Journal of Visualized Experiments

Designing a bio-responsive robot from DNA origami

--Manuscript Draft--

Manuscript Number:	JoVE50268R3
Article Type:	Invited Methods Article - JoVE Produced Video
Corresponding Author:	Ido Bachelet, Ph.D. Bar Ilan University Ramat Gan, ISRAEL
First Author:	Eldad Ben-Ishay, Ph.D.
Order of Authors:	Eldad Ben-Ishay, Ph.D.
	Almogit Abu-Horowitz, Ph.D.
	Ido Bachelet, Ph.D.

Designing a bio-responsive robot from DNA origami

Authors:

Eldad Ben-Ishay, Almogit Abu-Horowitz, Ido Bachelet

Author institutional affiliations:

Eldad Ben-Ishay, Ph.D.
Faculty of Life Sciences and the Institute for Nanotechnology & Advanced Materials
Bar-Ilan University, Ramat-Gan, Israel
eldadbi@gmail.com

Almogit Abu-Horowitz, Ph.D.
Faculty of Life Sciences and the Institute for Nanotechnology & Advanced Materials
Bar-Ilan University, Ramat-Gan, Israel
almogit@gmail.com

Ido Bachelet, Ph.D.
Faculty of Life Sciences and the Institute for Nanotechnology & Advanced Materials
Bar-Ilan University, Ramat-Gan, Israel
Ido.bachelet@biu.ac.il

Corresponding author:

Ido Bachelet

Keywords:

DNA origami, nanorobotics

Short abstract: (50 words maximum)

DNA origami is a powerful method for fabricating precise nanoscale objects by programming the self-assembly of DNA molecules. Here we describe how DNA origami can be utilized to design a robotic robot capable of sensing biological cues and responding by shape shifting, subsequently relayed to a desired effect.

Long abstract: (150 words minimum, 400 words maximum)

Nucleic acids are astonishingly versatile. In addition to their natural role as storage medium for biological information[1], they can be utilized in parallel computing[2,3], recognize and bind molecular or cellular targets[4,5], catalyze chemical reactions[6,7], and generate calculated responses in a biological system[8,9]. Importantly, nucleic acids can be programmed to self-assemble into 2D and 3D structures[10-12], enabling the integration of all these remarkable features in a single robot linking the sensing of biological cues to a preset response in order to exert a desired effect.

Creating shapes from nucleic acids was first proposed by Seeman[13], and several variations on this theme have since been realized using various techniques[11,12,14,15]. However, perhaps the most significant is the one proposed by Rothemund, termed scaffolded DNA origami[16]. In this technique, the folding of a long (>7,000 bases) single-stranded DNA 'scaffold' is directed to a desired shape by hundreds of short complementary strands termed 'staples'. Folding is carried out by temperature annealing ramp. This technique was successfully demonstrated in the creation of a diverse array of 2D shapes with remarkable precision and robustness. DNA origami was later extended to 3D as well[17,18].

The current paper will focus on the caDNAno 2.0 software[19] developed by Douglas and colleagues. caDNAno is a robust, user-friendly CAD tool enabling the design of 2D and 3D DNA origami shapes with versatile features. The design process relies on a systematic and accurate abstraction scheme for DNA structures, making it relatively straightforward and efficient.

In this paper we demonstrate the design of a DNA origami nanorobot that has been recently described[20]. This robot is 'robotic' in the sense that it links sensing to actuation, in order to perform a task. We explain how various sensing schemes can be integrated into the structure, and how this can be relayed to a desired effect. Finally we use Cando[21] to simulate the mechanical properties of the designed shape. The concept we discuss can be adapted to multiple tasks and settings.

Protocol Text:

The robot we will design in this paper responds to a protein *P* by making a cargo *C* available to bind to receptors on the surface of a chosen target cell. The robot is shown in **Figure 1**. *C* may be a receptor-blocking drug; a growth factor etc., and a way to chemically link it to a DNA oligonucleotide must be available that does not destroy its function.
The robot has two states. When inactive, DNA gates on the two external 'lips' are hybridized, making sure the robot remains closed such that any cargo loaded within it is securely sequestered. In the presence of protein *P*, the gates open by either one of several mechanisms (discussed below) allowing the robot to open and expose the cargo.
When designing the structure, consider that the robot has to be flexible enough to close onto itself in the closed state, and spring to the open state when the gates enable it to do so. Modeling the behavior of a DNA structure integrating thermodynamic and mechanical components is difficult, and the actual object might require some iterative improvement. Nevertheless, here we focus on the design process using a general working model, which can be built upon.

Note:
For a more comprehensive understanding of the process of DNA origami design and folding, we highly recommend reading the original caDNAno paper by Douglas and colleagues[19] which explains the abstract representation of DNA in the design interface and how it relates to the actual molecular structure of a 3D DNA shape. This paper is accompanied by two video tutorials describing the caDNAno representation and interface in a very clear way. Additionally, we recommend reading the more recent paper by Dietz and colleagues describing many important aspects and detailed protocols of the folding process, including the Cando analysis tool[21].

1. Download and install caDNAno 2.0 and Autodesk Maya 2012

Note: Autodesk software is free for students and academic use. The instructions below include setting up an academic account at Autodesk.

1.1. Create an academic account at http://students.autodesk.com/. After receiving the account setup e-mail, click the activation link and fill in your preferences as desired.

1.2. Download the free version of Maya 2012 from the download center.

1.3. Install Maya 2012 on your computer.

1.4. Run Maya once before installing caDNAno 2.0.

1.5. Download and install the latest version of caDNAno 2.0 from http://cadnano.org/.

1.6. Run Maya 2012. A caDNAno icon should appear at the top right corner of the graphical user interface. Click the icon to go into caDNAno.

2. Outline the desired shape and scaffold strand path

2.1. The design interface of caDNAno within Maya includes 3 panels **(Figure 2)**:
1. Top panel: lattice view, where the shape is initially outlined. This panel enables double helix-level actions and provides a section view of the shape.
2. Bottom panel: editing panel, enabling single base-level actions.
3. Right panel: a Maya-generated real time 3D model of the shape

2.2. Click the "Honeycomb" icon. Zooming in on and out of the lattice in the top panel can be done by mouse scroll up and down, respectively.
caDNAno enables two possible design lattices, honeycomb and square; in this paper we will use the honeycomb layout, although the square lattice could be generally used as well[22].

2.3. Start by drawing the section of the desired shape on the left panel.
Each circle represents a double DNA helix. To choose the helices which build the shape, simply left-click on their center **(Figure 3)**. Continue helix by helix until the entire shape is outlined. Alternatively, the shape can be drawn by pressing the left mouse button and continuously drawing the shape's outline. Any action can be undone by clicking Edit menu and "Undo", or by the keyboard shortcut CTRL+Z (PC) or CMD+Z (Mac).
At this point, the selected helices will appear yellow. At the same time, the bottom panel will display a side view of the shape, comprised of these helices. The helix numbering in the bottom panel is consistent with numbering in the top one.

2.4. Observe the bottom panel. Each helix is represented by two rows of squares: the rows are the two strands of the double helix, with each square representing a base **(Figure 4)**.
The orange vertical bar determines where editing actions take place along a helix. The base position along the grid appears as a number above the orange bar.
The helix framework's default length is 42 bases. The length can be extended by clicking one of the grey arrow icons at the top right corner of the editing panel and choosing the extension length (in multiples of 21, which correspond to two full turns of the DNA helix, in which one turn spans 10.5 bases) **(Figure 4).** The grid will extend to the direction of the chosen arrow.

2.5. To plot the actual scaffold strand path throughout the shape, press the mouse button, start from the first helix and go continuously over all the helices following the same order they were initially selected in section 2.3. Note that:
1. The helices selected this time will change color to orange.
2. In the bottom panel, scaffold strand fragments will be automatically drawn at the selected helices.
3. The right panel will show the 3D model of the shape being built in real time.

At the end of this process, a draft of the scaffold strand path will be automatically drawn in the bottom panel **(Figure 5)**.

2.6. Draw a rectangle around all the leftmost edges of the scaffold path. Note that edges so selected will appear red **(Figure 6)**.

2.7. Extend the scaffold path by dragging the selected edges as a group to the left side of the grid. Repeat this process for the right edges until the path is properly extended. Note that scaffold extension also extends the 3D shape in the right panel **(Figure 7)**.

2.8. Locate the scaffold path parts that are isolated from the rest, and connect them. In our shape, for example, helices 0-9 form an isolated part. Helix 9 needs to be connected to helix 12 (note that helices 9 and 10 are not adjacent in the shape [top panel] so they cannot be connected).

2.9. Zoom in on the strands to be connected, and using the "Select" tool click on any point on one of the strands. Upon clicking any point along a blue scaffold fragment, 'bridge' icons appear between helices, denoting the positions where crossovers are allowed. In these positions, bases in adjacent helices face each other directly, allowing the strands to cross over from helix to helix without deforming or twisting the DNA. The number appearing next to each bridge icon indicates the number of helix it will crossover to **(Figure 8)**.

2.10. To create crossovers, left click the bridge icon of choice. A scaffold crossover will be generated, meaning the scaffold crosses at this point from helix to helix **(Figure 9)**. Repeat this process until the scaffold traverses all helices and creates a closed loop that spans the entire shape, leaving no regions that are isolated from the rest of the shape.
Note that while crossovers appear to span a distance in the software, in reality they do not include any DNA base. Physically, the crossover "bridge" contains only one phosphate unit of the DNA backbone that links the two bases from the adjacent helices together.

2.11. Before moving on to the next step, make sure the entire scaffold is continuous, and no part of it is isolated from the others.

3. Define opening mechanism axes

The described robot opens in response to a defined biological input to expose its payload. Opening takes place in a shell-like manner, with two halves (helices 0-29 make up one half, helices 30-61 make up the second half) revolving around two axes. The axes are formed by crossovers between helices 29-30 and 61-0, which are the only crossovers between those halves and are positioned only in or close to the left edge of the grid. The right edge will contain the gate strands (discussed below).

3.1. Erase the existing crossover between helices 29-30. To erase the crossover, click the "knee" point in either strand. This leaves a nick in both strands where the crossover used to be. To seam the nicks, press SHIFT and click each nick.

3.2. Create a new crossover between helices 29-30 as close as possible to the left edge of the grid **(Figure 10)**.

3.3. Create a new crossover between helices 61 and 0 as close as possible to the left edge of the grid.

4. Define payload attachment sites

After we finish plotting the scaffold strand path, we need to define the payload attachment (loading) sites. Loading sites are in fact staple strands that extend out of their helices as single stranded 'branches'. It is therefore important to define very precisely where along the helix this branching occurs, to make sure it extends to the desired direction. If we define staple extensions arbitrarily, loading sites might occur on the external side of the robot instead of the internal side.
To make sure a staple extends to a specific direction only, we plot an additional helix, which serves as guides for the directional branching of the staple from the main body. After extending the desired loading site staple, the guide helix is removed.

4.1. Let us define 4 loading sites facing towards the internal side of the robot. The loading sites will branch out of helices 3, 27, 34, and 58. For each site, in the top panel click the helix immediately adjacent to these helices that faces the internal side **(Figure 11)**. This will add the helices to the grid in the bottom panel. Do not second-click these helices yet.

5. Add and edit staples

5.1. Click "AutoStaple". The software will automatically add staple sequences in various colors **(Figure 12).** Note that staples have been added to the 3D shape in the right panel. Staple colors are consistent for the bottom and right panels. In addition, there is an indicator on the bottom left corner of the interface, which indicates a staple.
Note: staples cannot be too long, too short or circular. Most of the staples generated here do not meet these criteria, and have to be edited. The first step in editing them is automatic (see next step).

5.2. Click "AutoBreak". A dialogue box will open **(Figure 13)**, asking for user-defined parameters for this action:

1. Target length (bp): anticipated length of staple if possible
2. Min length (bp): minimal length allowed for a staple
3. Max length (bp): maximal length allowed for a staple
4. Min dist to xover (bp): the minimal number of base pairs a staple can traverse between its edge and a crossover or between two crossovers.

Use the default parameters, click OK. The software will break the staples according to these parameters to the best of its ability **(Figure 14)**.

5.3. Erase all the staple crossovers between helices 29-30 and 61-0, to enable these helices to separate and enable the robot to open. Erasing staple crossovers will require some manual editing to correct staples that become too short or irrational as a result of this action. To do this properly, follow the instructions in the following sections.
Make sure to leave the scaffold crossovers created in sections 3.2 and 3.3 intact.

5.4. Consider, for example, the first staple crossover (cyan and black staples) from the left between helices 29 and 30 **(Figure 15)**. Erase both bridges of this crossover by clicking each knee point or bridge so it appears red, then hitting DELETE **(Figure 16)**.

5.5. Seam the two staples on helix 29 by pressing SHIFT and clicking the nick between them. Similarly, seam the three staples on strand 30 to a single staple **(Figure 17)**. Staples can be manually extended or shortened by clicking an edge and dragging it as desired. Take care not to circularize any staple. **Figure 18** shows the gap between helices 29-30 after complete editing of staple crossovers. Repeat this process for helices 0 and 61, and manually edit all the staples in each helix.

5.6. Locate staples that are drawn by a thick line, meaning they require further editing. Examine each one and correct as necessary. For example, staples that are too short can be erased **(Figure 19)** or extended if possible.

6. Create loading sites and gates

6.1. Second-click the loading site helices in the top panel, and extend the resulting scaffold strand fragments in the bottom panel by clicking an edge and dragging it as desired **(Figure 20)**.

6.2. Manually add staples to these scaffold fragments by placing the orange vertical bar at the desired position along the scaffold, going over the guide helices on the left panel, holding SHIFT and clicking. This will add a staple precursor at each helix **(Figure 21)**.

6.3. Extend the staple precursors to full length as well by clicking and dragging.

6.4. Locate the red bridge icons, denoting allowed crossover positions between the guide strand (for example, helix 62) and the chassis (for example, helix 3).

6.5. Choose the most convenient location to introduce a crossover and click the bridge icon **(Figure 22)**. A convenient location requires minimal editing of existing staples in the chassis.

6.6. In the guide helix (helix 62), delete the staple part that is not a part of the loading site, and shorten the participating part to the desired length. The desired length should provide both specificity for loading different types of cargo, and binding strength. Typically, an 18-mer tail should be fine. Make sure the staple remains drawn by a thin line, otherwise edit it until it is.

6.7. In the chassis, edit the changed staples as necessary.

6.8. Erase the guide (helix 62) leaving only the staple extension.

6.9. Repeat steps 6.4-6.8 for all loading sites **(Figure 23)**.

7. Designing gate strands

The gate strands are the only strands, except for the axes, linking helices 29-30 and 61-0. In contrast to the axes, the gate strands are not crossovers. Rather, they hybridize to form a double stranded segment that serves as the sensor for the biological input of choice. Once the gate duplexes are displaced, the entire robot can entropically revolve around the axes and open.

7.1. Locate the proper positions for gate strands. These will be staples on helices 29, 30, 61, and 0.

7.2. For example, examine the 29-30 gate region. There are convenient staple strands flanking helices 29 and 30 on the right side of the grid, which can be used as gate strands. Note that they face opposite directions.

