when many indenters are used, and the prescribed stage displacement amplitude is large (> 3 mm). However, while the actual displacement is less than the prescribed displacement at large amplitudes, it remains repeatable (**Figure 2B**). Therefore, large, actual displacements amplitudes can be reliably obtained by entering a prescribed value in excess of the desired value. Displacement amplitude matters only because it is an easily recorded proxy for the peak membrane strain, which directly measures the mechanical insult that induces pathology. Therefore, the procedure described for determining membrane strain from stage displacement is critical. This process should be repeated if any major changes are made to the system that affect the interaction between the plate and the indenters, for example if different diameter indenters, different indenter materials or coatings, or different types of silicone bottomed plate are used. The process of realigning the indenter block and determining the zero position should be repeated at the start of each experiment. A schematic of the stretching device is shown in **Figure 1**. CAD models required to reproduce the device are provided as supplemental materials: '**Injury Device - FULL ASSEMBLY - Generic 3D.STEP**'; the associated bill of materials provided as '**Supplementary Table 1: Custom Built Devices - BOM.xlsx**. Also see **Supplementary Table 2 96 Well Plate _loader – Pinout Wiring Diagram.xlsx**, which describes the cabling connections that connect various components of the systems. **Interconnector_circuit_board.dip** describes a circuit board that interconnects the cables.

If the device is deactivated with the stage near the middle of its travel, the stage will move after the power is cut off because it is spring-loaded. When the power is restored, the feedback loop will detect a large difference between the last known prescribed position and the actual position. This will cause the stage to move suddenly to the position it was in when the device was deactivated. This sudden motion can cause errors in the output of the encoder, so care should

be taken to deactivate the device only when it is in its unpowered resting position at the top of its travel.

The fabrication clamp is designed to bring the plate body and silicone bottom together in a manner that allows optimal bonding. To this end, there are three key features in the design presented in the supplemental file '**Press Die - Generic 3D.STEP'**. First, the clamp plate body holder is parallel to the silicone bottom. If this is properly built, it will require no adjustment after the initial setup. Second, the layer of foam rubber in the clamp provides a small amount of compliance under the plate, as a completely rigid system would theoretically experience a sudden increase from zero clamping force to infinite clamping force when the clamp was closed. The position of the crossbar and set screw of the clamp are adjustable so that the distance between the two sides of the clamp can be fine-tuned.

Every effort should be made to provide a bright, white background behind the dot on the well bottom during strain characterization experiments. The better the contrast in these images, the easier it will be to automate the process of measuring the height and width of the dot, which can become tedious for a human operator analyzing a large experiment. High-speed videography of the bottom of a well in a 96-well plate presents challenges because the walls of the well tend to cast shadows. The use of a dome light or diffuse axial light that can illuminate along the line of sight of the camera without obscuring the image eliminates shadows or specular reflections that would arise with a conventional light source. The brightest available light source should be used because bright illumination allows images to be acquired with a short exposure time. Short exposure times minimize motion blur. Upgrading the light emitting diodes (LEDs) in the diffuse axial light allows shorter exposure times during high-speed video acquisition. The LEDs can be upgraded by opening the diffuse axial light, removing the stock LEDs, mounting 4 high power LED arrays to the back pane using LEDs holders, connecting them to a constant current power supply, and reassembling the diffuse axial light (see **Table of Materials** for catalog numbers). The disadvantage of upgrading the LEDs is that the passively cooled LEDs cannot be kept on for more than a few seconds due to the risk of overheating. Therefore, a different light is needed for alignment of the post-block and camera adjustment.

The presented method of quantifying membrane strain by measuring the dilation of a dot stamped on to the membrane is relatively crude, but it can scale up to multiple wells in a robust manner. The strain field across the well bottom can be characterized in more detail using digital image correlation. This technique involves spraying a speckled pattern onto the base of the well and then imaging it at high-speed during deformation. Commercial software can then be used to quantify strain at every point in the image by tracking the evolution of the speckled pattern.

This protocol produces a multi-faceted, clinically relevant, stretch injury phenotype in hiPSCNs. Cell death, neurite degeneration, and neurite beading are all well-documented sequelae of TBI in humans and animal models[15]. The key to success in this model is establishing and maintaining healthy cultures. Generally speaking, a cell culture protocol developed with conventional rigid plates is a worthwhile starting point for stretchable plate culture. However, the possibility that the cells in question may respond differently on silicone must always be considered. This is

particularly true of hiPSCNs, which are very sensitive to culture conditions. Some examples of optimizing cell density and laminin concentration are supplied in the **Representative Results** section (**Figure 3**, **Figure 4**). Activation of the silicone with plasma treatment is vital. Silicone is hydrophobic and unreactive; in its natural state, it will not bind to laminin or other molecules used to promote cell attachment. Plasma treatment renders the surface hydrophilic and exposes reactive groups. These changes allow adhesion molecules to bind to the silicone and promote cell attachment. It is important to note that the plasma treatment effect dissipates within minutes unless the surface is submerged in liquid, and so procedures that involve drying the activated surface should be performed as quickly as possible. A simple way to check if the effect of plasma treatment has worn off is to place a droplet of water on the surface. On untreated silicone, the droplet will bead up whereas on plasma treated silicone, it will spread out. With the hiPSCNs that we used (see **Table of Materials**), the manufacturer recommends adding the laminin with the cell suspension rather than pre-coating. This protocol has incorporated this approach successfully. While segmentation can, in theory, be accomplished with open source software or general-purpose programming languages, a high degree of proficiency with these tools is required to obtain good results. Neurites are frequently difficult to distinguish from background signal because they are so slender. Therefore, we recommend the use of commercial software tools distributed by high content microscopy companies with dedicated modules for segmentation and quantification of neurons, if they are available. Even with commercial software, it is wise to export images of the segmentation to visually verify accuracy.

There are some limitations associated with working in stretchable plates compared to working with conventional, rigid plates. Stretchable plates can be imaged as normal with air objectives. However, imaging with immersion objectives is very difficult. Lens oil may damage the silicone. Additionally, the objective exerts pressure on the silicone membrane as it moves upwards. This pressure displaces the membrane vertically, making it difficult to bring the sample into focus. The silicone membranes currently used in fabricating the plates are approximately 250 µm thick. This thickness exceeds the focal distance of many high power, immersion objectives. Special care must be taken to lay the membranes perfectly flat before clamping to achieve the flatness required for microscopy. Autofocus systems can compensate for deviations in the flatness of the finished plate to some extent. Future versions of the protocol may pre-tension the membrane before it is bonded to the plate top to ensure flatness. The adhesive-free procedure for bonding the silicone membrane to the plate top[14] is considered an important strength of the current protocol. It eliminates the risk of neurotoxicity from the adhesive as well as any deviations in flatness due to non-uniform thickness of the adhesive layer.

Multi-electrode arrays are commonly used in experiments with hiPSCNs to assess their maturity and functionality. Unfortunately, these systems are incompatible with this model because the cell culture substrate is rigid. It is possible to create a stretchable multi-electrode array, although this has so far only been demonstrated in a single well format[16,17]. Note that indenters can be removed individually from the indenter block so that some wells are not indented and can serve as shams. Removing the indenter prevents indentation but does not completely eliminate mechanical loading since there is still inertial motion of the fluid in the wells while the stage is moving. It is worth comparing *these* wells to wells in plates that were never subject to stage

motion to measure any pathological influence of fluid motion. Also, the array of indenters in the block should be bisymmetric (symmetrical from front to back and side to side). This precaution ensures that the plate is evenly loaded during indentation, so that the stage does not tilt sideways and cause the rods to bind in their bearings.

One of the primary challenges to therapeutic innovation in neurotrauma is the complexity and heterogeneity of the condition. Trauma applies multi-modal stress to every cell type in the central nervous system simultaneously. Neurons have been reliably generated from human induced pluripotent stem cells (hiPSCs) and are now widely available from commercial vendors. Innovation is proceeding quickly in this field, and other neural cell types such as astrocytes[18] and microglia[19] are also being derived from hiPSCs. It may soon be possible to isolate the cell-autonomous responses of each of these cell types to trauma *in vitro* and then to co-culture different cell types to understand how they communicate after trauma. In this way, it may ultimately be possible to recreate the clinical challenge from the bottom up to thoroughly understand it in a human system. This approach is distinct from the conventional approach relying on rodent models and has the potential to generate novel insights that lead to the first therapies for this common, devastating, and intractable condition.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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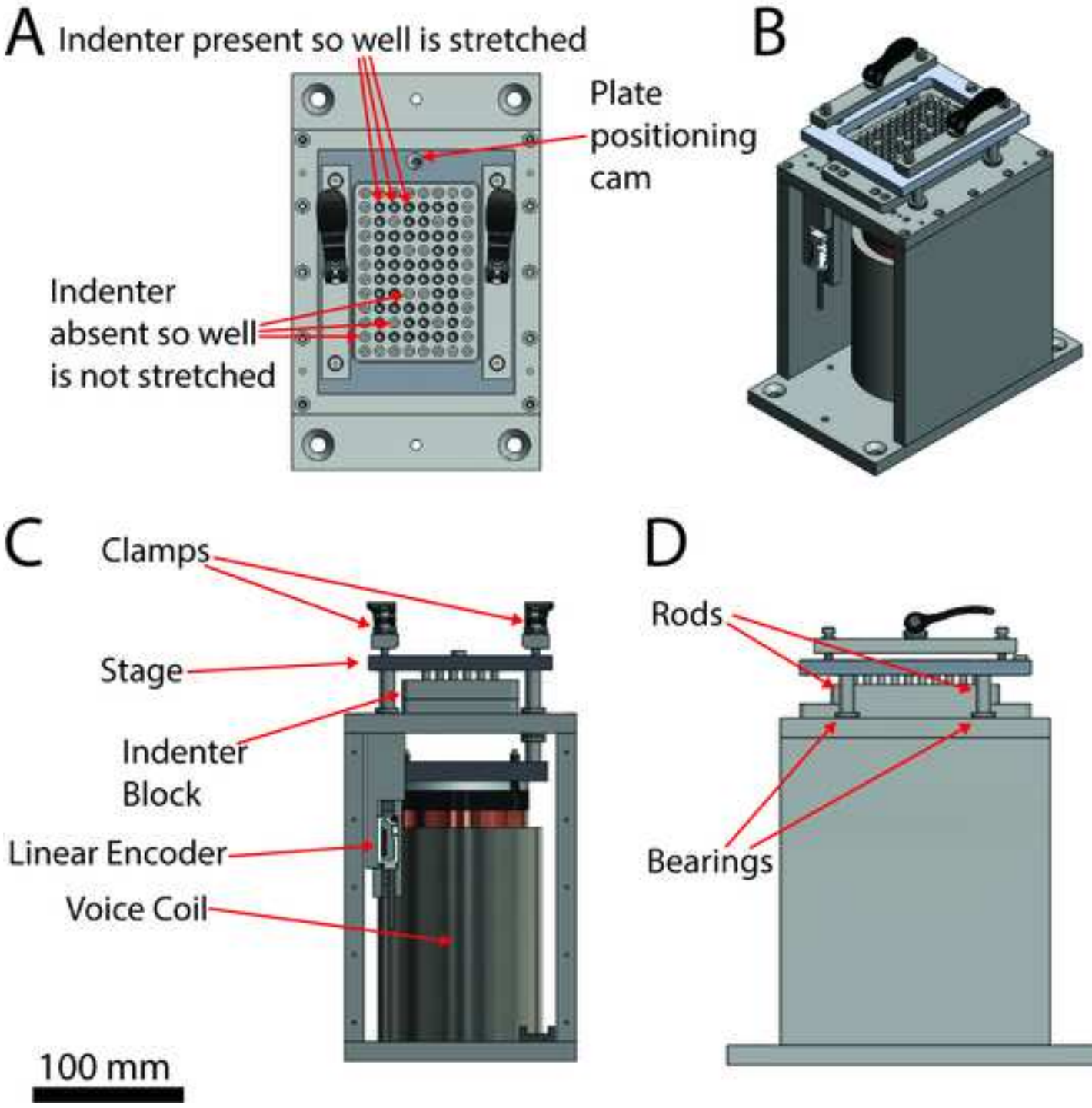