7.3. Click the edge of one of the potential gate strands to extend it outside of the shape. If the edge lies over a scaffold crossover, its selection could be simplified by making sure only "Stap"(les) are selectable, by clicking off "Scaf"(fold) in the "Selectable" toolbar on the top right side of the interface.

7.4. Extend both staples to form the gate strands. Edit the staples if this extension requires it **(Figure 24)**. Repeat this for the gate strands of helices 0 and 61.

Note that for now, the actual length does not matter, since the sensor DNA (e.g. aptamer) will replace the gate strand sequences at the sequence completion step.

8. Choose scaffold sequence

8.1. Click the "Seq" tool. Place the cursor anywhere on the scaffold strand and click. A dialog box will open asking us to choose the scaffold DNA source **(Figure 25)**.

8.2. Choosing the source DNA greatly depends on the robot size. For example, M13mp18 ssDNA (p7249), and its extended derivatives (p7308 etc.) which have generallybeen the choice for large DNA origami shapes, fit when the scaffold strand is ~7 kb long. If the scaffold of the designed shape is significantly shorter than the chosen source, the excess scaffold strand that is not hybridized to any staple will create a loop of ssDNA protruding from the folded shape. While this usually poses little problem for relatively short loops, multi-kb long loops could drastically interfere with folding and function of the robot. Therefore it is important to fit the chosen source to the shape scaffold length.
For example, if the scaffold strand needed to fold a small shape is ~1,600 bases long, which is significantly shorter than the preset sources in the dialog box, a custom sequence can be used as scaffold. Several sources can be considered. For

example, the M13mp18 can be digested with a specific restriction enzyme that produces a fragment of the desired length. Designing such a source can be done at NebCutter (http://tools.neb.com/NEBcutter2/) by pasting the M13mp18 sequence (http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/dna_sequences_maps.asp?#.UAygyzFWomR) in the NebCutter input window, and mapping restriction sites. Another option is to use pre-digested ssDNA, such as the phiX174 virion ssDNA HaeIII digest, available from New England Biolabs.

8.3. In the dialog box, click "M13mp18". Note that the chosen DNA sequence has been added to the scaffold and staple strands in the bottom panel.

9. Export staple sequence as a spreadsheet.

9.1. Click "Export" on the top toolbar, and choose a destination filename for the staple list. Click "Save".

9.2. Locate the destination .csv file and open it.

9.3. The spreadsheet shows the staple list, which can be sent as is to a DNA synthesis company. The first two columns display the start and end coordinates, with the number outside the brackets denoting helix number and the number inside brackets denoting base position.

10. Assign gate and loading sequences.

10.1. In the staple list, you will notice that some sequences begin or end with a string of question marks "?????". These question marks denote that since no scaffold strand hybridizes with these specific staple regions, they cannot be assigned complementary sequences. These are in fact the extensions we designed for the gate strands and loading sites, and therefore these need to be assigned manually now.

10.2. Gate:

10.2.1. The gates determine the nature of the biological input upon which the robot will switch from inactive to active state and expose its payload. Each single dsDNA gate can encode response to one biological input (or more), so a profile of inputs required for robot activation can be defined.
Let us assume for this example that the biological cue triggering robot activation is a restriction enzyme, which could indicate the presence of infectious bacteria.

10.2.2. First consider that the gate ssDNA strands do not hybridize immediately after branching out of their helices. Designing the gate otherwise might impede hybridization during folding. Therefore, each branch should start with a spacer string. We typically use poly-T as spacer strings, as this sequence provides flexibility.

10.2.3. We also assume that the length of the gate hybridization region is 20 bases, containing the target restriction site in its middle.

10.2.4. Therefore the gate might look like this:

```
[Helix 29]-5′-.....TTTTTTTGTGAGTTxxxxxxGCTAGAG-3′
[Helix 30]-3′-.....TTTTTTTCACTCAAxxxxxxCGATCTC-5′
```

The "….." denote the staple region that hybridizes with the scaffold strand, therefore it has a sequence already and should not be changed.
The random duplex "GTGAGTT" and its complement ensures the restriction site is not partly open, and provides some extra bases to ensure effective digestion by the enzyme.
The "x" denotes the restriction site.
The random duplex "GCTAGAG" and its complement provide some extra bases for the enzyme to work efficiently, but also makes sure the gate strand is sufficiently long to ensure good robot closure.
Before choosing a restriction site as a target, make sure the entire robot structure, loading sites and other part of the gate itself are not digested by the enzyme of choice. In this examination, the NEBCutter 0-cutter list (enzymes which do not cut the entire sequence) highlighted EagI, isolated from the Enterobacter Pantoea agglomerans, as a potential enzyme that could indicate the presence of an enterobacterial infectious.

10.2.5. The gate now looks like this (yellow marks EagI restriction site):

```
[Helix 29]-5′-.....TTTTTTTGTGAGTTCGGCCGGCTAGAG-3′
[Helix 30]-3′-.....TTTTTTTCACTCAAGCCGGCCGATCTC-5′
```

Note that this design assumes that after digestion, the sequence "GTGAGTTCGG" (T_m = 32°C) is not sufficiently long or thermodynamically stable to hold the robot closed anymore. This assumption will most likely need to be verified experimentally.

10.2.6. The second gate can be the same in which case the robot would only respond to one enzyme, or can be designed with a different site, increasing specificity of robot. More restriction sites can be added onto the same strand, increasing the complexity and specificity of the robot.

10.3 Loading sites:

10.3.1. The loading site can be a universal sequence. Alternatively, loading sites can be based on unique sequences, which will decrease modularity but improve control over cargo orientation and ratios (for different types of cargo).

10.3.2. Finally, the loading site oligonucleotides should include a chemical functional group enabling them to conjugate with any payload: protein,

nanoparticle, etc. Make sure the chemical group is mounted on the correct end (5' or 3'), according to the staple direction.

11. Simulate results in CANDO.

11.1. After the job is saved as a .json file, it can be uploaded to CANDO for analysis. CANDO is a finite-element based simulation of the DNA structure that can estimate its rigidity and stability in solution[21].

11.2. Go to http://cando-dna-origami.org/

11.3. Click "Submit a caDNAno file for analysis" and fill all the necessary information.

11.4. Analysis in CANDO usually takes up to 15-20 min. At the end, an e-mail message lets us know the analysis is complete, providing a link to download the simulation results **(Figure 26)**.

12. Order DNA and fold the robot.

Once the design process is complete and CANDO analysis shows satisfactory prediction of the product, the staple strand list generated in sections 9-10 can be ordered. Typically, staple strands do not require particular purification; however, it is recommended that special purpose strands such as gates or loading sites be purified by HPLC.
The steps following DNA order, namely folding, purification and evaluation of product, including visualization of the folded structure by either atomic force microscopy (AFM) or transmission electron microscopy (TEM) are outside the scope of this paper, and can be found in previous reports[17,18,20,21]. A TEM image of the robot designed here is brought as an example **(Figure 27)**. Sample preparation and staining was carried out exactly as described elsewhere[21].

Representative results:

Figures 1-25 are screenshots of the caDNAno 2.0 interface showing the design process step-by-step. The cross-section of the shape was first outlined **(Figure 3)**, followed by automatic addition of scaffold strand fragments and completion of the entire scaffold path **(Figure 7)**. Staple strands are automatically added **(Figure 12)**, broken according to user-defined parameters **(Figure 14)**, and manually edited to adapt the staples to the desired function of the device **(Figures 15-18)**. **Figures 23-24** describe how loading site and gate strands are added and edited. Finally, **Figure 27** shows a TEM image of the model designed here.

Figure 1: A 3D model of the finished robot, designed by caDNAno 2.0 and generated by Autodesk Maya 2012.

Figure 2: A view of the caDNAno 2.0/Autodesk Maya 2012 design interface. Top panel: lattice panel for outlining the initial shape. Bottom panel: editing panel. Right panel: 3D model generator (see section 2.1).

Figure 3: Drawing the section of the shape on the top panel (see section 2.3).

Figure 4: The bottom (editing) panel of caDNAno 2.0. The orange vertical bar determines where along the grid editing actions will occur. The grey arrows on the top right corner are used to extend the grid to either side (see section 2.4).

Figure 5: A draft of the scaffold strand after the initial outline in the top panel (see section 2.5).

Figure 6: Selecting all the scaffold strand path edges and extending the path to the desired length (see section 2.7).

Figure 7: A general view of the bottom and right panels demonstrating how the 3D model changes in real time along with editing actions.

Figure 8: The blue bridge icons between helices denote the positions where scaffold crossovers are allowed (red icons refer to staple crossovers and are not yet shown, see section 2.9).

Figure 9: Creating new scaffold crossovers by clicking the bridge icons of choice (see section 2.10).

Figure 10: Creating an axis (a crossover a close as possible to the left side of the grid) between helices 29 and 30 (see section 3.2).

Figure 11: Adding helices that guide the branching of loading sites (see section 4.1).

Figure 12: The blueprint after the "AutoStaple" action. The staple colors in the bottom panel and the right panel are consistent (see section 5.1).

Figure 13: The "AutoBreak" dialogue box in which the user can define AutoBreak parameters (see section 5.2).

Figure 14: The blueprint after the "AutoBreak" action (see section 5.2).

Figure 15: Manual editing of staples I: locating staples that cross over from helix 29 and 30 and should be deleted.

Figure 16: Manual editing of staples II: deleting the bridges between the located staples.

Figure 17: Manual editing of staples III: seaming the nicks along fragmented staples (see section 5.5).

Figure 18: The entire gap between helices 29-30 showing no crossovers link the two (see section 5.5).

Figure 19: Manual editing of staples drawn in thick line (denoting they are either too short, too long or circular, see section 5.6).

Figure 20: Adding guide helices for loading site branching (see section 6.1).

Figure 21: Manual addition of staple strands to the guide helices, so branching points can be located (see section 6.2).

Figure 22: Introducing a loading site crossover to the robot chassis scaffold in a convenient location (one that requires minimal editing of chassis staples, see section 6.5).

Figure 23: View of the loading site staples as seen in the bottom panel after removing the guide helices, which are no longer necessary (see section 6.9).

Figure 24: Extending two staples, which are going to be used as gate strands, from helices 29 and 30. Note that the two strands face opposite directions, which is mandatory for the formation of the gate duplex (see section 7.4).

Figure 25: The scaffold sequence addition ("Seq" tool) dialogue box, allowing to choose either one of pre-defined scaffolds, or to insert a custom sequence (see section 8.1).

Figure 26: Results of CANDO analysis of the design described here. The simulation generates a .zip archive containing the various files that provide the requested information. Here the RMSF (root mean square fluctuation) files (.png) are depicted, showing a model of the design from 3 view angles, colored according to the key detailed in the accompanying file named "HeatMap4RMSF.txt". In this case, minimum RMSF (bluest) is 1.03 nm, and 95% RMSF (redest) is 3.19 nm. The gradient of color across the model derives from the polarity of the robot (gates in 'front', axis in 'back') and the fact that there are no connecting staples along helices 29-30 and 61-0, causing the 'front' side to fluctuate more than the 'back' side.

Figure 27: TEM image of the robot designed in this article. Sample preparation and staining was carried out exactly as described elsewhere[21].

Discussion

DNA origami enables us to fabricate accurately defined objects with arbitrary features at the nanoscale. An important next step would be the integration of function into these designs. While many applications and challenges could be addressed with this technology, there is a particular interest in fabricating therapeutic and scientific robots from DNA origami, as these represent a natural milieu of DNA. DNA already interfaces with molecular machinery in cells as a genetic information storage medium. Interestingly, the folded DNA in a nanorobot or another machine can still serve as genetic information in addition to being a construction material, which can be relayed to the expression of a desired protein after the nanorobot disintegrates, as parts of a sequence of outputs.

In the example discussed in this paper, we use a restriction enzyme to operate the robot. However, additional mechanisms by which DNA robots can respond to inputs include the following.

Molecular recognition: we recently demonstrated aptamer-based gates for DNA robots that recognize protein molecules on the surface on target cells[20]. Aptamers can be selected in-vitro using methods such as SELEX[23], outsourced from companies, or used from the aptamer database (http://aptamer.icmb.utexas.edu/). When aptamers are employed, it is important to consider that the strand complementary to the aptamer, which together forms the gate, can be designed to include mismatches, which will facilitate binding of the aptamer's ligand and displacement of the complementary strand. While the mechanism allowing this is unknown, the sensitivity and specificity of an aptamer-based gate can be tuned by increasing or decreasing the % of mismatch between the two strands, to get either a very stringent but inefficient gate, or a fast but leaky one.

Enzymatic cleavage: for this, the gates should be designed such that they contain the substrate of that enzyme. For example, a small peptide substrate of a protease can be tethered from both sides to the gate, which will keep the robot closed in the absence of the enzyme.

Remote control: a potential approach that has not been applied to DNA machines is using a gold nanocrystal antenna in a high-frequency electromagnetic field to induce dsDNA melting[24]. This may provide a user-operated switch in addition to bio-responsive ones.

Although DNA origami robots are relatively straightforward to design and make, they pose several technical challenges as a therapeutic platform. DNA is not an ideal material for drug delivery as it is highly vulnerable to cleavage by nucleases. Moreover, it might precipitate an immune response. A thorough study of the behavior of DNA origami objects in an organism is needed to define their fate and make sure they do not aggregate in tissues or integrate into the host genome.

In summary, we presented the use of caDNAno, a straightforward, robust CAD tool to designing DNA origami shapes. We hope to start seeing application-driven research in DNA origami, in areas such as therapeutics, energy, metamaterials, and education. In all these places, caDNAno is expected to have a significant impact on realizing the solutions. In the future, it might become an industrial and design standard, which can be replaced (or parts of which can) by any user because they are all compatible.

Acknowledgements

The authors wish to thank S. Douglas for extremely valuable discussions and advice, and all the members of the Bachelet lab for helpful discussions and work. This work is supported by grants from the Faculty of Life Sciences and Institute of Nanotechnology & Advanced Materials at Bar-Ilan University.

Disclosure

The authors have nothing to disclose.

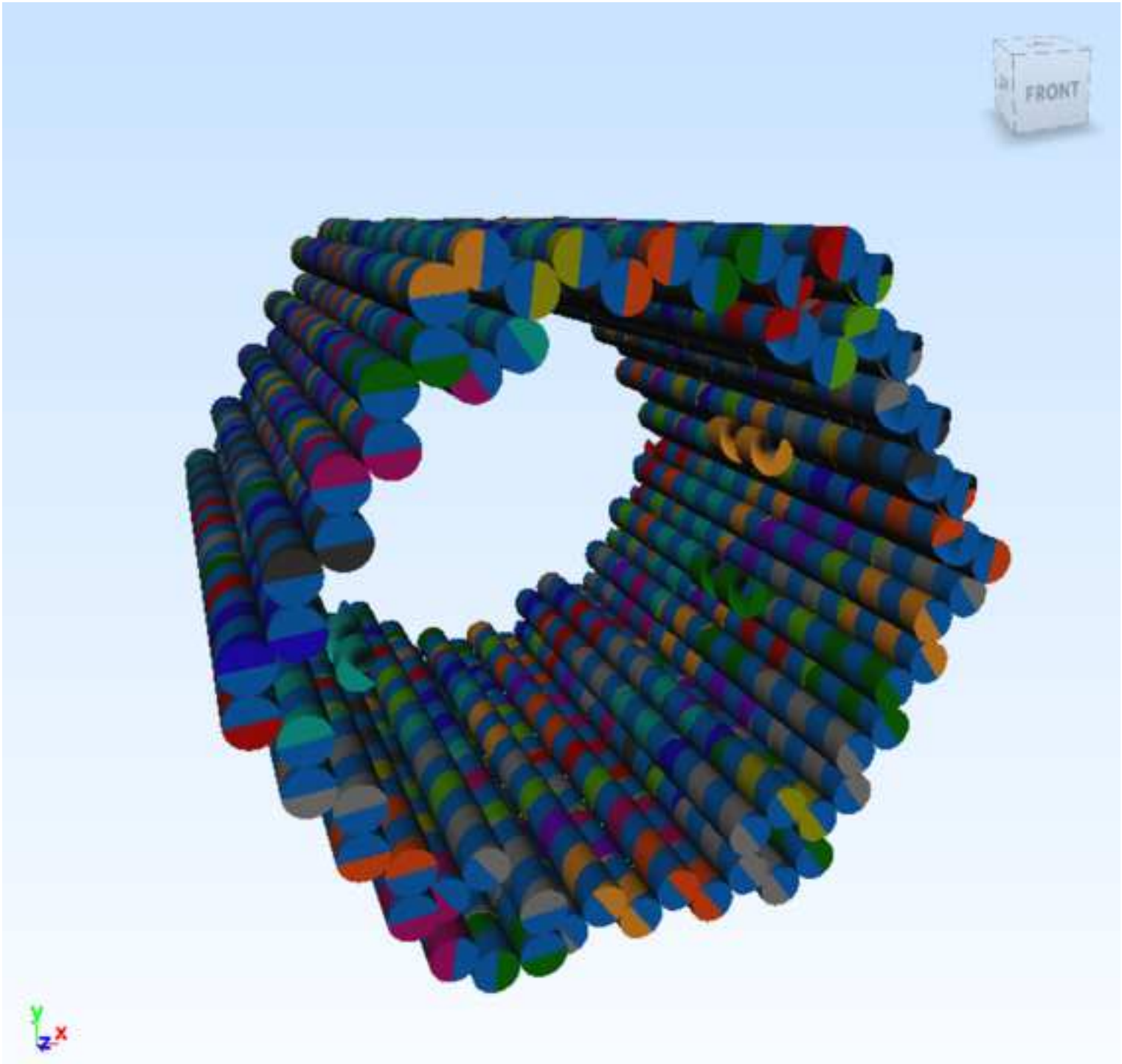
References

1 Watson, J. D. & Crick, F. H. Genetical implications of the structure of deoxyribonucleic acid. *Nature* **171**, 964-967 (1953).
2 Adleman, L. M. Molecular computation of solutions to combinatorial problems. *Science* **266**, 1021-1024 (1994).
3 Qian, L., Winfree, E. & Bruck, J. Neural network computation with DNA strand displacement cascades. *Nature* **475**, 368-372, doi:10.1038/nature10262 (2011).
4 Ellington, A. D. & Szostak, J. W. In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-822, doi:10.1038/346818a0 (1990).
5 Tang, Z., Parekh, P., Turner, P., Moyer, R. W. & Tan, W. Generating aptamers for recognition of virus-infected cells. *Clin Chem* **55**, 813-822, doi:10.1373/clinchem.2008.113514 (2009).
6 Baskerville, S. & Bartel, D. P. A ribozyme that ligates RNA to protein. *Proc Natl Acad Sci U S A* **99**, 9154-9159, doi:10.1073/pnas.142153799 (2002).
7 Bartel, D. P. & Szostak, J. W. Isolation of new ribozymes from a large pool of random sequences [see comment]. *Science* **261**, 1411-1418 (1993).
8 Benenson, Y., Gil, B., Ben-Dor, U., Adar, R. & Shapiro, E. An autonomous molecular computer for logical control of gene expression. *Nature* **429**, 423-429, doi:10.1038/nature02551 (2004).
9 Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R. & Benenson, Y. Multi-input RNAi-based logic circuit for identification of specific cancer cells. *Science* **333**, 1307-1311, doi:10.1126/science.1205527 (2011).
10 Rothemund, P. W., Papadakis, N. & Winfree, E. Algorithmic self-assembly of DNA Sierpinski triangles. *PLoS Biol* **2**, e424, doi:10.1371/journal.pbio.0020424 (2004).
11 Chen, J. H. & Seeman, N. C. Synthesis from DNA of a molecule with the connectivity of a cube. *Nature* **350**, 631-633, doi:10.1038/350631a0 (1991).
12 He, Y. *et al.* Hierarchical self-assembly of DNA into symmetric supramolecular polyhedra. *Nature* **452**, 198-201, doi:10.1038/nature06597 (2008).
13 Seeman, N. C. Nucleic acid junctions and lattices. *J Theor Biol* **99**, 237-247 (1982).
14 Wei, B., Dai, M. & Yin, P. Complex shapes self-assembled from single-stranded DNA tiles. *Nature* **485**, 623-626, doi:10.1038/nature11075 (2012).
15 Yin, P. *et al.* Programming DNA tube circumferences. *Science* **321**, 824-826, doi:10.1126/science.1157312 (2008).
16 Rothemund, P. W. Folding DNA to create nanoscale shapes and patterns. *Nature* **440**, 297-302, doi:10.1038/nature04586 (2006).

17 Dietz, H., Douglas, S. M. & Shih, W. M. Folding DNA into twisted and curved nanoscale shapes. *Science* **325**, 725-730, doi:10.1126/science.1174251 (2009).
18 Douglas, S. M. *et al.* Self-assembly of DNA into nanoscale three-dimensional shapes. *Nature* **459**, 414-418, doi:10.1038/nature08016 (2009).
19 Douglas, S. M. *et al.* Rapid prototyping of 3D DNA-origami shapes with caDNAno. *Nucleic Acids Res* **37**, 5001-5006, doi:10.1093/nar/gkp436 (2009).
20 Douglas, S. M., Bachelet, I. & Church, G. M. A logic-gated nanorobot for targeted transport of molecular payloads. *Science* **335**, 831-834, doi:10.1126/science.1214081 (2012).
21 Castro, C. E. *et al.* A primer to scaffolded DNA origami. *Nature methods* **8**, 221-229, doi:10.1038/nmeth.1570 (2011).
22 Ke, Y. *et al.* Multilayer DNA origami packed on a square lattice. *Journal of the American Chemical Society* **131**, 15903-15908, doi:10.1021/ja906381y (2009).
23 Mallikaratchy, P. *et al.* Using aptamers evolved from cell-SELEX to engineer a molecular delivery platform. *Chem Commun (Camb)*, 3056-3058, doi:10.1039/b823258j (2009).
24 Hamad-Schifferli, K., Schwartz, J. J., Santos, A. T., Zhang, S. & Jacobson, J. M. Remote electronic control of DNA hybridization through inductive coupling to an attached metal nanocrystal antenna. *Nature* **415**, 152-155, doi:10.1038/415152a (2002).