Figure

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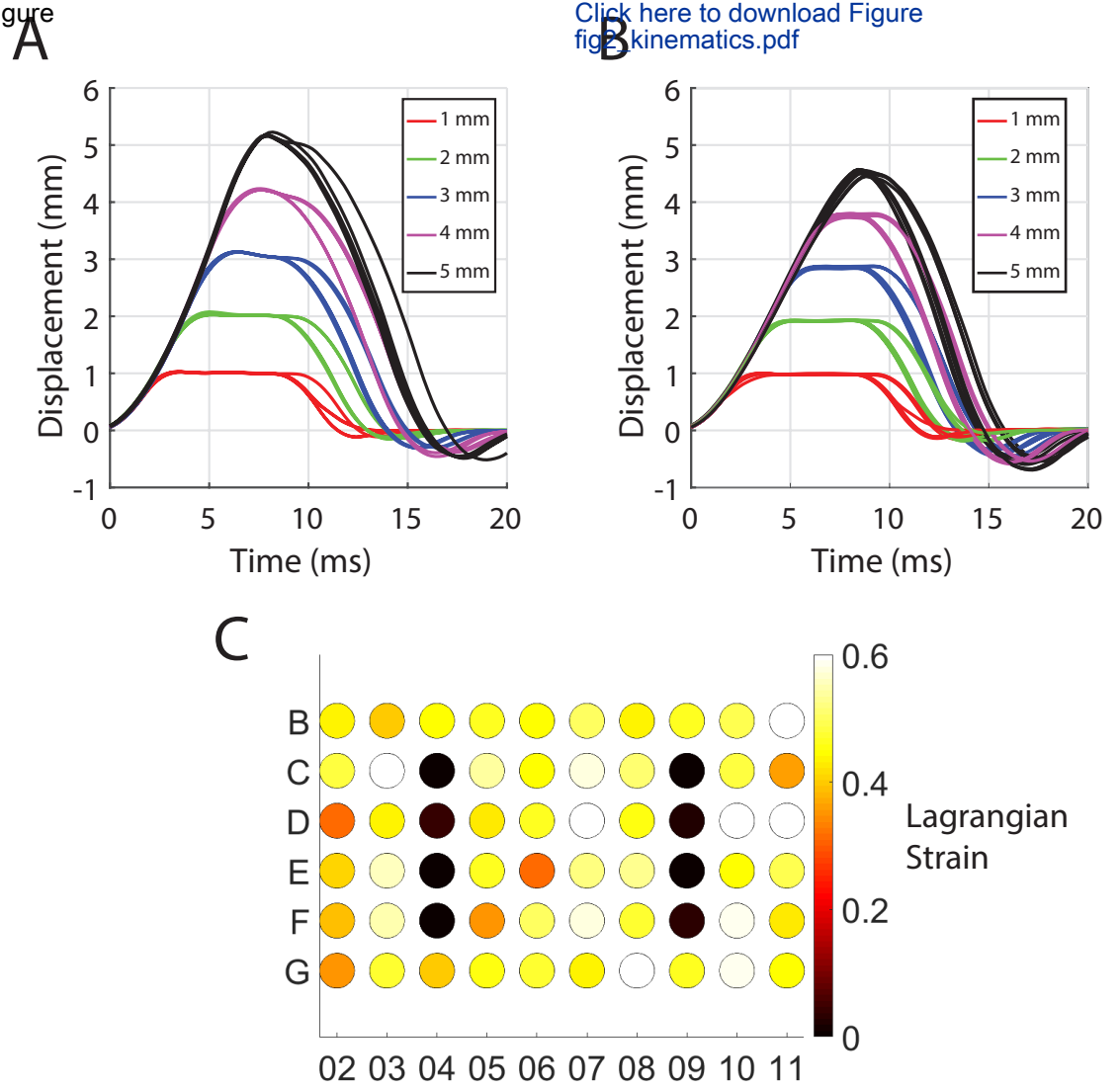

Figure

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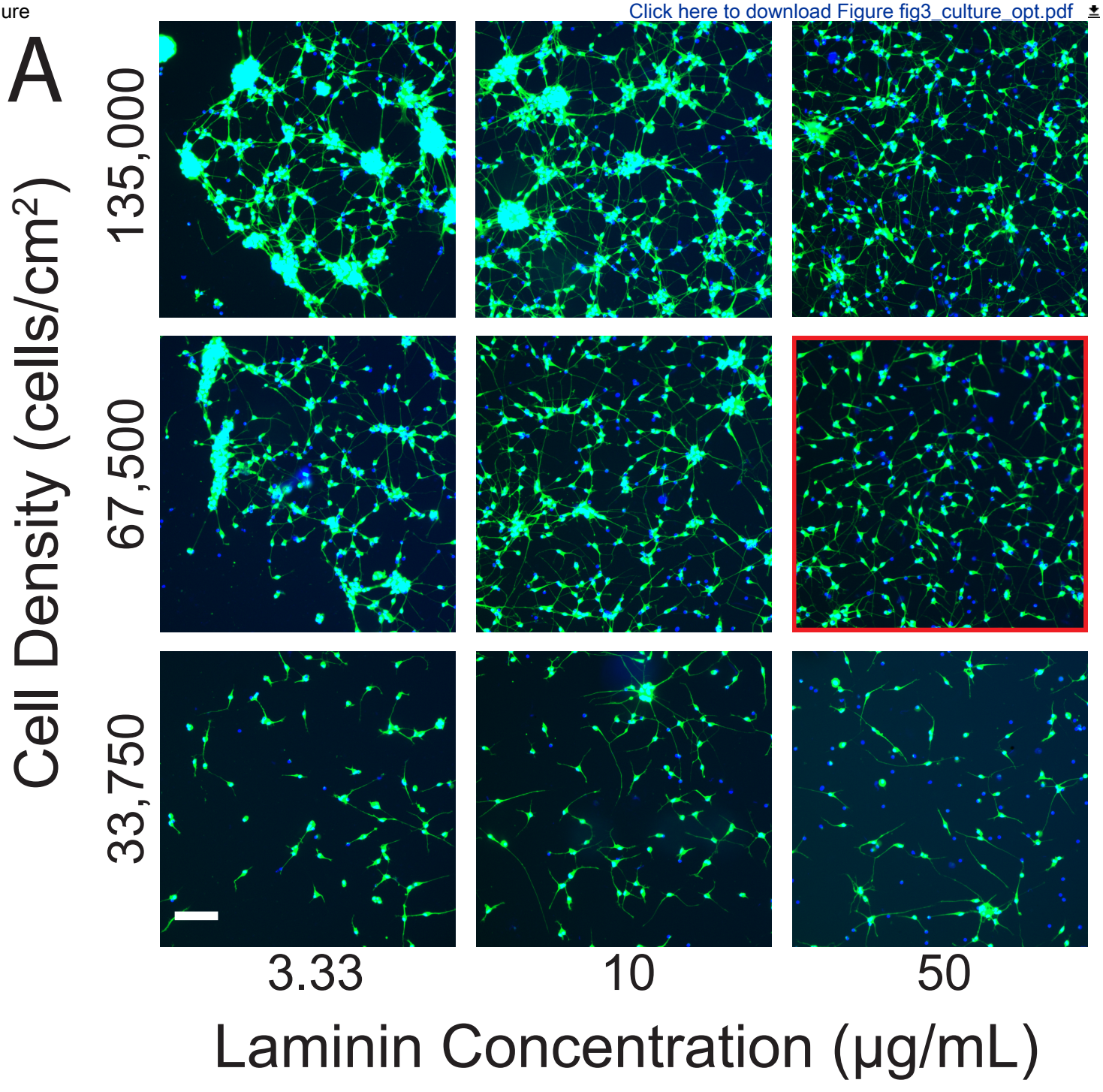