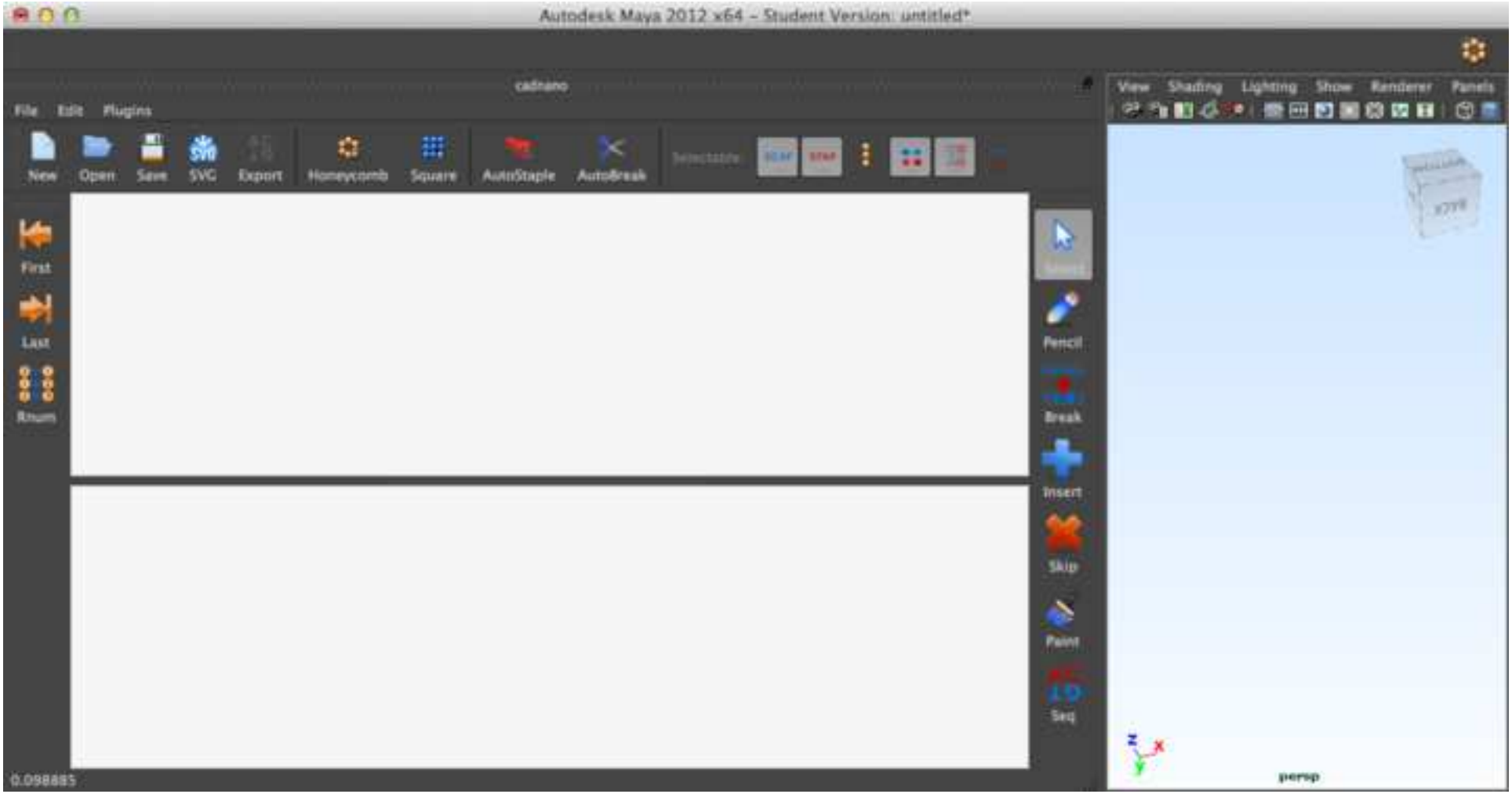
1

Click here to download high resolution image



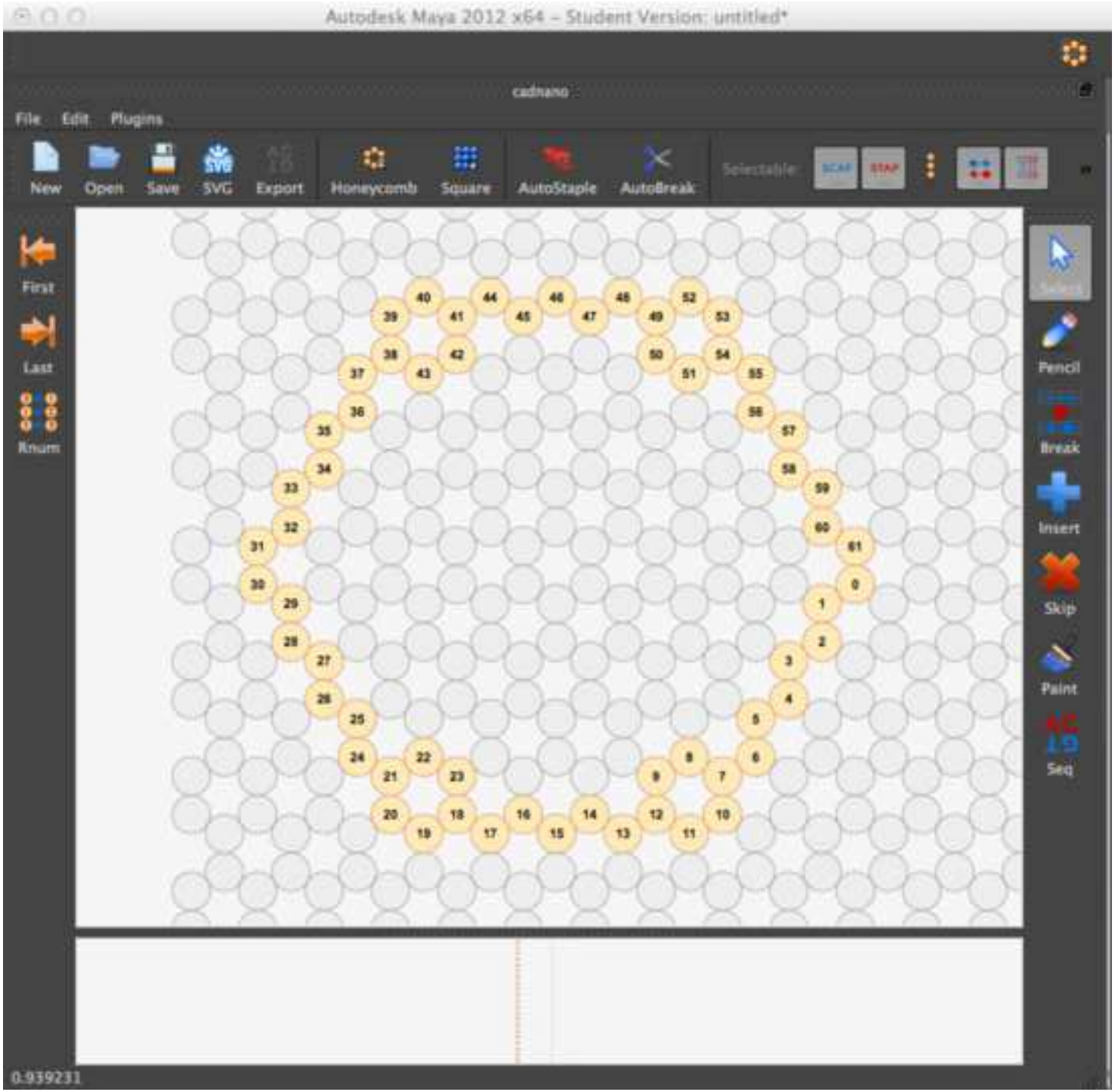
2

Click here to download high resolution image

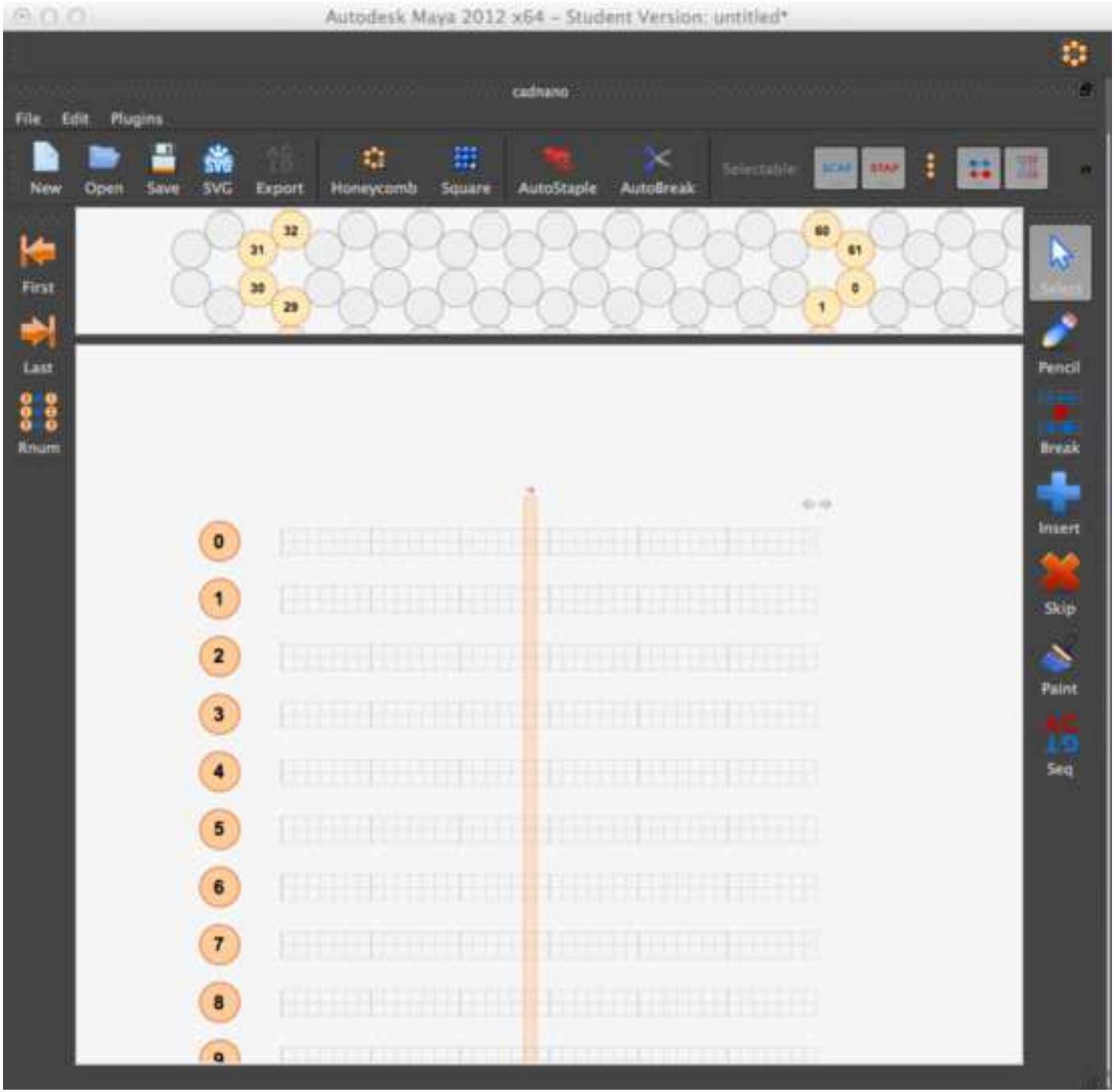


3

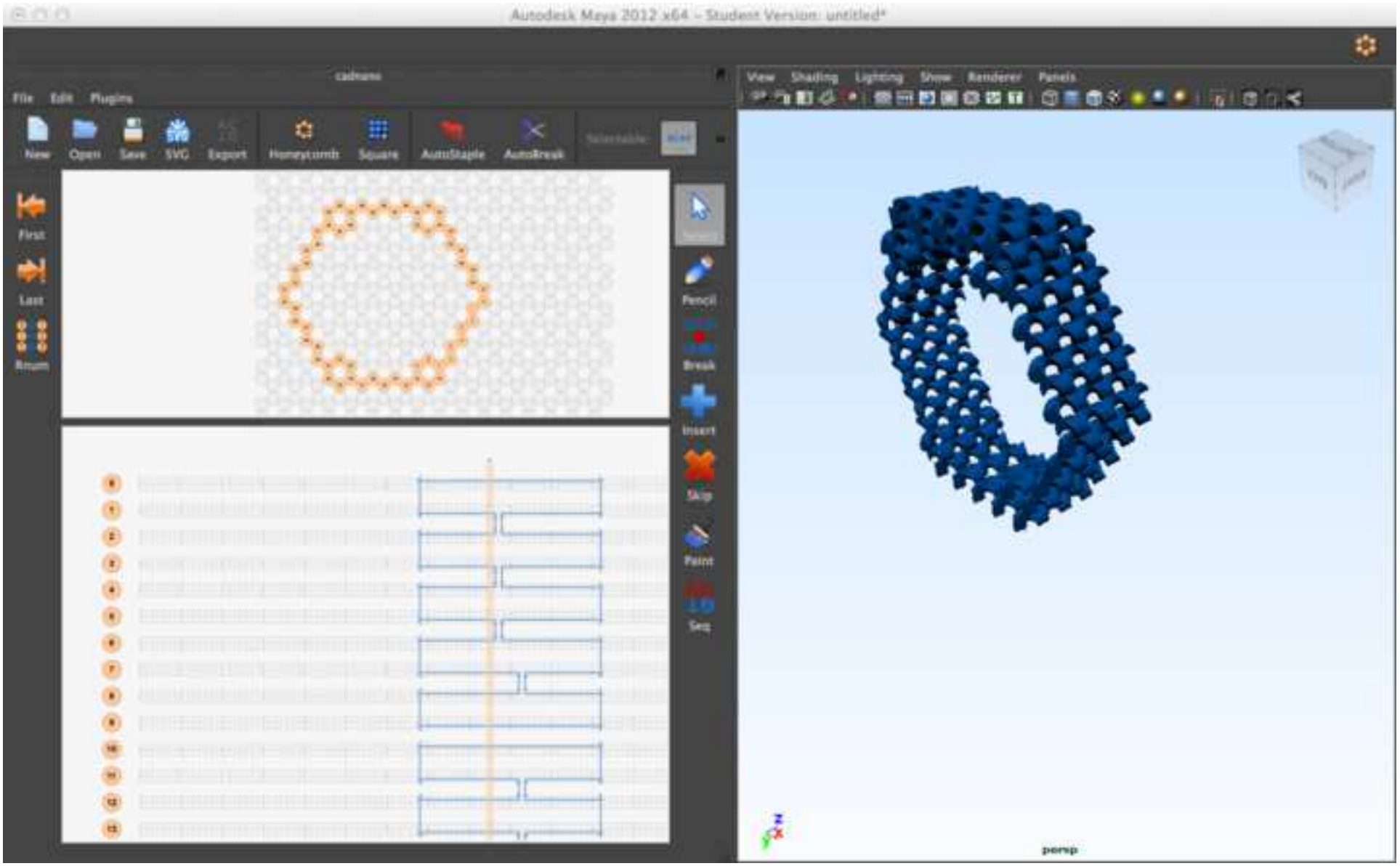
Click here to download high resolution image



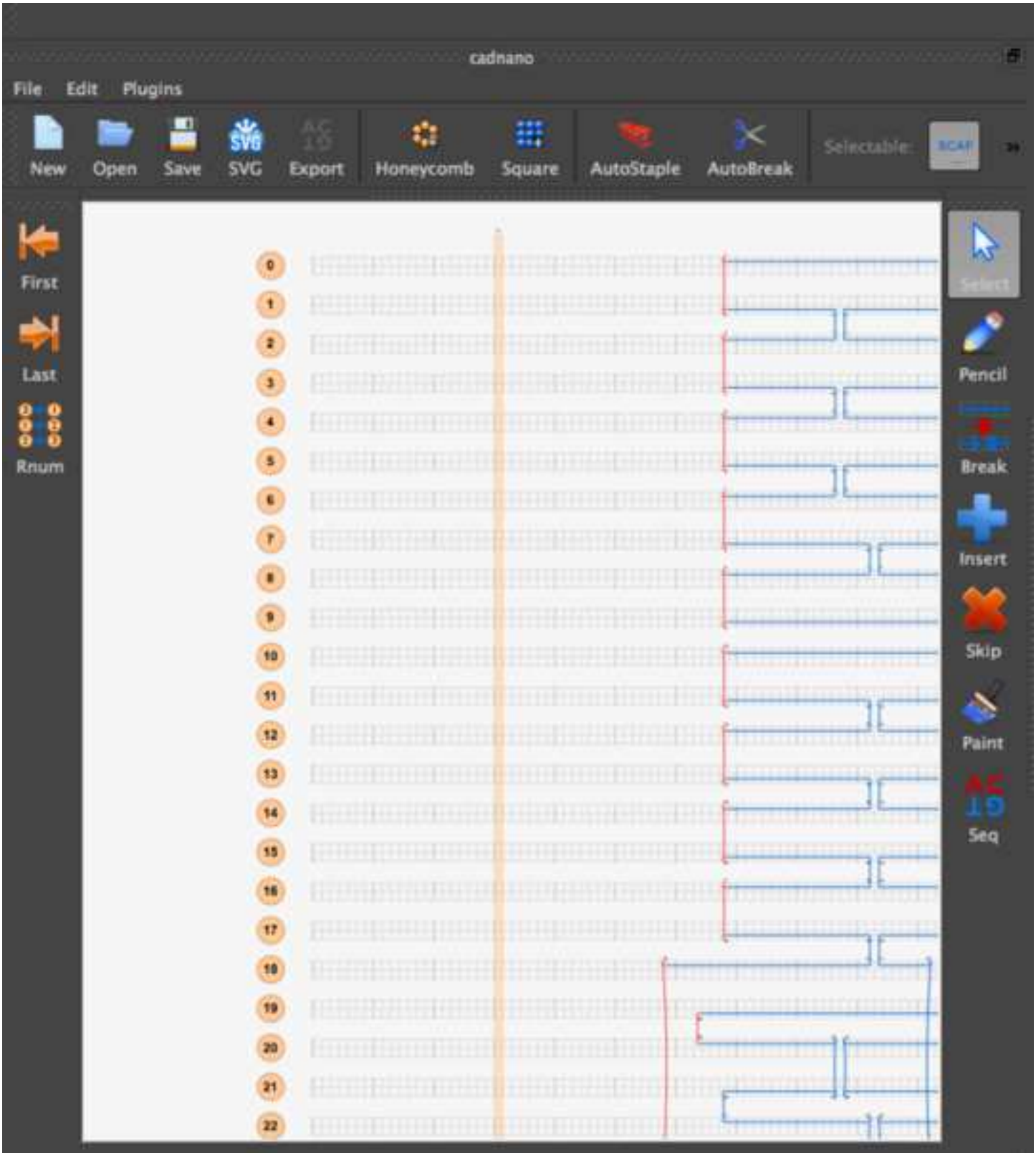
Click here to download high resolution image



Click here to download high resolution image

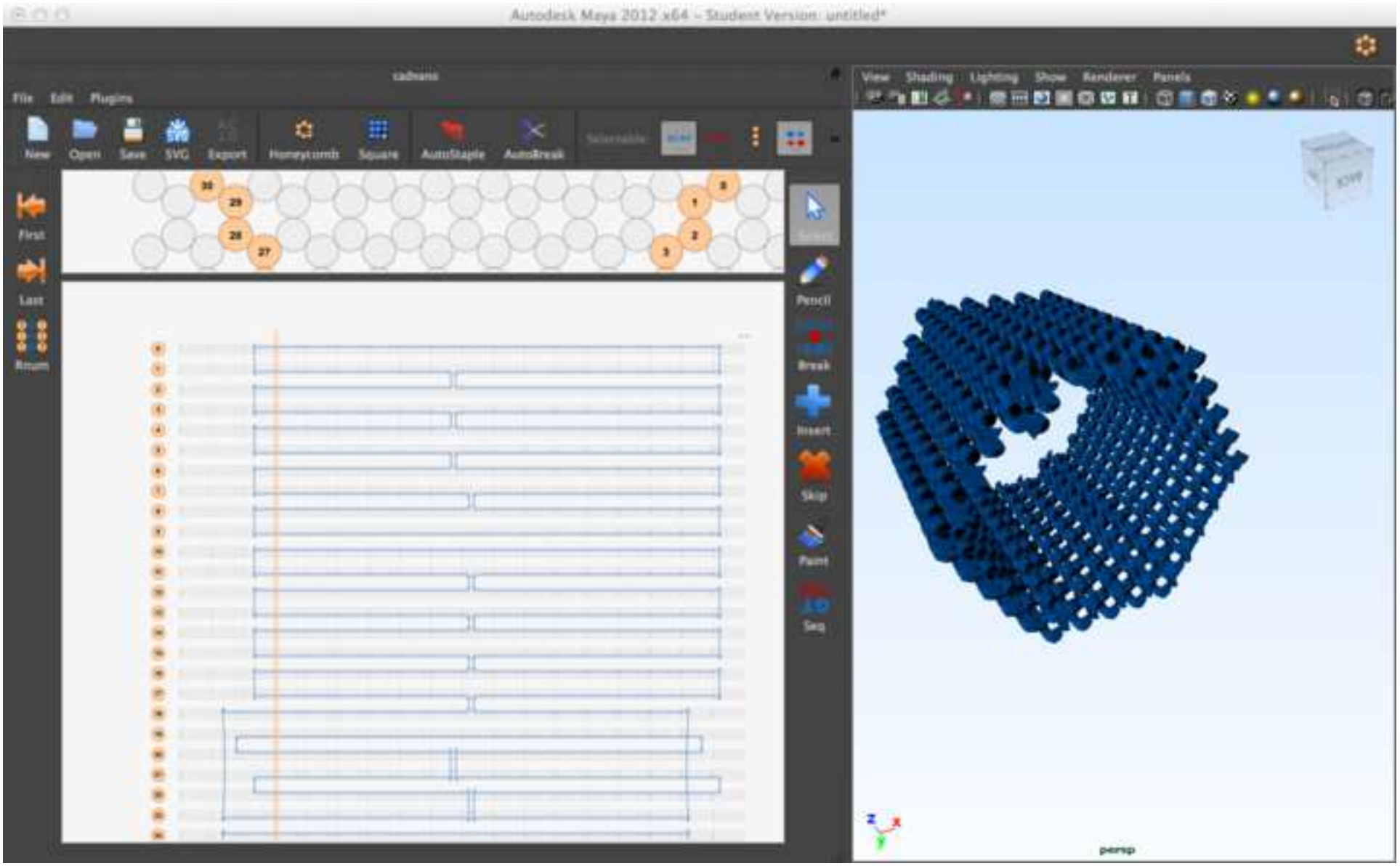


Click here to download high resolution image

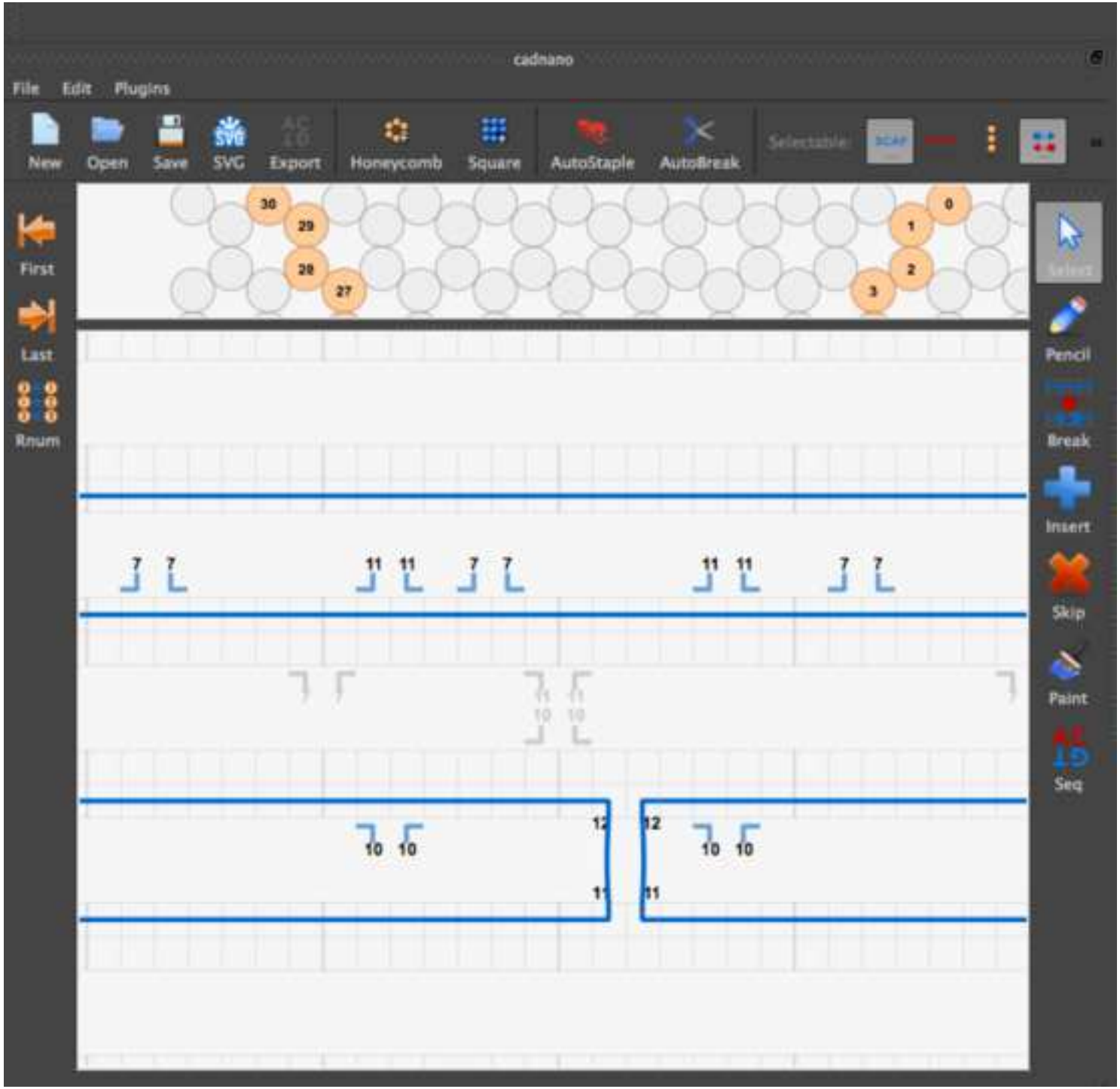


7

Click here to download high resolution image

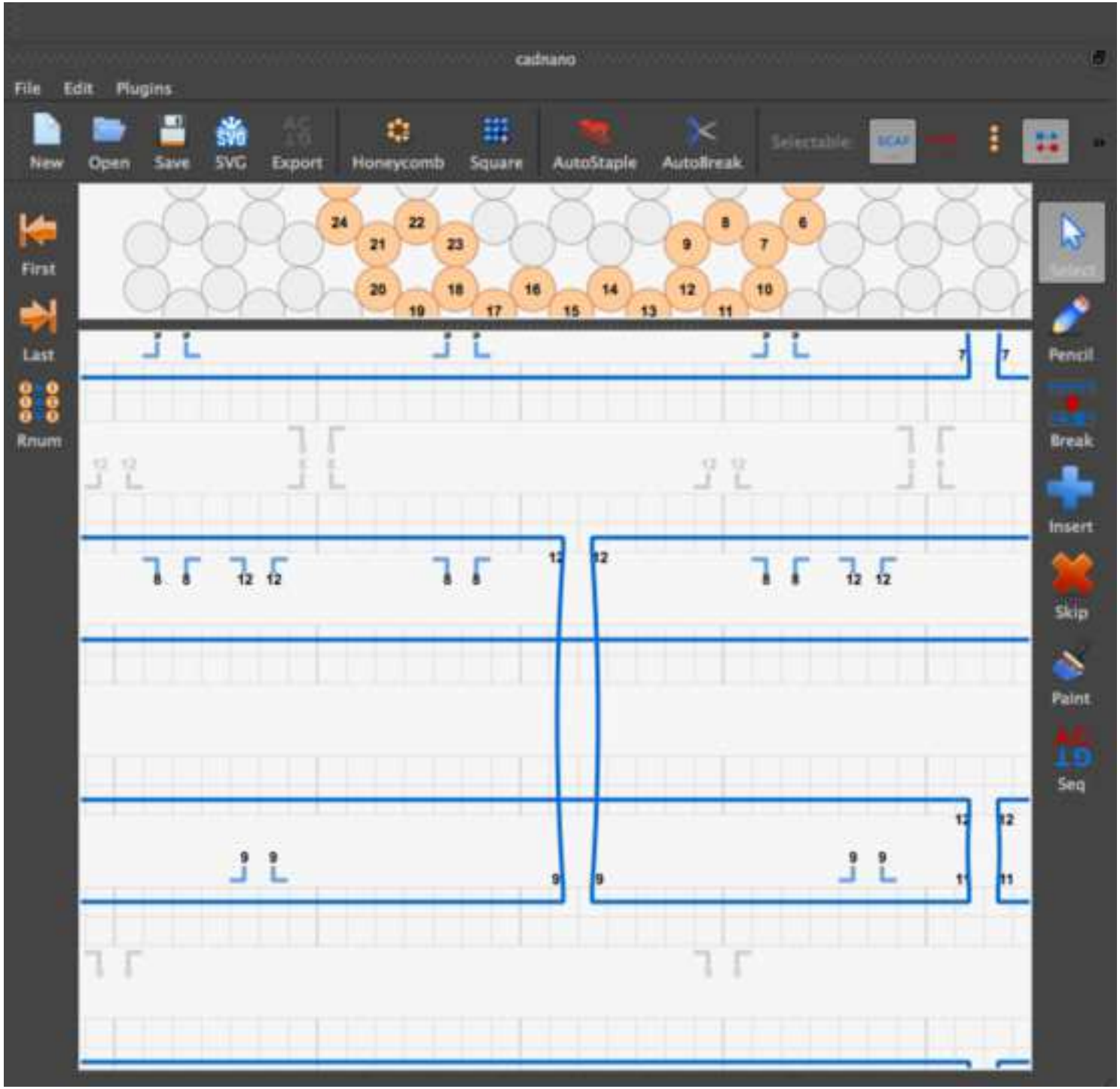


Click here to download high resolution image

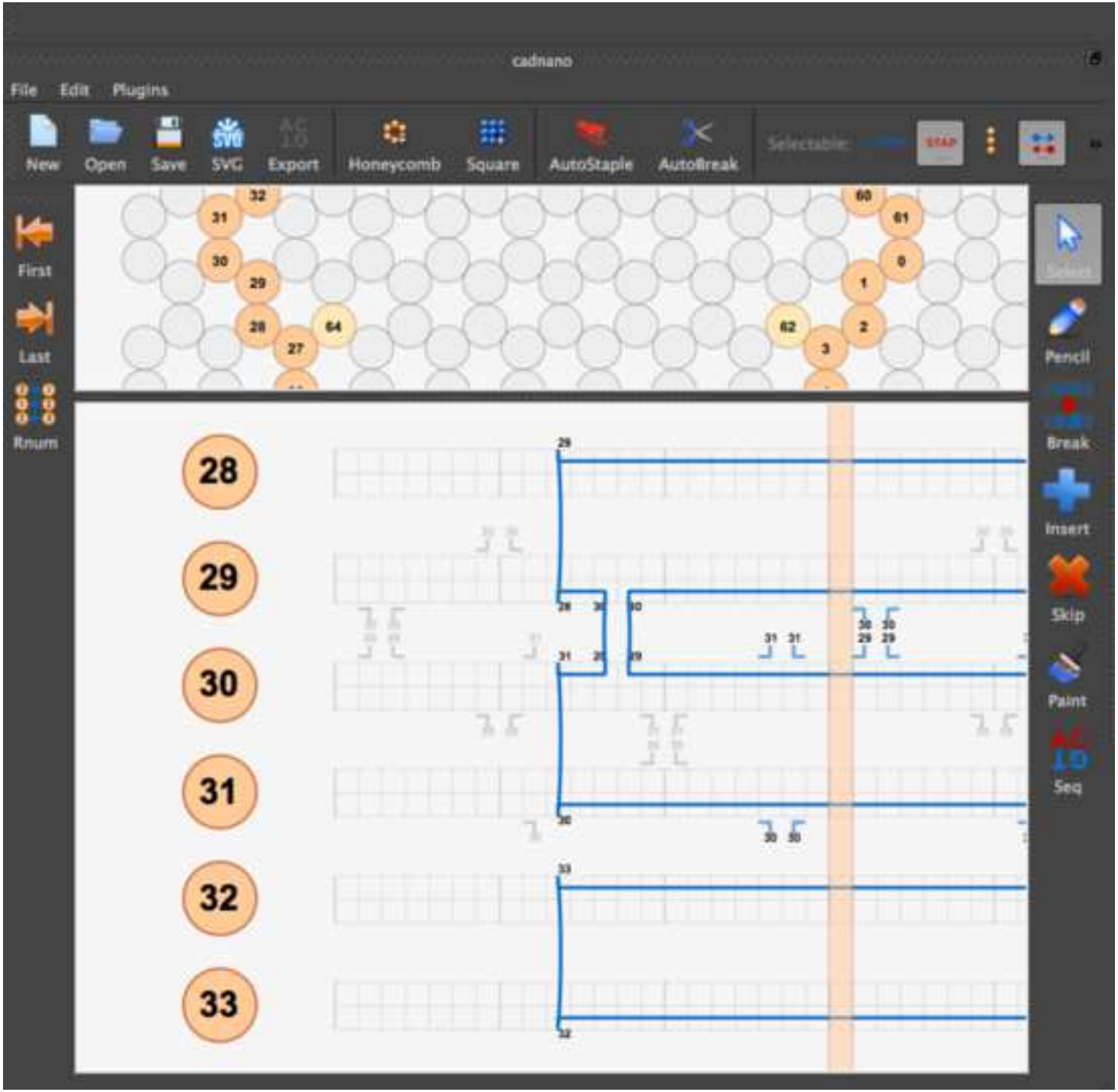


9

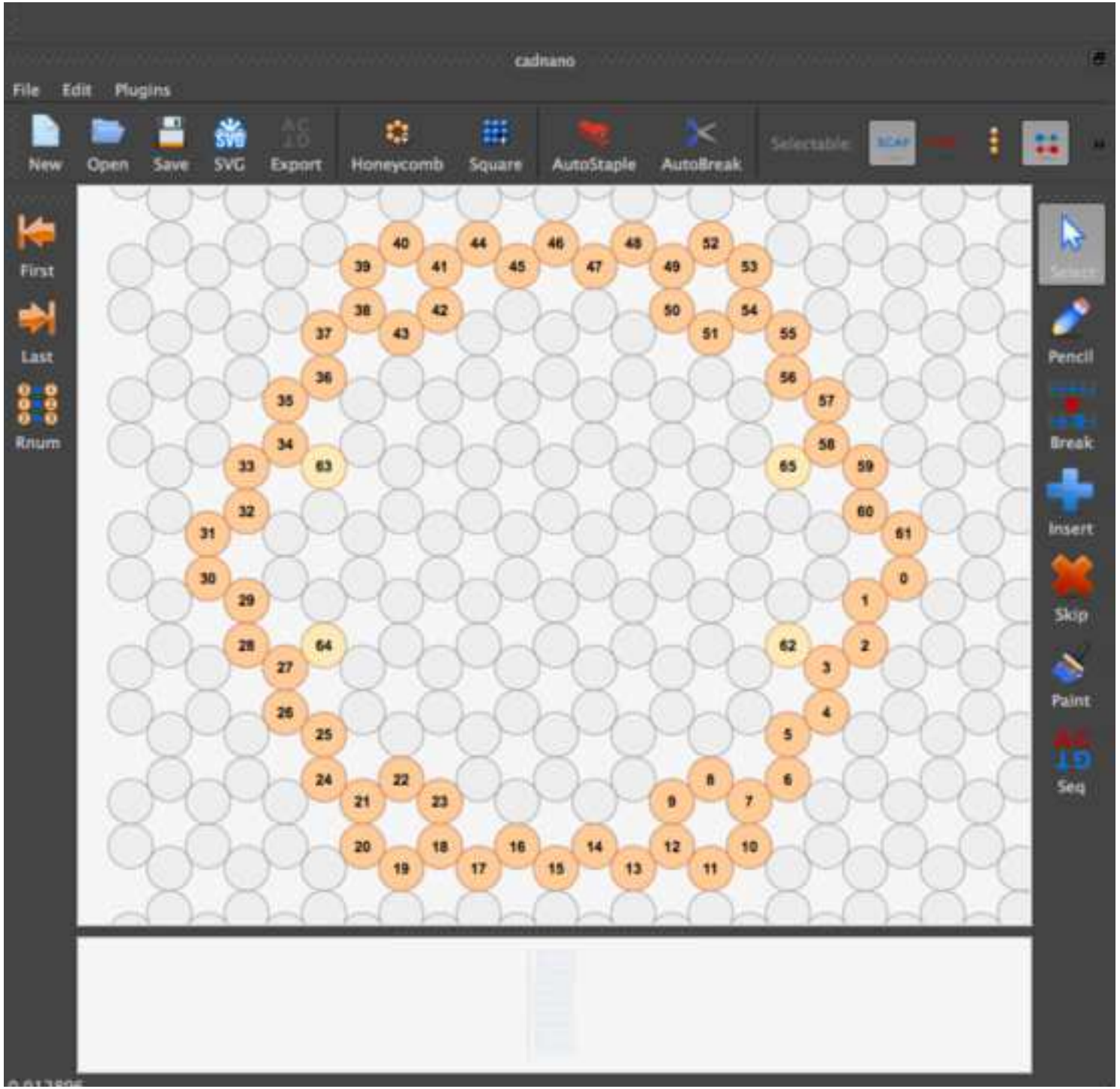
Click here to download high resolution image