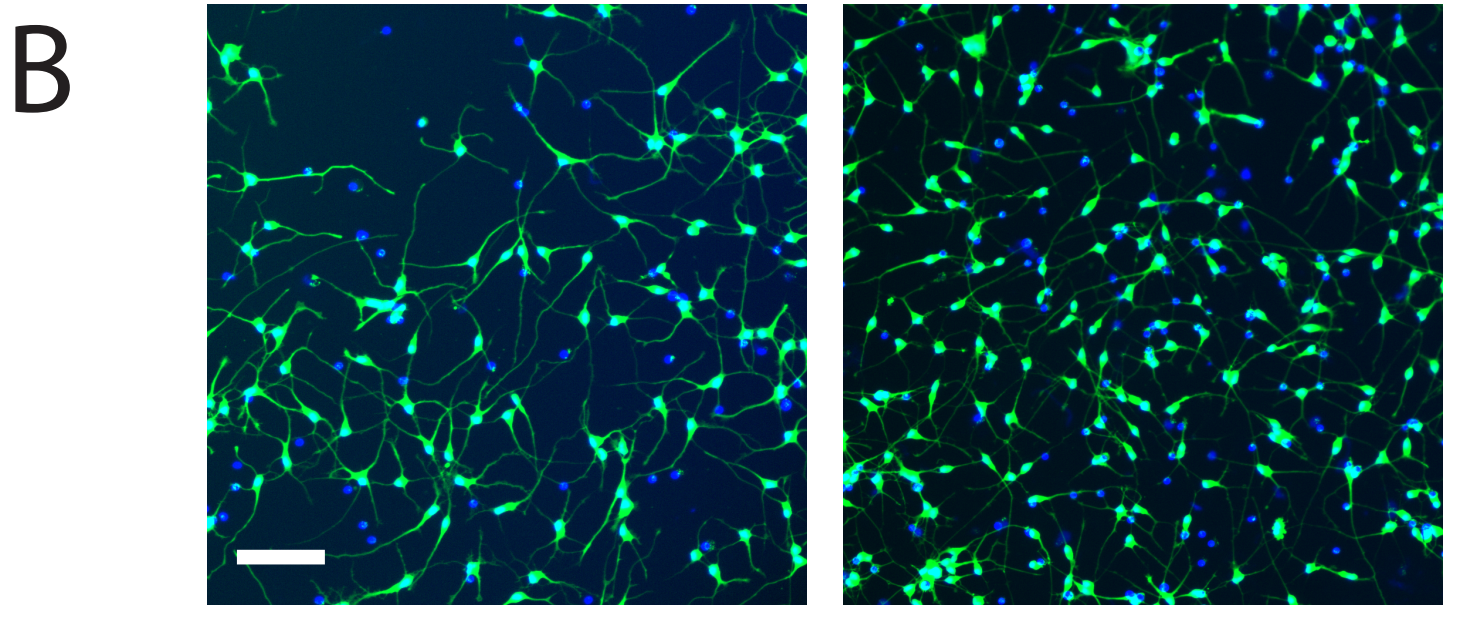

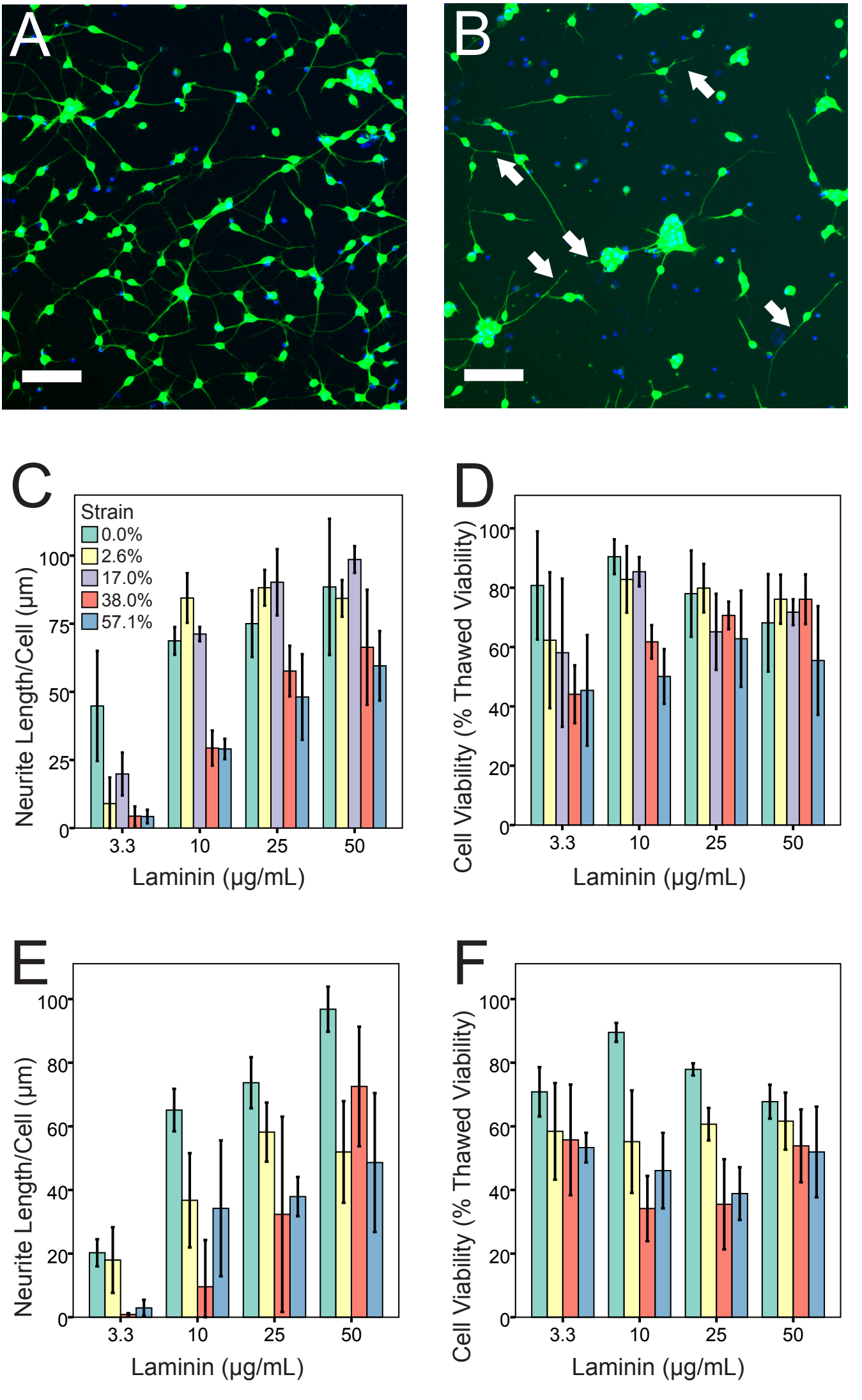

Materials Table

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Name of Material/ Equipment	Company
.010" Silicone Sheet	Specialty Manufacturing, Inc
Sparkleen	Fisher Scientific
Nunc 256665	Fisher Scientific
Kim Wipes	ULINE
Plasma Cleaner	Harrick Plasma
(3-Aminopropyl) triethoxysilane	Sigma-Aldrich
Parchment Paper	Reynolds
Dome Light	CCS inc
Dome Light Power Supply	CCS inc
Axial Diffuse Lighting Unit	Siemens
High Power LED Array	CREE
LED holder	Molex
LED power supply	Mean Well
FastCam Viewer software	Photron
Fastcam Mini UX50	Photron
Micro-NIKKOR 105mm f/2.8	Nikon
0.1 mg/mL Poly-L-Ornithine	Sigma-Aldrich
iCells	Cellular Dynamics International
iCell media	Cellular Dynamics International
iCell supplement	Cellular Dynamics International
Laminin	Sigma-Aldrich
Hoechst 33342	Fisher Scientific
Calcein AM	Fisher Scientific
voice coil actuator	BEI Kimco
optical linear encoder	Renishaw
servo drive	Copley Controls
Controller	National Instruments
cRIO chassis	National Instruments
digital input module	National Instruments
data acquistion chassis	National Instruments
LabVIEW	National Instruments
hiPSCNs	Cellular Dynamics International

Catalog Number	Comments/Description
#70P001200010	Polydimethylsiloxane (PDMS) sheet
#043204	
#12-565-600	Bottomless 96 Well Plate
S-8115	
#PDC-001-HC	
#440140	APTES
N/A	
LFX2-100SW	
PSB-1024VB	
Nerlite DOAL-75-LED	Diffuse axial light
XLamp CXA2540	High Power LED Array
1807200001	LED Holder
HLG-320H-36B	Constant Current Power Supply
	camera softeware
N/A	High Speed Camera
#1455	High Speed Camera Lens
#P4597	
#NRC-100-010-001	
#NRM-100-121-001	
#NRM-100-031-001	
#L2020	
#H3570	
#C3099	
LA43-67-000A	
T1031-30A	
Xenus XTL	
cRIO 9024 Real Time PowerPC Controller	
cRIO 9113	
NI 9411	
NI 9113	
	instrument control software

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Article Title: Method for High Speed Stretch Injury of Human Induced Pluripotent Stem Cell-Derived Neurons in a 96 Well Format

Signature:  Date: 9/1/2017

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Editorial comments:

1. The manuscript has been modified. Please read it carefully and revise if necessary. Enclosed please find the top copy. Please do not change the current format/font.

2. Please rephrase the Summary to clearly describe the protocol and its applications in COMPLETE SENTENCES between 10-50 words: “Here, we present a protocol to …”.

The summary has been revised for style.

3. Please remove citations (1 and 2) from the Abstract.

These citations have been removed.

4. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. If you are using a commercial software, for example, the term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language. However, the details of using the software (commercial or non-commercial) should be provided for the reader to be able to reproduce your work.

The manuscript has been edited to add back in references to specific software and, by extension, instructions on how to use the specific commercial software we use.

5. For steps that involve software or analyzing tools, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. This is the level of detail we’re looking for. Please keep in mind that software steps without a graphical user interface cannot be filmed.

Specific, click-by-click instructions have been added throughout the manuscript.

6. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

7. Please leave a blank line between all protocol steps as well as Notes.

8. Protocol: 1.3: “Rinse” with how much water? How to wash? Which pipette size used?

This is performed with running DI water from the DI water tap. This has been noted.

9. Protocol: 1.6: How to transfer? Using what?

Transfer with gloved hands. This has been noted.

10. Protocol: 2.1: Please provide an appropriate reference for plasma treat.

A reference has been added to the paper that inspired our approach to bonding silicone. Additional details have also been added regarding the plasma treatment.