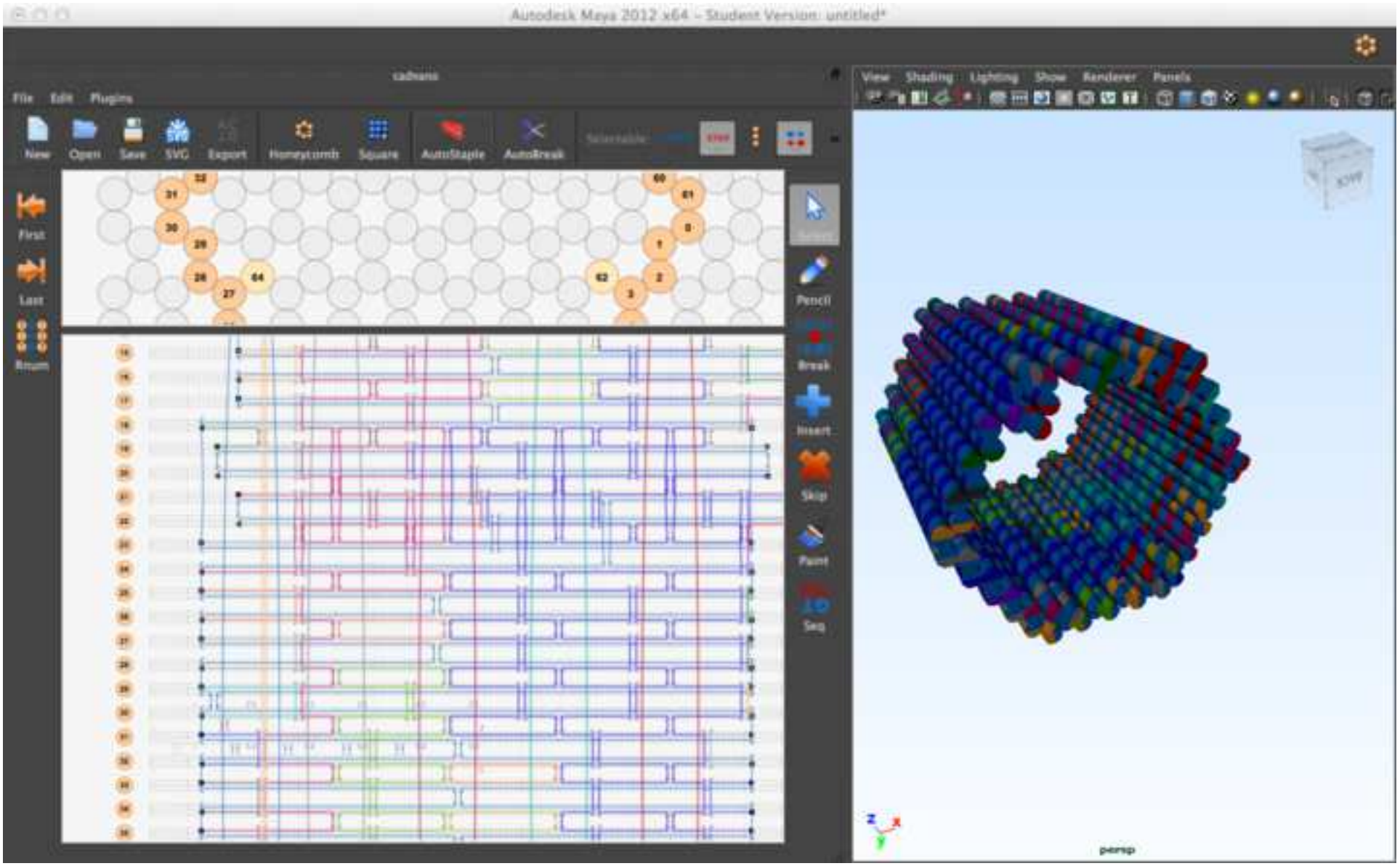
Click here to download high resolution image



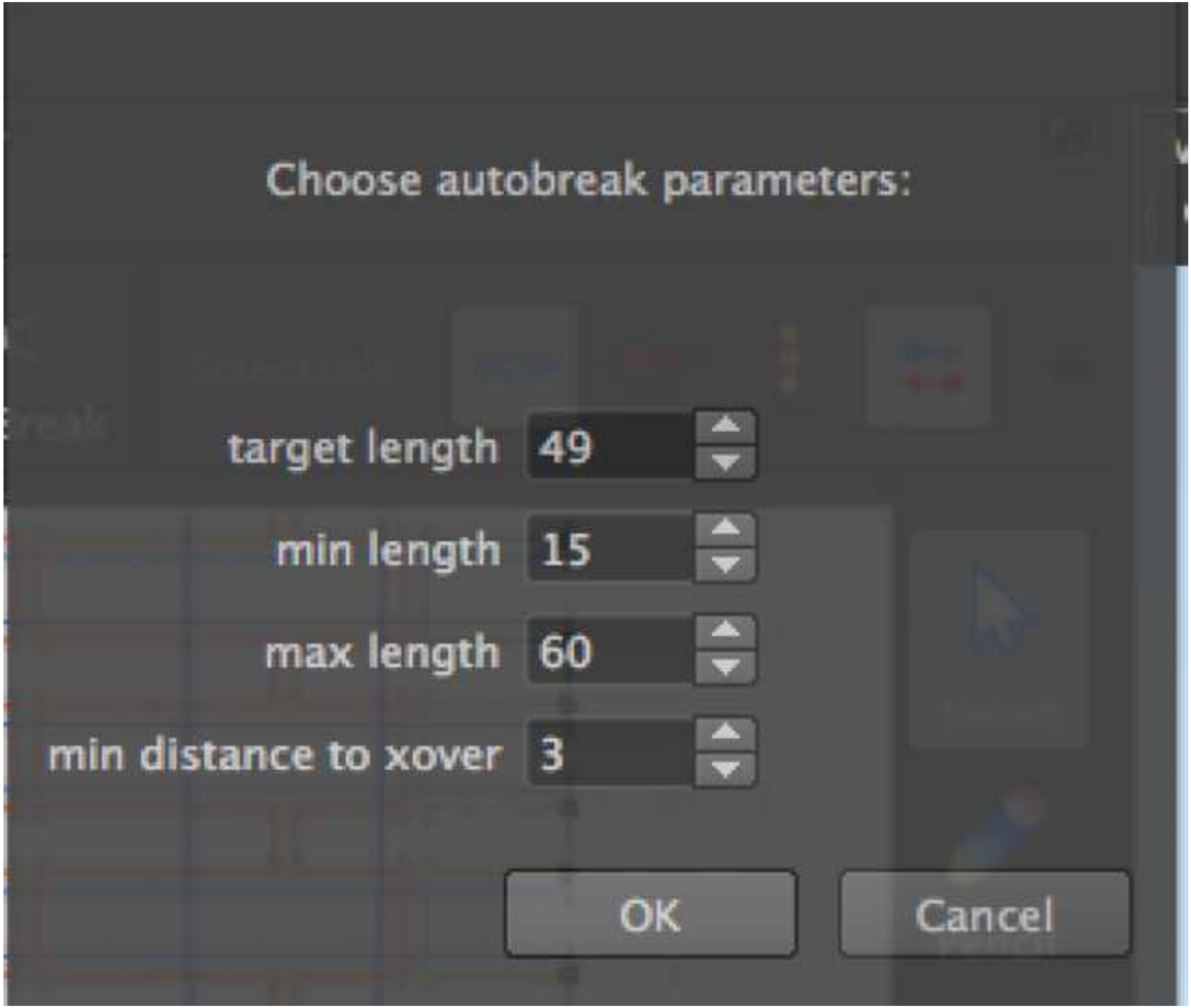
Click here to download high resolution image



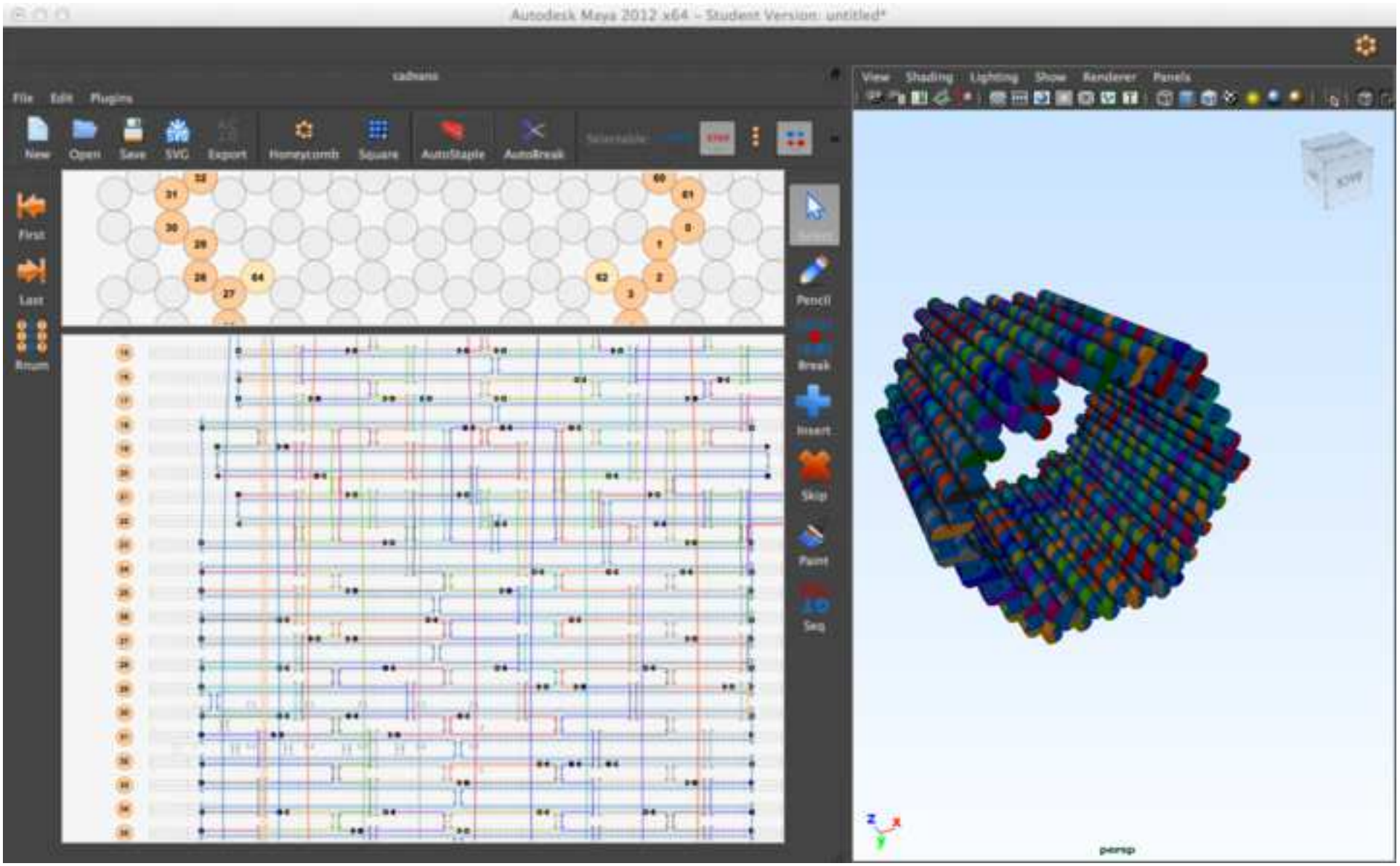
Click here to download high resolution image



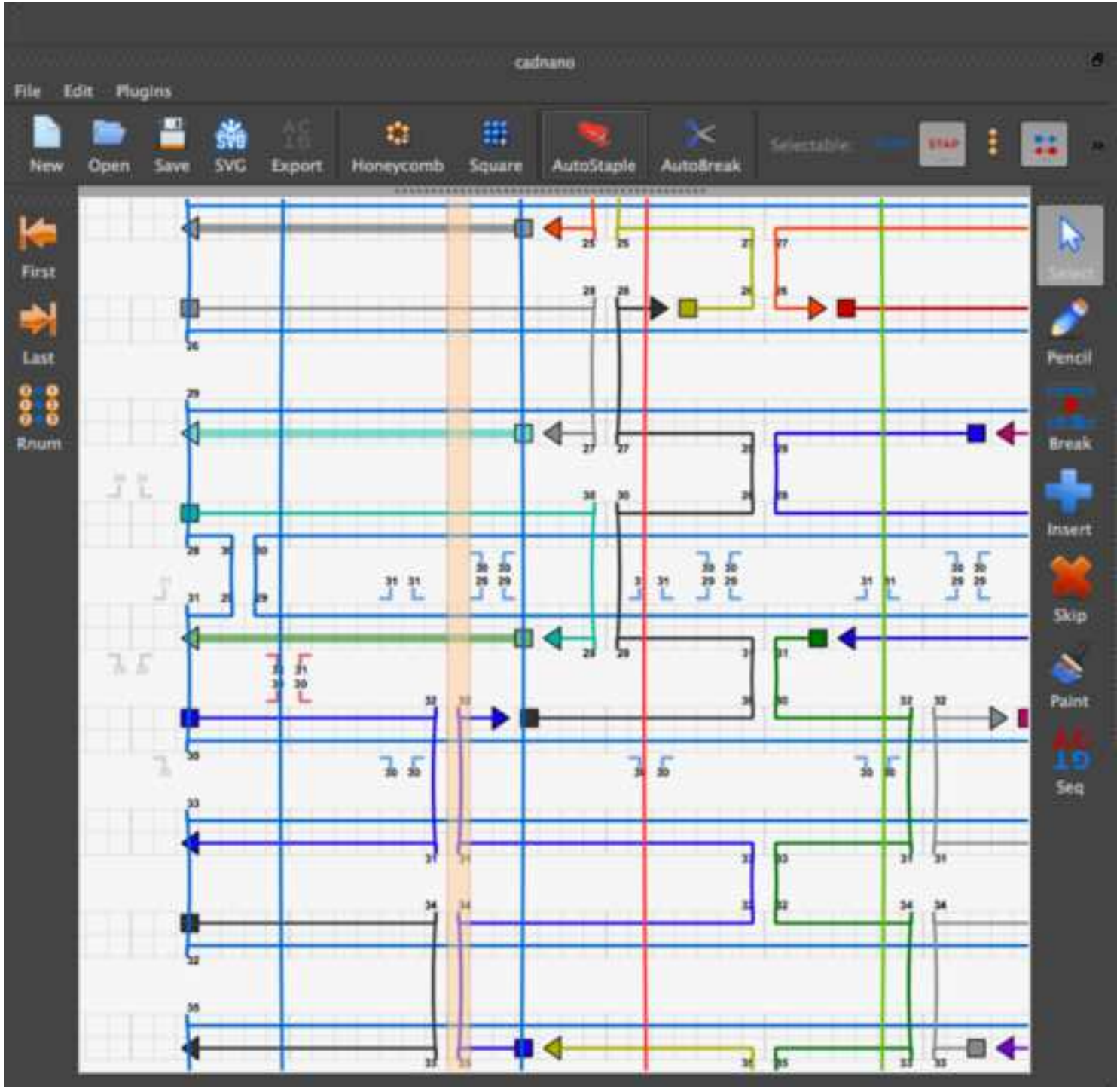
Click here to download high resolution image