11. Protocol: 2.7: Please provide enough guide for the reader to be able to build similar device.

CAD drawings and a dedicated bill of materials have been added to the supplemental files to allow the reader to build the device.

12. Protocol: 3.2.3: Please include all the buttons clicked in the software.

The button sequence has been added.

13. Protocol: 3.2.4, 3.2.5, etc.: If using any software, please include all the buttons clicked and all the setting selected.

The button sequence has been added.

14. Protocol: 3.26: Please clearly describe the actions. “Raise” how?

The button sequence has been added.

15. Protocol: 3.2.7: “Deactivate the device” how is that done?

The device is deactivated by opening the door. This has been noted.

16. Protocol: 3.2.8: How is the adjustment done?

As in the preceding step. This has been noted.

17. Protocol: 3.2.9: Insert what? Please clearly describe the actions.

Insert the tie down screws. The word order has been changed to disambiguate this.

18. Protocol: 3.4.1: How is that done? With what?

Lubricating the indenters is described in the preceding step, “lubricating the indenters.” This has been noted.

19. Protocol: 3.4.3: Please describe how to use the software, please include all the buttons clicked.

The reader has been directed to 3.2.

20. Protocol: 3.4.4: How to run? Which button clicked?

The button sequence has been added.

21. Protocol: 3.4.5: How to do that? Please include all the buttons clicked and all the icons selected.

The button sequence has been added.

22. Protocol: 3.4.6: How is that done?

The button sequence has been added.

23. Protocol: 3.4.7: Caution: Please move this Caution to the Discussion.

The caution statement has been moved to the discussion.

24. Protocol: 3.4.8: How is the software used? How to save the displacement trace? Please include all the buttons clicked.

The button sequence has been added.

25. Protocol: 4.2: Please guide the reader to the supplementary materials, please mention the .stl file you provided.

Mention of the supplementary file has been added.

26. Protocol: 4.9: Please describe the actions of using the software, please include all the buttons clicked. We need all the details, this protocol will be used later by our script writers for the video production, the details of all actions are necessary.

The button sequence has been added.

27. Protocol: 4.13: How to save the file. Which button clicked?

The button sequence has been added.

28. Protocol: 4.15: Please describe using the software or refer to appropriate references.

A description of this using ImageJ has been added.

29. Protocol: 5.2: “as above”? Please include the step number.

2.1. The step number has been noted.

30. Protocol: 5.6: How is that done?

Step has been clarified: Add the PLO, let sit for one hour.

31. Protocol: 5.8: using what?

Safety gloves. This has been noted.

32. Protocol: 5.15: How? Using what?

By adding media. The exact amount will vary depending on the number of cells and desired volume. This has been noted.

33. Protocol: 5.16: How is that done? How to mix? Which pipette size used?

A 1000 µL micropipete is used and this is now stated in the text. The step has been changed to clarify that 10 µg/mL final concentration of laminin is achieved with 10 µL of 1 mg/mL stock per mL of cell suspension. It has also been updated to instruct the reader to mix by inverting the tube.

34. Protocol: 6.3: Please describe how to use the software? Please include all the buttons clicked.

The reader has been referred to steps 3.4.3 and 3.4.4.

35. Protocol: 6.5, 6.6, 6.9: How is the software used?

The reader has been referred to steps 3.4.3, 3.4.4, 3.4.6, 3.4.7, and 3.4.8.

36. Protocol: 7.5: Note: Please move the discussion to the Discussion section. Please move this Note to the Discussion.

This note has been moved to the discussion. 37. After revising the protocol, please highlight 2.75 pages or less (currently that is more than 4 pages) of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

38. 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.

39. Please discuss all figures in the Representative Results. However, for figures showing the

experimental set-up, please reference them in the Protocol. Figure 1 is not mentioned in the representative results. It is discussed in the Discussion after other figures. Please revise accordingly.

Figure 1 is now referenced in the Protocol before the other figures referenced by the representative results.

40. Please provide a title and description for the SI file (.stl).

A title and description have been added during submission

41. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]".

An email thread with the nature permissions desk, along with the full text of the Creative Commons Attribution 4.0 International license, was attached. In the figure 2 description, "Portions of this figure and this legend were reproduced from a prior publication by Sherman *et al*. [13]" has been changed to "This figure has been modified from Sherman *et al.* [13]"

42. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

Materials for building the injury device from the initial submission have been added back in.

We thank both the Editor and the Reviewers for their thoughtful and insightful comments. We feel that the process of addressing their concerns has considerably strengthened the manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have made minor changes throughout the document.

2. Please remove all commercial language: Sparkleen, catalog numbers, PowerPC, National instruments, LabVIEW VI, Kimwipe, etc.

We have removed commercial language. Section 8 of the original manuscript was specific to the commercial high content microscopy software that we used to analyze our images. To accommodate the prohibition of commercial language, we have rephrased this in more general terms and included a caution statement recommending the use of a commercial tool generated by professional software engineers for this step without specifying any of the several viable choices in the marketplace.

3. Manuscript, Figure 1 and 2: Please use SI units, e.g. use “µL” instead of “µl”. Please ensure that the L in these abbreviations are capitalized.

We have corrected liters to a capital L, and corrected imperial units.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have made minor changes throughout the document, and rearranged some text to conform.

5. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have made minor changes throughout the document.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have made changes throughout the document and moved some text to the discussion.

6. Some additional details are needed in the protocol:
1.6: Please use the Celsius grade.

Done

2.10: Cure plate where and at what temperature?

At room temperature. This has been stated specifically in the text.

3.2.3: What other parameters for sonication are used?

8 minutes in at 42,000 Hz in a 60W device in DI water. This has been stated specifically in the text.

3.3.2: Secure the plate how?

The plate is secured with clamps. Figure 1 has been added to illustrate the clamps on the device. References to securing the plate throughout the protocol have been updated to make clear that the plate is secured with clamps.

3.4.3: How many seconds exactly?

10. This has been added.

Remove what?

The foam pad and the components that weight it down. This step has been reworded for greater clarity.

4.2: Please provide an stl file.

An .stl file is being provided.

4.3: What are the plasma treatment parameters? Same as before?

Yes. This has been clarified in the text.

7. In the JoVE Protocol format, “Notes” 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. Any text that provides details about how to perform a particular step should either be

included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

The text block at the start of 3.1.1 has been cut in light of this comment and a related comment from Reviewer #1 and moved to the end of the first paragraph of the Discussion.

8. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

An appropriate subset of the protocol has been highlighted.

9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

As above.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The paper titled "Method for High Speed Stretch Injury of Human Induced Pluripotent Stem Cell-Derived Neurons in a 96 Well Format" aims to present a platform for studying brain injury in vitro with human-derived neuronal cultures. While stretch injury models have been used for many years to study mechanisms of trauma in neuronal cultures, this paper focuses on two specific challenges in this field which would make the technique attractive to researchers: (1) Increased throughput and (2) incorporation of Human cell lines. In general, the paper describes the necessary components required for this method to be reproduced successfully, and therefore I recommend it for publication, given that the subsequent issues are addressed.

Major Concerns:
1. There needs to be a general increase in the amount of quantification of mechanics associated with the model. Previous stretch devices were designed to generate highly reproducible strain and strain rates as these have been shown to be critical for neuronal injury. The major limitation of moving to high throughput is the risk of losing precision in the injury across samples. In its current form, I have no idea of how precise this instrument is and this would be my single hesitation in using it. There are many instances in which the authors describe potential pitfalls and subsequently offer suggestions as to how to avoid these, however, these are not sufficient. Expanding on this, the use of a voice coil actuator is a clever design, however, plate trajectories and induced substrate strain traces need to be included. I would want to see that the PID controller combined with the voice coil is sufficient to accurately produce specific strain and strain velocity profiles. Furthermore, to be complete I would also include traces with indenters

partially and fully loaded in order to vary the load on the coil.

Figure 2 has been added to quantify the kinematics of the device in detail. Panels B and C are reproduced from a prior publication[1] under the terms of the Creative Commons license governing that publication. Panel A is a new figure of freshly generated data that allows the reader to compare the motion of the device when the plate is loaded with 52 posts to the motion when not loaded. This addresses the concern raised in the last sentence of the above paragraph. Also, numerical results have been added to the first paragraph of the Representative Results quantifying the variability in the mechanical strain across wells and plates.

2. The figures included to support this technique are fairly weak. The multi-panel indicating the relationship between Laminin concentration and cell density is minimally helpful.

Cell density and laminin concentration are the two experimental parameters that most strongly influence the health of the cultures on silicone. These parameters cannot be optimized independently because one influences the other, as explained by the figures. Furthermore, optimizing uninjured cultures is not sufficient. Cell density and laminin concentration also influence the injury phenotype. This fact is also illustrated by the figures. These trends are vital to the design of successful experiments. We have included these figures to illustrate these trends to those who wish to use this technique in the hope that it will accelerate their efforts to optimize their cultures.

It is also over-saturated.

While calcein AM evenly labels the entire cytoplasm, the neurites are much fainter structures than the soma because their volume is much smaller. In these images, the dynamic range is intentionally adjusted to optimize visualization of the neurites. This adjustment inevitably leads to some saturation of the soma but this is worthwhile because the morphology of the neurites is more important in this context than the internal features of the soma. This practice is standard in the field[2-4]. The reviewer's concern is understandable because we neglected to explain this in our previous draft. We have explained this choice in the legend for Figure 3 of the revised manuscript. This point is also addressed in step 7.4 in the revised manuscript.

The inclusion of time points or comparison to controls would be informative.

We have added data from the 24 hour post-injury time point so that it can be compared to the 4 hour post-injury time point that was originally presented.

For example, you might include an example of an optimal culture at several time points next to a control dish using the manufacturer's protocol to illustrate minimal differences in culture quality metrics such as those provided in the following figure. Such a figure would provide an example for the appearance of a quality sample as well as provide evidence for the optimization of the culture protocol on the required flexible silicone substrate.

Figure 3B in the revised manuscript has been added to allow comparison of cultures on conventional rigid substrates with cultures on the required, flexible, silicone substrate.

The second figure is also fairly weak. Even if we are not reviewing for quality of data, my primary concern is that this figure doesn't emphasize the strength of the technique that is being described in the paper. I am assuming that this entire dataset is generated from 1 well, which should be emphasized if true in order to highlight the high throughput nature of the device.

This entire data set was not generated from a single well. It was also not generated from a single plate. All the wells in a plate must be injured with the same strain. Therefore, an experimental design that requires multiple strain levels must include multiple plates.

Proper statistics should also be included, again to highlight the shear volume of data that can be generated from a single plate.

We have added ANOVAs identifying which main and interaction effects in each data set are statistically significant. The results of these statistical tests are presented in the third paragraph of the Representative Results in the revised manuscript.

The injury levels should also be presented in proper units. Why indicate a 1 mm vs 2 mm indentation, which tells me nothing, when the actual numbers of strain have been measured.

The categorization of data by displacement has been replaced with categorization of data by strain in Figure 4 of the revised manuscript.

Also, it is unclear how neurite length was calculated. Clarifying this with an image would be helpful.

It is difficult to be highly specific about how neurite length was calculated because we used commercial software for this purpose and the Editor has asked us to eliminate all commercial language from the revised manuscript. We have rewritten section 7 to address this concern while also respecting the Editor's wishes on this point.

Minor Concerns:
1. Implementation of this technique requires multiple custom fabricated parts. It is understood that these parts are difficult to describe in the text alone, but it will be essential to include drawings with necessary dimensions. For instance, section 3.1.1 describing the stretching plate is not sufficient to reproduce the device. From the text, it is also unclear if one will have to write the necessary supporting LabVIEW scripts or if they are provided.

The reviewer raises an important point about the challenges of describing a protocol that requires custom-built tools. The process of building the device is not a part of this protocol and the editor has specifically instructed us not to include material that is beyond the scope of the protocol. We have included a schematic of the device to better orient the reader as Figure 1 in the revised manuscript. We consider a complete set of technical documents necessary to reproduce the device (technical drawings with tolerances, bills of materials, LabVIEW code etc.) inappropriate for publication in the peer-reviewed literature so we will instead make them available to interested groups by informal communication. We have specified in the first paragraph in the Discussion of the revised manuscript that these documents are available upon request. When we

share these documents, we will do so on an “as is” basis to assist those interested in reproducing our device. However, if we included these numerous, detailed documents as part of this publication, we would commit ourselves to guaranteeing that every single document is without error and might in theory have to retract the paper or publish an erratum over an inconsistency in the drawings or a bug in the code. Guaranteeing this level of accuracy across all these numerous documents would substantially delay publication of the paper. Therefore, we feel that informal communication is the more appropriate method for disseminating this information.

2. Section 4 may be one of the most important for producing consistent injuries across laboratories, and the depth of the text is appreciated. However, I suggest including a table of measured values and expected variability.

Figure 2 in the revised manuscript provides results on measured strains and variability. These parameters are also quantified in the first paragraph of the Representative Results section in the revised manuscript.

Please also include a statement about the expected calibration requirements - how often, is the full procedure with high-speed camera necessary each time.

Our practice is to repeat the indenter-block alignment step (3.3) every time we wash the indenter (i.e. for every experiment) and to repeat characterization of membrane stretch (section 4) any time we alter the indenters or the plates. This practice is now described at the end of the first paragraph in the Discussion in the revised manuscript.

3. Given the importance presented within the paper on substrate preparation, it would be informative to provide control images of neurons cultured using the manufacturer’s protocol. It would be helpful to clarify in section 5 the differences in culture methodology for the iCell neurons on glass vs stretchable surfaces. In my experience, culturing neurons on stretchable surfaces is always more difficult compared to rigid surfaces, so this section may be best utilized to highlight these differences and simply direct the researcher to the iCell protocol for steps that adhere to the manufacturer’s protocol.

Figure 3B in the revised manuscript allows comparison of cells cultured on conventional rigid substrates according to the manufacturer’s protocol with cells cultured on silicone using our optimized protocol.

4. The discussion addresses the "…primary physical driver of variability in the system…" and there are suggestions for minimizing this error. However, there needs to be numbers included to inform the researcher how much variability to expect. The primary allure to this type of technique is the ability to injure many wells simultaneously. However, I don't have any idea what sort of variability to expect across wells within a single stretch as well as within a well across multiple stretches. These numbers will be useful both when assembling this type of device as well as for proper experimental design.

The newly added Figure 2 provides quantitative results describing measured strains and their variability. Numerical results quantifying the variability in strains across wells in a single plate and from plate to plate have been added to the first paragraph of the Representative Results section of the revised manuscript.

Reviewer #2:
Manuscript Summary:
This is a very nice piece of work, which will constitute an important contribution to the literature. The authors are commended for their efforts. A number of minor points need addressing before the paper can be fully accepted for publication.

Major Concerns:
None

Minor Concerns:
(1) Protocol 1.2. Was this washing only with H2O?

Membranes are washed with soapy water. Section 1.2 has been reworded to be more explicit on this point.

(2) Plate Fabrication steps 2.2 and 2.3. What is the actual time (text says "at the last minute")? What is the time between rinses?

APTES solution should be diluted no more than 60 seconds before the introduction of the plate. The text has been updated to clarify this point.

(3) Section 3. A picture or schematic of the apparatus would be helpful.

A schematic of the apparatus has been added to the revised manuscript as Figure 1.

(4) Section 3.4.1. Was the water de-ionised?

Yes, but it does not need to be. The tubes of water simply function as ballast in this step. This passage has been reworded for greater clarity on this point.

(5) Section 3.5.5. What is the full range? The typical range is interesting too.

The typical range of plate displacements is 1-4mm. The maximum is 5mm. These values have been added to the text.

(6) Section 4.2. 3D printing us stated. Is this necessary or could it be fabricated alternatively?

3D printing is not necessary although it is the approach we employed. The text has been adjusted to clarify this point.

(7) Section 4.17.1. Can a schematic of the wells C05-E08 be provided?

In reflecting on this comment, we concluded that the description of the process for choosing how many wells to image was too prescriptive. We have made the language in this section more general so the reader can make their own choices for their experimental design in characterizing the induced strain. The choice of how many wells to image at a time, and how many times to image them, will depend on the reader's judgement and understanding of their application.

(8) Section 5.17. Better to say "0.033". Do the authors want to mix SI and Imperial units?

.33 has been replaced with 0.33. All imperial units have been converted to SI.

References

1 Sherman, S. A. *et al.* Stretch Injury of Human Induced Pluripotent Stem Cell Derived Neurons in a 96 Well Format. *Sci Rep.* **6** 34097, doi:10.1038/srep34097, (2016).

2 Morrison, G. *et al.* Evaluation of inter-batch differences in stem-cell derived neurons. *Stem Cell Res.* **16** (1), 140-148, doi:10.1016/j.scr.2015.12.025, (2016).

3 Wheeler, H. E., Wing, C., Delaney, S. M., Komatsu, M. & Dolan, M. E. Modeling chemotherapeutic neurotoxicity with human induced pluripotent stem cell-derived neuronal cells. *PLoS One.* **10** (2), e0118020, doi:10.1371/journal.pone.0118020, (2015).

4 Sirenko, O., Hesley, J., Rusyn, I. & Cromwell, E. F. High-content high-throughput assays for characterizing the viability and morphology of human iPSC-derived neuronal cultures. *Assay Drug Dev Technol.* **12** (9-10), 536-547, doi:10.1089/adt.2014.592, (2014).

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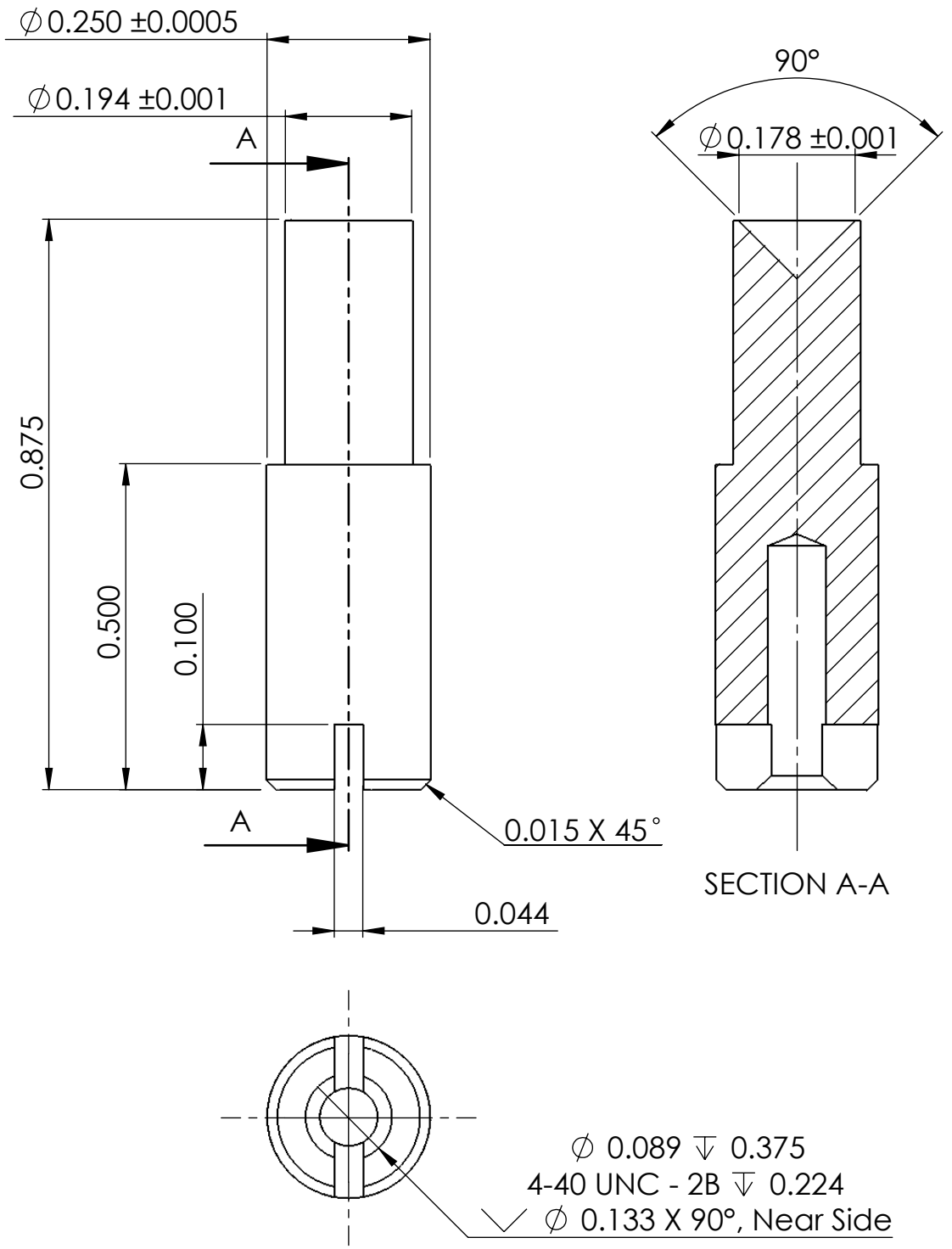