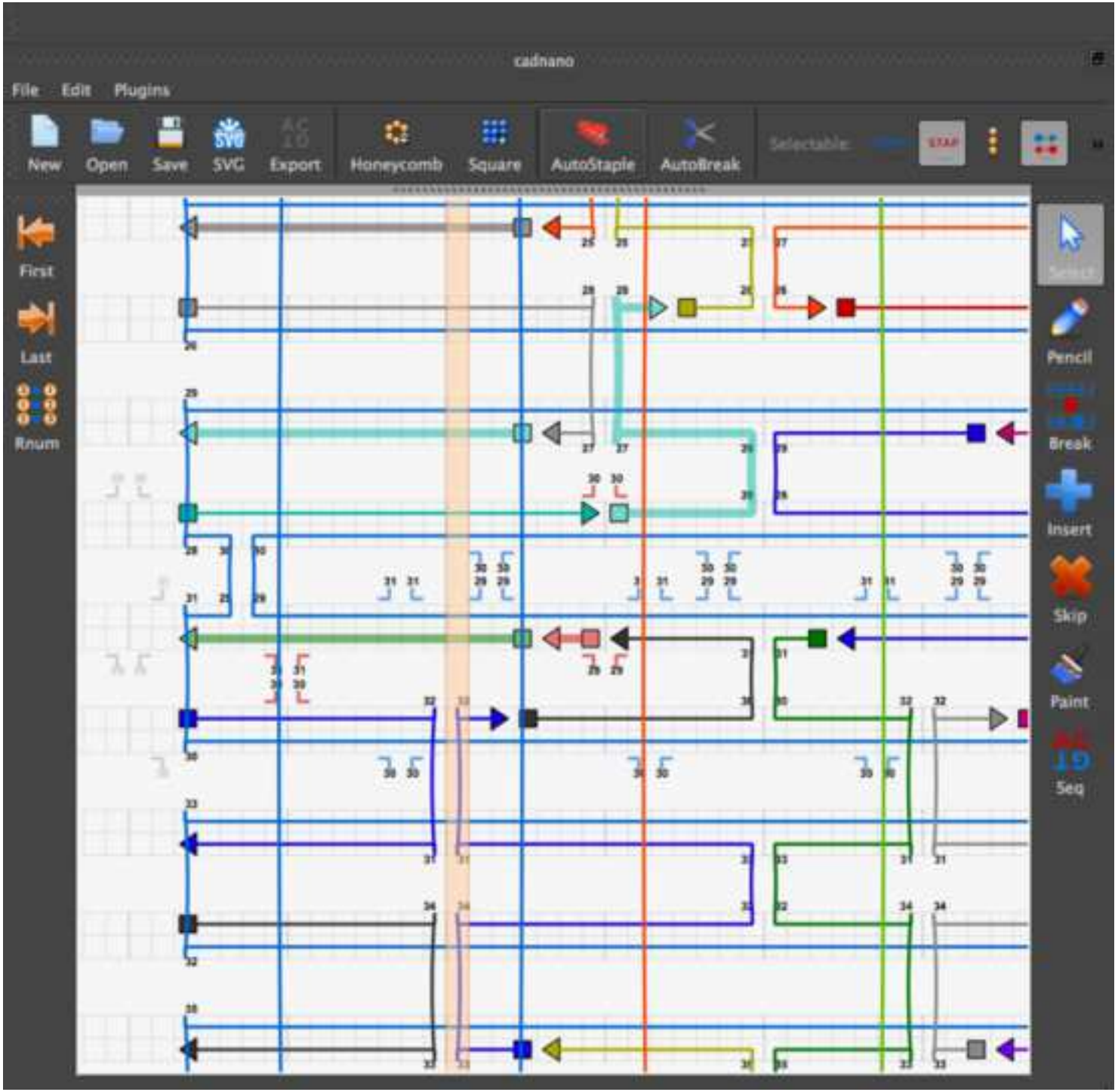
Click here to download high resolution image



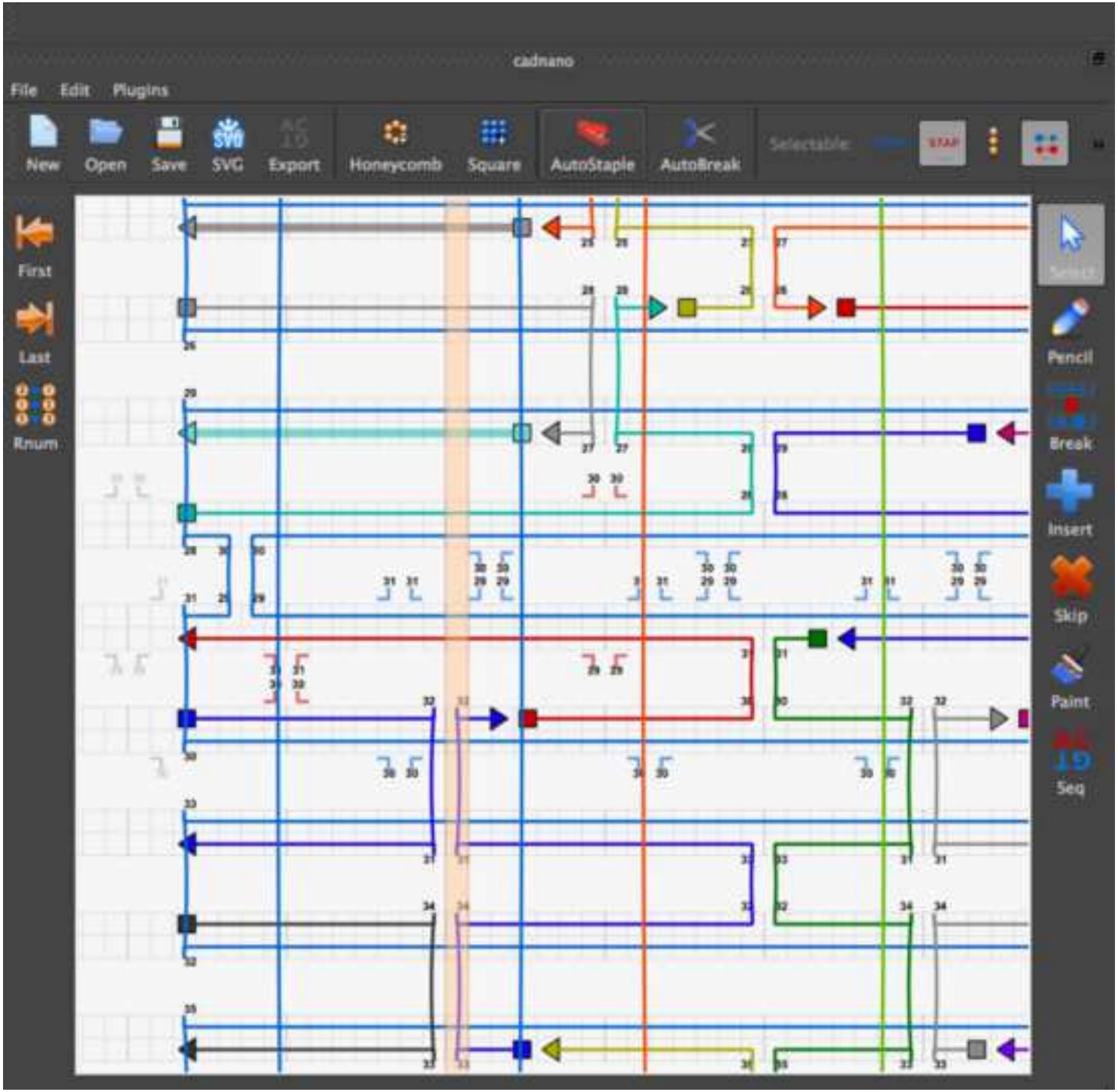
Click here to download high resolution image



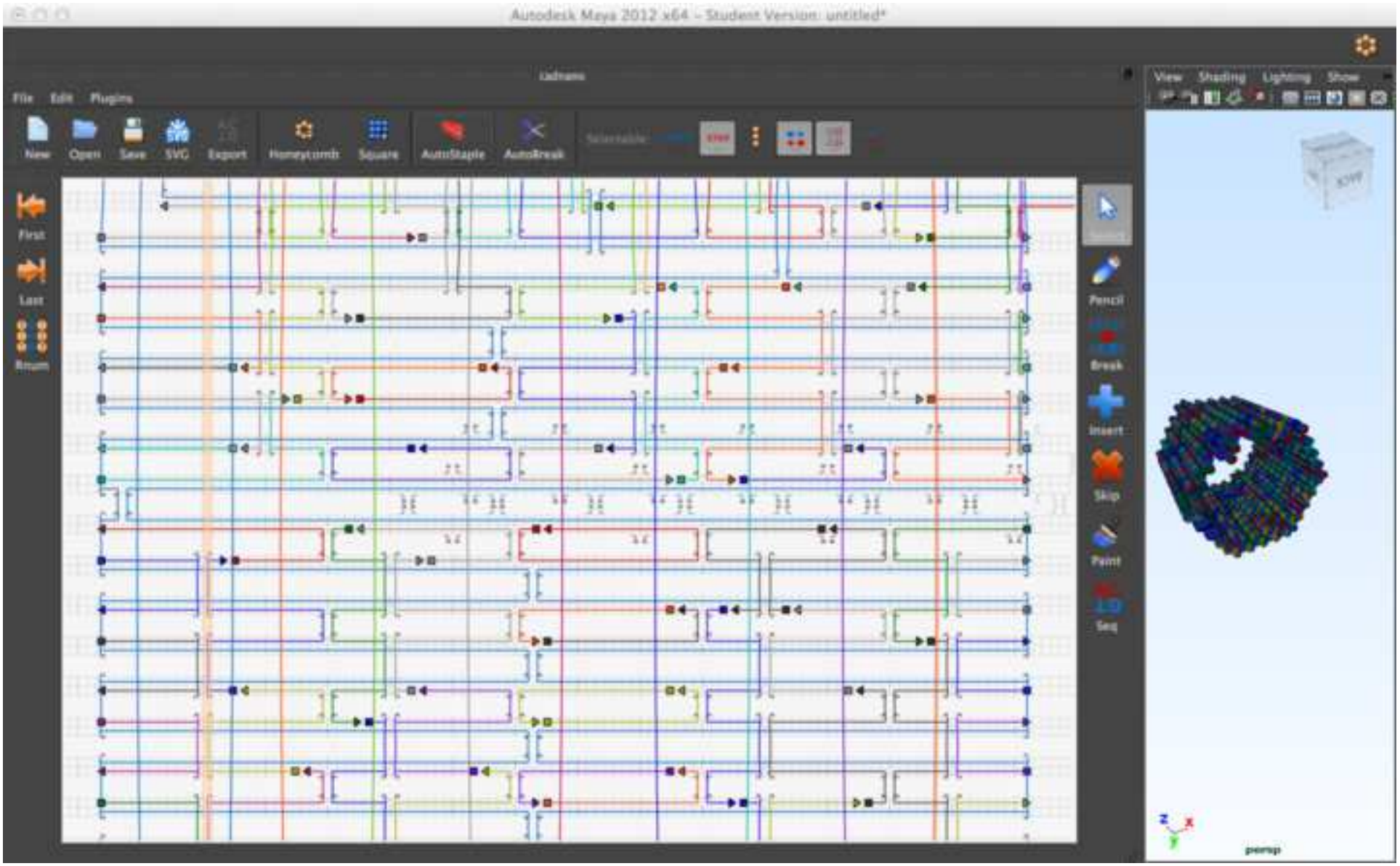
Click here to download high resolution image



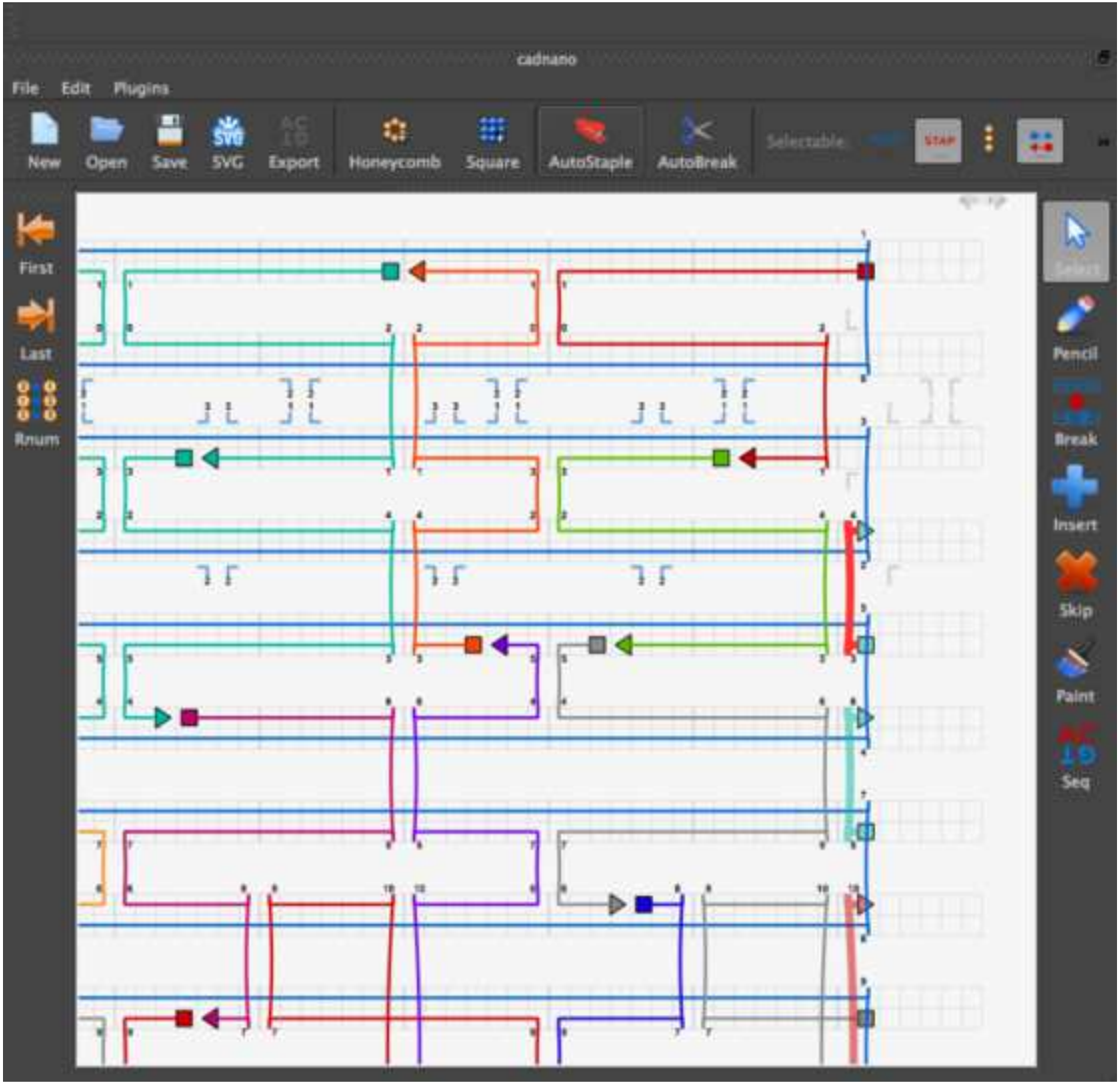
Click here to download high resolution image