UNLESS OTHERWISE SPECIFIED: DIMENSIONS ARE IN INCHES SURFACE FINISH: TOLERANCES: LINEAR: ± 0.002 ANGULAR: ± 0.1	FINISH: MACHINE FINISH TUMBLE DEBURR		DEBUR AND BREAK SHARP EDGES		DO NOT SCALE DRAWING	REVISION 07
	NAME	SIGNATURE	DATE			TITLE:
DRAWN	SRO		01/07/15			REMOVABLE PIN
CHK'D						
APPV'D						
MFG						
Q.A				MATERIAL: 6061-T6 ALUMINUM		DWG NO. SRO - 3021 A4
				WEIGHT:		SCALE:4:1 SHEET 1 OF 1

BOM-Injury Device, Plate press

Category	Vendor	Catalog No.
Computer	Advantech	SYS-2U2320-7A01
Computer	Advantech	96OT-MT-690120
Computer	Advantech	96KB-104U-KT-B-US
Computer	Advantech	96MS-OP2-USB-LT2
Controller	DigiKey	SW1963-ND
Controller	DigiKey	Z3749-ND
Controller	DigiKey	HM1362-ND
Controller	DigiKey	HM1360-ND
Controller	National Instruments	781174--01
Controller	National Instruments	780917--01
Controller	National Instruments	780110-01
Controller	National Instruments	779351-01
Controller	National Instruments	779005-01
Controller	National Instruments	196917-01
Controller	National Instruments	779019-01
Controller	National Instruments	182219--05
Controller	National Instruments	784005-35
Controller	National Instruments	779953-35
Controller	National Instruments	781233-01
Controller	National Instruments	782803-01
Enclosure	ServerRack.com	3141-3-001-06
Enclosure	ServerRack.com	1922-3-400-01
Enclosure	ServerRack.com	3150-3-001-06
Enclosure	ServerRack.com	HW1032-50-Cage
Motion Platform	BEI Kimco	LA43-67-000A
Motion Platform	Copley Controls	XSL-230-40
Motion Platform	Hitek Hardware	KC3824
Motion Platform	Hitek Hardware	KF3824
Motion Platform	Hitek Hardware	KS3824
Motion Platform	Hitek Hardware	BES3824-16
Motion Platform	McMaster	1374N35
Motion Platform	McMaster	1435A470
Motion Platform	McMaster	2030N14
Motion Platform	McMaster	3935K11
Motion Platform	McMaster	5779K652
Motion Platform	McMaster	5986K672
Motion Platform	McMaster	6294K433
Motion Platform	McMaster	85175K84
Motion Platform	McMaster	8685K47
Motion Platform	McMaster	8975K149
Motion Platform	McMaster	9002T120
Motion Platform	McMaster	90145A475
Motion Platform	McMaster	90145A540
Motion Platform	McMaster	91292A013
Motion Platform	McMaster	9151K510
Motion Platform	McMaster	92196A110
Motion Platform	McMaster	92196A113
Motion Platform	McMaster	92196A269
Motion Platform	McMaster	92196A272
Motion Platform	McMaster	92196A544

Motion Platform	McMaster	92210A108
Motion Platform	McMaster	92210A622
Motion Platform	McMaster	93340A305
Motion Platform	McMaster	94035A167
Motion Platform	McMaster	94115A539
Motion Platform	McMaster	94115A547
Motion Platform	McMaster	9663K750
Motion Platform	Misumi	A5052P-6F-INCHA-N
Motion Platform	Misumi	A5052P-4F2G-INCHA
Motion Platform	Misumi	A5052P-6F-INCHA-N
Motion Platform	Misumi	U-PBFU0.38-L2.25-T
Motion Platform	Misumi	A5052P-4F2G-INCHA
Motion Platform	Misumi	A5052P-4F2G-INCHA
Motion Platform	Misumi	A5052P-6F-INCHA-N
Motion Platform	Misumi	A5052P-4F2G-INCHA
Motion Platform	Misumi	A5052P-6F-INCHA-N
Motion Platform	Misumi	A5052P-6F-INCHA-N
Motion Platform	Renishaw	T1031-30A
Motion Platform	Renishaw	Ti0200A20A
Motion Platform	Renishaw	A-9653-0138
Motion Platform	Renishaw	A-9653-0139
Motion Platform	Renishaw	A-9531-0342
Motion Platform	ThorLabs	CMS010
Motion Platform	ThorLabs	CMS011
Wiring	DigiKey	A2076-ND
Wiring	DigiKey	A31944-ND
Wiring	DigiKey	A31948-ND
Wiring	DigiKey	A31949-ND
Wiring	DigiKey	A31954-ND
Wiring	DigiKey	A31955-ND
Wiring	DigiKey	A1038-ND
Wiring	DigiKey	A1039-ND
Wiring	DigiKey	A1040-ND
Wiring	DigiKey	A1031-ND
Wiring	DigiKey	A1033-ND
Wiring	DigiKey	A1035-ND
Wiring	DigiKey	A31909CT-ND
Wiring	DigiKey	A31910CT-ND
Wiring	DigiKey	A31906CT-ND
Wiring	DigiKey	A31905CT-ND
Wiring	DigiKey	A34126-ND
Wiring	DigiKey	A34128-ND
Wiring	DigiKey	A34131-ND
Wiring	DigiKey	3M1768-ND
Wiring	DigiKey	3M1769-ND
Wiring	DigiKey	3M10686-ND
Wiring	DigiKey	3M10687-ND
Wiring	WAGO Products (On-Line Electronics, Inc.)	721-204/026-000
Wiring	WAGO Products (On-Line Electronics, Inc.)	721-104/026-000
Wiring	WAGO Products (On-Line Electronics, Inc.)	721-605/000-042
Wiring	WAGO Products (On-Line Electronics, Inc.)	721-103/026-000

BOM-Injury Device, Plate press

Wiring	WAGO Products (On-Line Electronics, Inc.)	231-131
Plate Press	McMaster	8975K39
Plate Press	McMaster	8975K352
Plate Press	McMaster	8975K251
Plate Press	McMaster	8975K979
Plate Press	McMaster	8934K18
Plate Press	McMaster	96242A734
Plate Press	McMaster	92196A587
Plate Press	McMaster	90669A622
Plate Press	McMaster	91847A031
Plate Press	McMaster	92949A267
Plate Press	McMaster	90669A537
Plate Press	McMaster	90145A546
Plate Press	McMaster	97155A737
Plate Press	McMaster	2610T34
Plate Press	McMaster	9540K782
Plate Press	McMaster	5093A76
Plate Press	McMaster	92196A199
Plate Press	McMaster	92949A108
Plate Press	McMaster	8610K93
Plate Press	McMaster	3723T63
Plate Press	W.B. Jones	788

BOM-Injury Device, Plate press

Description	Qty
High Performance Intel® Core™ i7/i5/i3 2U Rackmount System with up to 3 PCI/PCIe Expansion Slots	1
GENERAL DEVICES 20" RACK MOUNT SLIDE(G)	1
KEYTRONIC USB 104 KEY KEYBOARD BLACK(G)	1
INPUT DEVICE, LOGITECH M100 MOUSE OPTICAL USB(G)	1
AC/DC CONVERTER 5V 30W	1
PWR SUPPLY 60W 24V2.5AVAC100-240	1
ALUMINUM DIN CLIP 2.95"	4
ALUMINUM DIN CLIP 3.94"	4
cRIO--9024, Real--Time PowerPC Controller for cRIO, 800 MHz	1
cRIO--9113, 4--slot Virtex--5 LX50 Reconfigurable Chassis for cRIO	1
NI 9514 - C Series Servo Drive Interface with Encoder Feedback	1
NI 9401 - 8 Ch, 5 V/TTL High-Speed Bidirectional Digital I/O Module	1
NI 9411 6-Ch ±5-24 V, 1 MHz, Diff./SE DI Module	1
NI 9977 C Series filler module	2
NI 9912: DIN Rail Kit for 4-Slot Chassis	2
E1 Ethernet Cable, Twisted--pair, 5 m	1
LabVIEW Embedded Control and Monitoring Suite, USB, Include 1 Year SSP	1
NI LabVIEW Statechart Module, Includes 1 Year SSP	1
EMI Suppression Ferrite, 7.0mm	4
EMI Suppression Ferrite for NI 9401	2
Kendall Howard 3141-3-001-06 - 6U LINIER Fixed Wall Mount Cabinet - Solid Door	1
Kendall Howard 1922-3-400-01 - 1U 20" Vented Rack Mountable Sliding Shelf	1
Kendall Howard 3150-3-001-06 - 6U LINIER Wall Mount Vertical Rail Kit - Cage Nut	1
Rackmount Solutions HW1032-50-Cage - 10-32 Hardware Kit, 50 count screws and cage nuts	1
Voice Coil	1
Servo Drive	1
Kinematic Vee Groove	1
Kinematic Flat	2
Kinematic Cone	1
Ball End Set Screw	4
Multipurpose Neoprene Rubber Sheet, Adhesive-Back, with Certificate, 12" x 24", 1/8" Thick	1
Round Grip Pull Handle with Threaded Holes, Anodized Aluminum, 10" Wide Center-to-Center	2
CLAMPING HANDLE WITH INTERNAL THREAD 10-32 SIZE	2
Single Scale Vacuum Gauge with ABS Plastic Case, 1-1/2" Dial, 1/8 NPT Male Bottom Connection, 30-0	1
Push-to-Connect Tube Fitting for Air, 90 Degree Elbow, for 1/4" Tube OD x 1/8 NPT Male	1
Fixed Alignment Linear Sleeve Bearing 6061 Aluminum, for 3/8" Shaft Diameter, 0.0005" Shaft Cleara	4
Bushing	4
Oil-Resistant Fire-Retardant Blended Foam Sheet, Soft Buna-N, 1/4" Thick	1
12.00" X 12.00" X 0.125" - Ultem	1
6061 Aluminum, 3/4" Thick x 12" Wide, 2 Feet Long	1
Helical Spring	4
18-8 Stainless Steel Dowel Pin 1/8" Diameter, 1" Long	1
18-8 Stainless Steel Dowel Pin 1/4" Diameter, 3/4" Long	1
18-8 Stainless Steel Socket Head Screw M2 x 0.4 mm Thread, 20 mm Long	1
High-Pressure Nickel-Plated Brass Pipe Fitting, Right-Angle Tee Adapter, 1/8 NPTF Female x Male	1
18-8 Stainless Steel Socket Head Screw 4-40 Thread Size, 1/2" Long	1
18-8 Stainless Steel Socket Head Screw 4-40 Thread Size, 3/4" Long	1
18-8 Stainless Steel Socket Head Screw 10-32 Thread Size, 1/2" Long	1
18-8 Stainless Steel Socket Head Screw 10-32 Thread Size, 3/4" Long	1
18-8 Stainless Steel Socket Head Screw, 1/4"-20 Thread Size, 1-1/4" Long	1

18-8 Stainless Steel Hex Drive Flat Head Screw 4-40 Thread Size, 3/8" Long	1
18-8 Stainless Steel Hex Drive Flat Head Screw 3/8"-16 Thread Size, 3/4" Long	1
Threaded Insert for differential screw drive	4
18-8 Stainless Steel Precision Shoulder Screw, 3/16" Diameter 7/8" Long Shoulder, 8-32 Thread	4
Alloy Steel Nylon-Tip Set Screws, Black-Oxide, 1/4"-20 Thread, 5/8" Long	1
Alloy Steel Nylon-Tip Set Screws, Black-Oxide, 1/4"-20 Thread, 1" Long	1
Corrosion-Resistant Compression Spring Stock, 20" Overall Length, 0.375" OD, 0.305" ID	1
3.00" X 0.75" X 0.375" - Aluminum	1
7.00" X 6.00" X 0.50" - Aluminum	1
3.50" X 1.00" X 0.375" - Aluminum	1
2.375" X 0.375" OD - #10-32 Tap on Both Ends - Coated Aluminum	8
6.00 X 4.75" X 0.375" - Aluminum	1
6.00" X 3.00" X 0.875" - Aluminum	1
5.25" X 0.6875" X 0.375" - Aluminum	2
4.75" X 4.50" X 0.50" - Aluminum	1
9.75" X 6.00" X 0.50" - Aluminum	1
8.00" X 7.00" X 0.50" - Aluminum	2
Renishaw Tonic - Readhead	1
Renishaw Tonic - Interface Unit	1
P Limit Magnet	2
Q Limit Magnet	2
RGG-2 Epoxy Packet	2
Cable Tie Bases for 6 mm (1/4") Wide Ties (Qty. 250)	1
Releasable Cable Ties, 6 mm (1/4") wide. (Qty. 100)	1
CONN D-SUB HOUSING PLUG 15POS	4
CONN D-SUB HOUSING PLUG 26POS	4
CONN D-SUB HOUSING RECEPT 15POS	4
CONN D-SUB HOUSING RECEPT 26POS	4
CONN D-SUB PIN 22-28AWG GOLD	100
CONN D-SUB SOCKET 22-28AWG GOLD	100
CONN D-SUB HOUSING PLUG 9POS	4
CONN D-SUB HOUSING PLUG 15POS	4
CONN D-SUB HOUSING PLUG 25POS	4
CONN D-SUB HOUSING RECEPT 9POS	4
CONN D-SUB HOUSING RECEPT 15POS	4
CONN D-SUB HOUSING RECEPT 25POS	4
CONTACT PIN 24-28AWG CRIMP GOLD	100
CONN PIN 20-24AWG GOLD CRIMP	100
CONN SOCKET 24-28AWG GOLD CRIMP	100
CONN SOCKET 20-24AWG GOLD CRIMP	100
CONN BACKSHELL DB9 DIE CAST	4
CONN BACKSHELL DB15 DIE CAST	4
CONN BACKSHELL DB25 DIE CAST	4
CONN MDR PLUG 20POS SLD CUP	4
CONN MDR PLUG 26POS SLD CUP	4
CONN BACKSHELL 20POS 180DEG SHLD	4
CONN BACKSHELL 26POS 180DEG SHLD	4
MOD F-CONN V0 POLARIZED	2
MOD F-CONN V0 POLARIZED	2
HDR W/C-C V0 w/GROUND	2
MOD F-CONN V0 POLARIZED	2

BOM-Injury Device, Plate press

PUSH BUTTON FOR F-CONN/M-CONN;LOOSE	2
Multipurpose 6061 Aluminum, Rectangular Bar, 1" x 2-1/2", 2' Long	1
Multipurpose 6061 Aluminum, Rectangular Bar, 1" x 5", 2' Long	1
Multipurpose 6061 Aluminum, Rectangular Bar, 1-1/4" x 4", 2' Long	1
Multipurpose 6061 Aluminum, Rectangular Bar, 1-1/4" x 6", 2' Long	1
Tight-Tolerance 304 Stainless Steel Rod, Precision Ground Finish, 3/4" Diameter, 2' Long	1
12 Point Flange Head Cap Screw, Type 304 Stainless Steel, 3/8"-16 Thread, 1/2" Long, Fully	1
18-8 Stainless Steel Socket Head Cap Screw, 5/16"-18 Thread, 1-1/2" Length, packs of 10	1
18-8 Stainless Steel Brass-Tip Set Screws 3/8"-16 Thread, 3/4" Long	1
18-8 Stainless Steel Thin Hex Nut, 3/8"-16 Thread Size, 9/16" Width, 7/32" Height, packs of 1	1
18-8 Stainless Steel Button-Head Socket Cap Screw, 10-32 Thread, 5/8" Length, packs of 10(	1
Conformable Soft Brass-Tip Set Screw, 18-8 Stainless Steel, 1/4"-20 Thread, 1/2" Long, pack	1
18-8 Stainless Steel Dowel Pin, 1/4" Diameter, 1-1/2" Length, packs of 10	1
Plastic Dowel Pin, 5/16" Diameter, 3/4" Length, Acetal, packs of 50	1
Acetal Sleeve Bearing for 3/4" Shaft Diameter, 1" OD, 3/4" Length	4
Rubber Bumper with Unthreaded Hole, Heavy Duty, SBR, Steel Washer, 13/16" OD, 13/16" H	1
Push/Pull Action Toggle Clamp, Hole Mounted, 700 lb Maximum Hold Capacity, 5-5/8" Height	1
18-8 Stainless Steel Socket Head Screw 8-32 Thread Size, 1" Long	1
18-8 Stainless Steel Button Head Hex Drive Screw 4-40 Thread Size, 3/8" Long	1
Weather-Resistant EPDM Rubber Sheet with Adhesive-Back, 12" x 12", 1/8" Thick	1
17-4 PH Stainless Steel Shim Stock 4" x 60" Roll, 0.031" Thick	1
Compression Spring - P/N 788, 0.968OD, 0.080 302/304 stainless steel, 20.000 overall length	1

Supplemental Table 2

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National Instruments - cRIO 9514 - Control Signals - MDR 20

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Austin, TX 78759-3504
1-888-280-7645
1-512-683-8411
http://www.ni.com/

MDR - 20
From: National Instruments NI - cRIO Servo Interface - 9514 - Operating Instructions - 374988e.pdf
Page: 10

1	Forward Limit	●	RENISHAW - DSUB	10	Limits, Q	HDR 11-12
2	Home	-	-	-	-	
3	COM	●	System Power	2	24Vdc GND	HDR 03-04
4	Digital Input 0	-	-	-	-	
5	COM	●	System Power	2	24Vdc GND	HDR 03-04
6	Encoder Index+	●	Copley Xenus - J7 (XSL)	20	Multi-Mode Encoder X	HDR 23-24
7	Encoder Index-	●	Copley Xenus - J7 (XSL)	21	Multi-Mode Encoder /X	HDR 25-26
8	COM	●	System Power	2	24Vdc GND	HDR 03-04
9	+5V OUT	-	-	-	-	
10	Position Compare	-	-	-	-	
11	Reverse Limit	●	RENISHAW - DSUB	11	Limits, P	HDR 13-14
12	Reserved	-	-	-	-	
13	Vsup	●	System Power	1	+24Vdc	HDR 01-02
14	Reserved	-	-	-	-	
15	COM	-	System Power	2	24Vdc GND	HDR 03-04
16	Encoder Phase A+	●	Copley Xenus - J7 (XSL)	16	Multi-Mode Encoder A	HDR 15-16
17	Encoder Phase A-	●	Copley Xenus - J7 (XSL)	17	Multi-Mode Encoder /A	HDR 17-18
18	Encoder Phase B+	●	Copley Xenus - J7 (XSL)	18	Multi-Mode Encoder B	HDR 19-20
19	Position Capture	-	-	-	-	
20	Encoder Phase B-	●	Copley Xenus - J7 (XSL)	19	Multi-Mode Encoder /B	HDR 21-22

HDR 07-08

HDR 07-08 DISCONNECT

HDR 07-08 DISCONNECT

HDR 07-08 DISCONNECT

National Instruments - cRIO 9514 - Control Signals - HD DSUB 15

National Instruments Corp.
11500 N Mopac Expressway
Austin, TX 78759-3504
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http://www.ni.com/

HD DSUB - 15
From: National Instruments NI - cRIO Servo Interface - 9514 - Operating Instructions - 374988e.pdf
Page: 9

1	Drive Command COM	●	Copley Xenus - J7 (XSL)	25	Ref(-) Input	HDR 33-34	
2	Drive Enable	●	ESTOP - DSUB 9	1	DPDT	HDR 37-38	
3	Reserved	-	-	-	-		
4	Reserved	-	-	-	-		
5	Reserved	-	-	-	-		
6	Drive Command	●	Copley Xenus - J7 (XSL)	24	Ref(+) Input	HDR 31-32	
7	COM	●	System Power	2	24Vdc GND	HDR 03-04	HDR 07-08
8	Digital Input 1	-	-	-	-		
9	Reserved	-	-	-	-		
10	Reserved	-	-	-	-		
11	Reserved	-	-	-	-		
12	Vsup	●	System Power	1	+24Vdc	HDR 01-02	
13	Reserved	-	-	-	-		
14	COM	●	System Power	2	24Vdc GND	HDR 03-04	HDR 07-08
15	NC	-	-	-	-		

DISCONNECT

NA

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DSUB 9
From: NA
Page: NA

1		•	NI 9514 - HD DSUB 15	2	Drive Enable	HDR 37-38
2		•	Copley Xenus - J7 (XSL)	3	Enable [IN1]	HDR 39-40
3	+5VDC					HDR 05-06
4	GND					HDR 07-08
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

Rensihaw Tonic - Feedback Connector

Renishaw Inc.
5277 Trillium Blvd
Hoffman Estates, IL 60192
1-847-286-9953

www.renishaw.com

DB15 - Female

From Dc Renishaw Tonic - Linear Encoder - Tonic Installation - tonic_t1000_m-9653-9225-01-a(en).pdf

Page 13

1	Set-up	-	-		-	-	
2	Power, 0V (Pin 2,9)	●	System Power		4	5Vdc GND	HDR 07-08
3	Alarm, E-	-	-		-	-	
4	Reference Mark, Z-	●	Copley Xenus - J8 (XSL)		9	Encoder /X Input	
5	Incremental, B-	●	Copley Xenus - J8 (XSL)		7	Encoder /B Input	
6	Incremental, A-	●	Copley Xenus - J8 (XSL)		5	Encoder /A Input	
7	Power, +5V (Pin 7,8)	●	System Power		3	+5Vdc	HDR 05-06
8	Power, +5V (Pin 7,8)	●	System Power		3	+5Vdc	HDR 05-06
9	Power, 0V (Pin 2,9)	●	System Power		4	5Vdc GND	HDR 07-08
10	Limits, Q	●	NI 9514 - MDR 20		1	Forward Limit	HDR 11-12
11	Limits, P	●	NI 9514 - MDR 20		11	Reverse Limit	HDR 13-14
12	Reference Mark, Z+	●	Copley Xenus - J8 (XSL)		8	Encoder X Input	
13	Incremental, B+	●	Copley Xenus - J8 (XSL)		6	Encoder B Input	
14	Incremental, A+	●	Copley Xenus - J8 (XSL)		4	Encoder A Input	
15	NC	-	-		-	-	

Copley Controls - Xenus XSL - Control Signals - J7

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MDR - 26
From: Copley Controls - Servo Drive - Xenus XSL - Xenus Datasheet - Xenus-datasheet.pdf
Page: 13

1	Frame Ground	●	CHASSIS					
2	Signal Ground	●	System Power	2	24Vdc GND	HDR 03-04		
3	Enable [IN1]	●	ESTOP - DSUB 9	2	DPDT	HDR 39-40		
4	GP Input [IN2]	-	-	-	-			
5	GP Input [IN3]	-	-	-	-			
6	GP Input [IN4]	-	-	-	-			
7	HS Input [IN6]	-	-	-	-			
8	HS Input [IN7]	-	-	-	-			
9	HS Input [IN8]	-	-	-	-			
10	HS Input [HS9]	-	-	-	-			
11	HS Input [IN10]	-	-	-	-			
12	GP Input [IN11]	-	-	-	-			
13	[OUT1]	-	-	-	-			
14	[OUT2]	-	-	-	-			
15	[OUT3]	-	-	-	-			
16	Multi-Mode Encoder A	●	NI 9514 - MDR 20	16	Encoder Phase A+	HDR 15-16		
17	Multi-Mode Encoder /A	●	NI 9514 - MDR 20	17	Encoder Phase A-	HDR 17-18		
18	Multi-Mode Encoder B	●	NI 9514 - MDR 20	18	Encoder Phase B+	HDR 19-20		
19	Multi-Mode Encoder /B	●	NI 9514 - MDR 20	20	Encoder Phase B-	HDR 21-22		
20	Multi-Mode Encoder X	●	NI 9514 - MDR 20	6	Encoder Index+	HDR 23-24		
21	Multi-Mode Encoder /X	●	NI 9514 - MDR 20	7	Encoder Index-	HDR 25-26		
22	+5 Vdc @ 400mA	-	-	-	-			
23	Signal Ground	●	System Power	2	24Vdc GND	HDR 03-04	DISCONNECT	
24	Ref(+) Input	●	NI 9514 - HD DSUB 15	6	Drive Command	HDR 31-32		
25	Ref(-) Input	●	NI 9514 - HD DSUB 15	1	Drive Command COM	HDR 33-34		
26	[IN12] GP Input	-	-	-	-			

Copley Controls - Xenus XSL - Motor Feedback - J8

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MDR - 20
From: Copley Controls - Servo Drive - Xenus XSL - Xenus Datasheet - Xenus-datasheet.pdf
Page: 13

1	Frame Ground	●	CHASSIS					
2	Signal Ground	●	System Power	2	24Vdc GND	HDR 03-04		
3	+5Vdc @ 400 mA	-	-	-	-			
4	Encoder A Input	●	RENISHAW - DSUB	14	Incremental, A+			
5	Encoder /A Input	●	RENISHAW - DSUB	6	Incremental, A-			
6	Encoder B Input	●	RENISHAW - DSUB	13	Incremental, B+			
7	Encoder /B Input	●	RENISHAW - DSUB	5	Incremental, B-			
8	Encoder X Input	●	RENISHAW - DSUB	12	Reference Mark, Z+			
9	Encoder /X Input	●	RENISHAW - DSUB	4	Reference Mark, Z-			
10	Signal Ground	●	System Power	2	24Vdc GND	HDR 03-04	DISCONNECT	
11	Digital Hall U	-	-	-	-			
12	Digital Hall V	-	-	-	-			
13	Digital Hall W	-	-	-	-			
14	[IN5] Temp Sensor	-	-	-	-			
15	Signal Ground	●	System Power	2	24Vdc GND	HDR 03-04	DISCONNECT	
16	Encoder Sin(+) Input	-	-	-	-			
17	Encoder Sin (-) Input	-	-	-	-			
18	Encoder Cos (+) Input	-	-	-	-			
19	Encoder Cos (-) Input	-	-	-	-			
20	Signal Ground	●	System Power	2	24Vdc GND	HDR 03-04	DISCONNECT	

System Power Connector

Phoenix

4 Terminal Screw Connector

From:

Page:

1	+24Vdc IN	●	NI 9514 - HD DSUB 15	12	Vsup	NI 9514 - MDR 20	13
2	24Vdc GND	●	NI 9514 - HD DSUB 15	7, 14	COM	NI 9514 - MDR 20	3, 5, 8, 15
3	+5Vdc In	●					
4	5Vdc GND	●					

Vsup						
COM	Copley Xenus - J7 (XSL)	2, 23	Signal Ground	Copley Xenus - J8 (XSL)	2, 10, 15, 20	Signal Ground

Copley Controls - Xenus XSL - Control Signals - J7

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HD DSUB - 26
From: Copley Controls - Servo Drive - Xenus XTL - DataSheet - Xenus_XTL.pdf
Page: 18

1	Frame Gnd	●				
2	Ref (-)	●				
3	Ref (+)	●				
4	[IN1] Enable	●				
5	[IN2] GP	-	-	-	-	
6	[IN3] GP	-	-	-	-	
7	[IN4] GP	-	-	-	-	
8	[IN11] GP	-	-	-	-	
9	[IN12] GP	-	-	-	-	
10	[IN6] HS	-	-	-	-	
11	[IN7] HS	-	-	-	-	
12	[IN8] HS	-	-	-	-	
13	[IN9] HS	-	-	-	-	
14	[IN10] HS	-	-	-	-	
15	Signal Gnd	●				
16	[OUT1]	-	-	-	-	
17	[OUT2]	-	-	-	-	
18	[OUT3]	-	-	-	-	
19	Signal Gnd	●				
20	+5 Vdc (Note 1)	-	Note 1: Total current drawn from +5 Vdc outputs cannot exceed 400 mA			
21	Multi Encoder /X	●				
22	Multi Encoder X	●				
23	Multi Encoder /B	●				
24	Multi Encoder B	●				
25	Multi Encoder /A	●				
26	Multi Encoder A	●				

Copley Controls - Xenus XSL - Motor Feedback - J8

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HD DSUB - 15
From: Copley Controls - Servo Drive - Xenus XTL - DataSheet - Xenus_XTL.pdf
Page: 18

1	Frame Gnd
2	+5 Vdc (Note 1)
3	Hall U
4	+5 Vdc (Note 1)
5	Signal Gnd
6	Hall V
7	Encoder /X
8	Encoder X
9	Hall W
10	[IN5] Motemp
11	Encoder /B
12	Encoder B
13	Encoder /A
14	Encoder A
15	Signal Gnd

Note 1: Total current drawn from +5 Vdc outputs cannot exceed 400 mA

Pins		Signal	Connected to
1	2	System Power (+24 V d.c.)	
3	4	System Power (ground)	
5	6	+5VDC	
7	8	System Power (ground)	
9	10	Empty	
11	12	Renishaw Encoder Forward Limit	
13	14	Renishaw Encoder Reverse Limit	
15	16	Renishaw Encoder Output A	
17	18	Renishaw Encoder Output A-	
19	20	Renishaw Encoder Output B	
21	22	Renishaw Encoder Output B-	
23	24	Renishaw Encoder Reference	
25	26	Renishaw Encoder Reference -	
27	28	Empty	
29	30	Empty	
31	32	Drive Command from NI 9514 (+)	
33	34	Drive Command from NI 9514 (-)	
35	36	Empty	
37	38	NI 9514 Enable	
39	40	Xenus Enable	