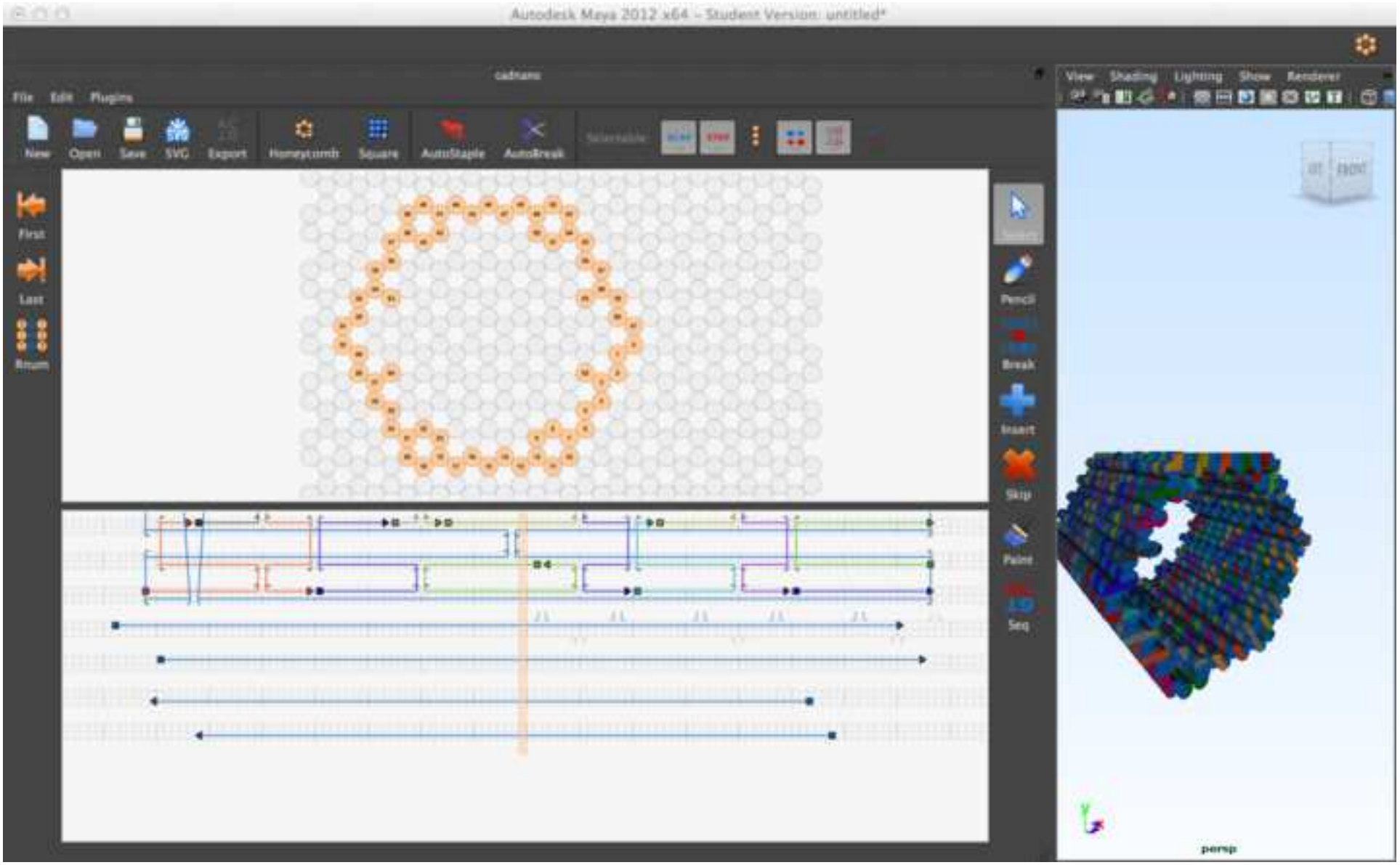
Click here to download high resolution image



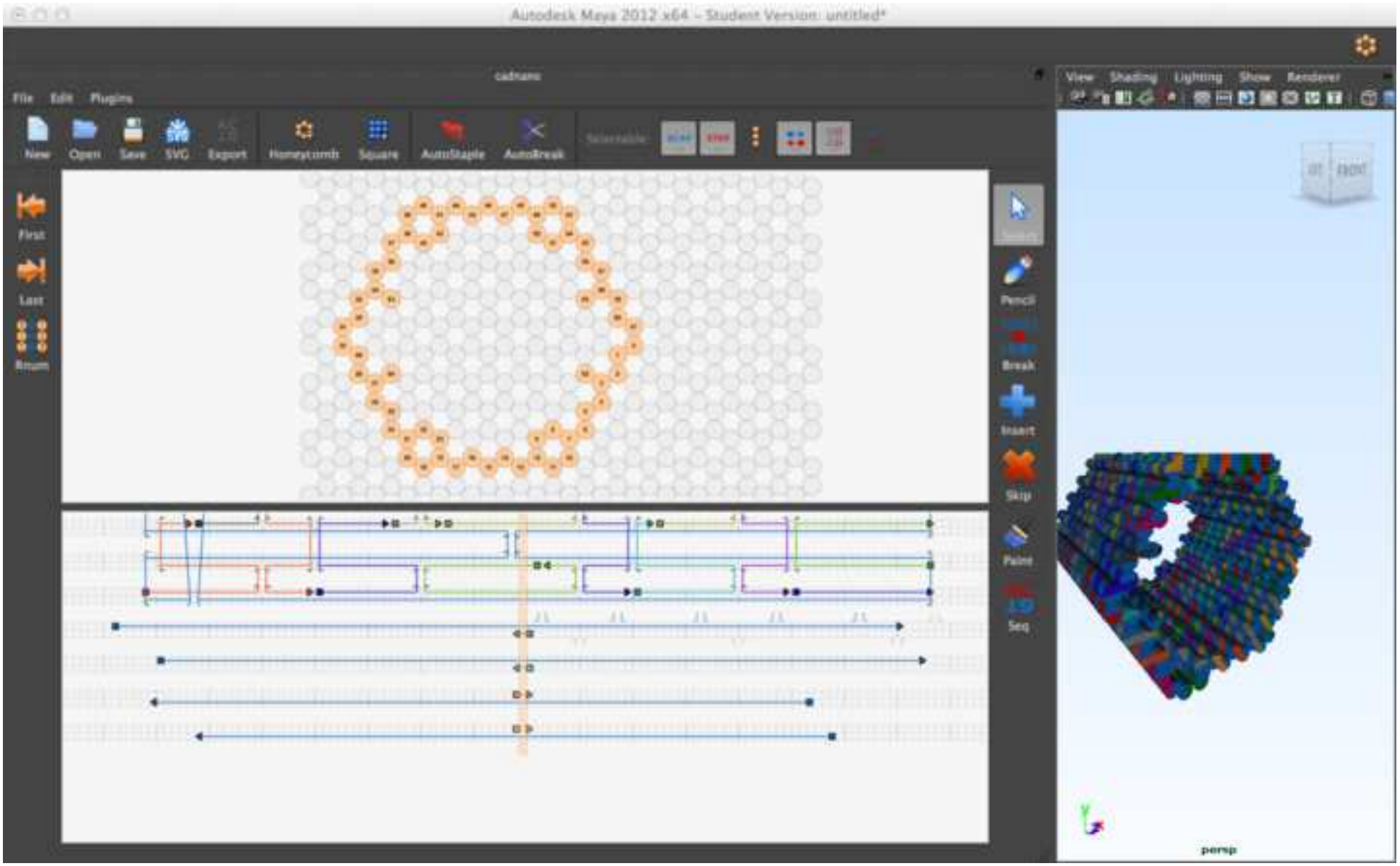
Click here to download high resolution image



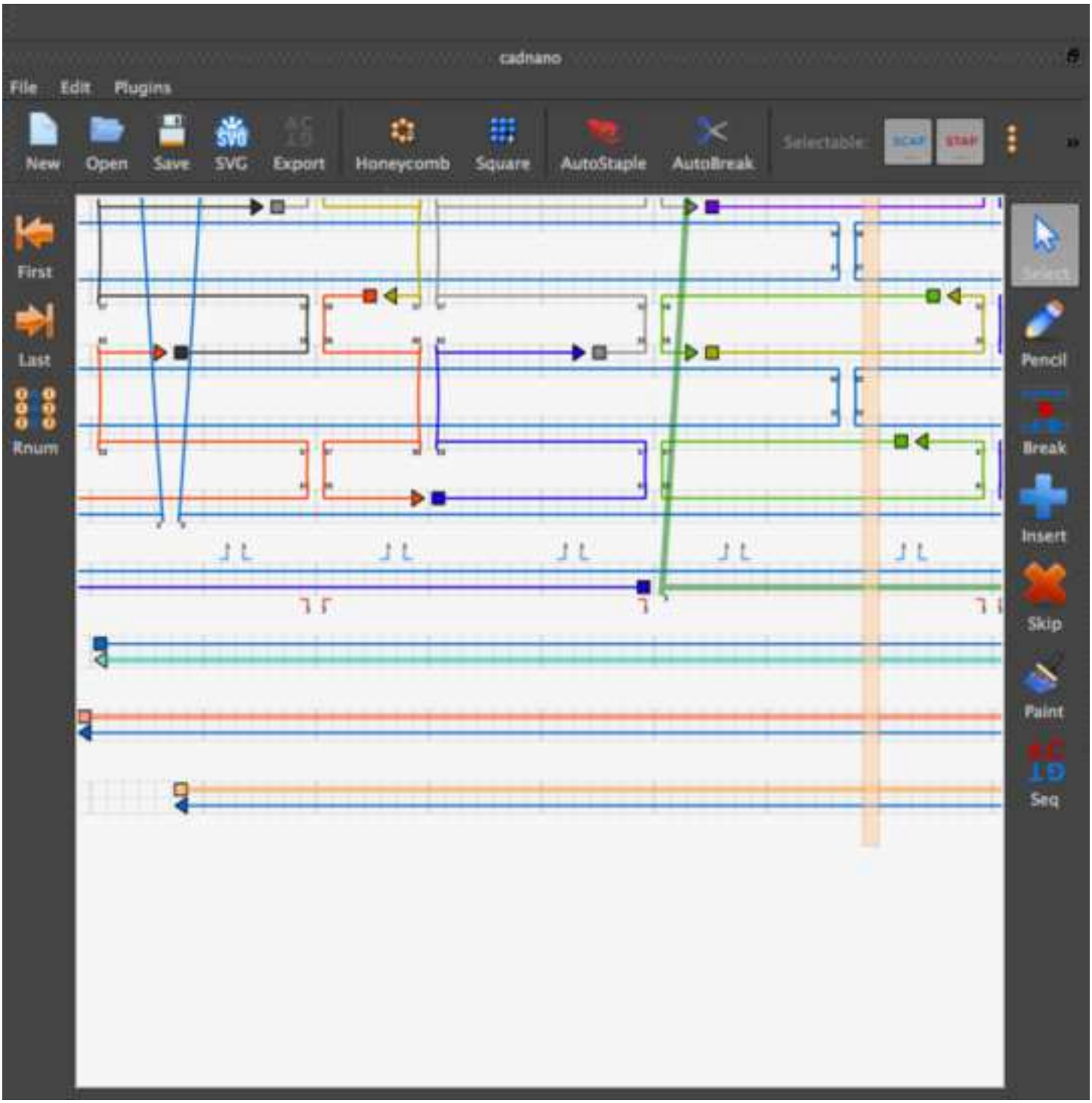
Click here to download high resolution image



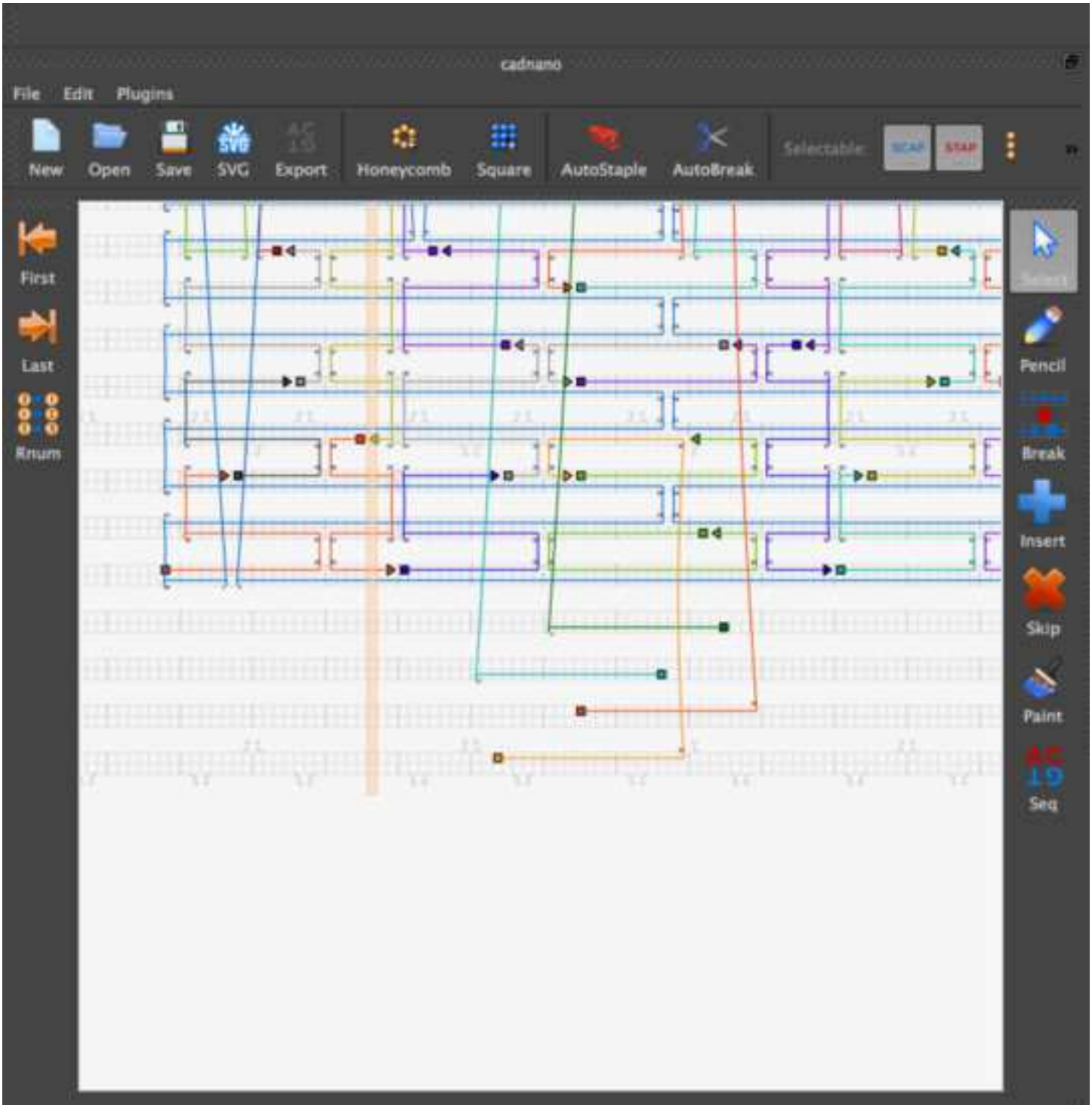
Click here to download high resolution image



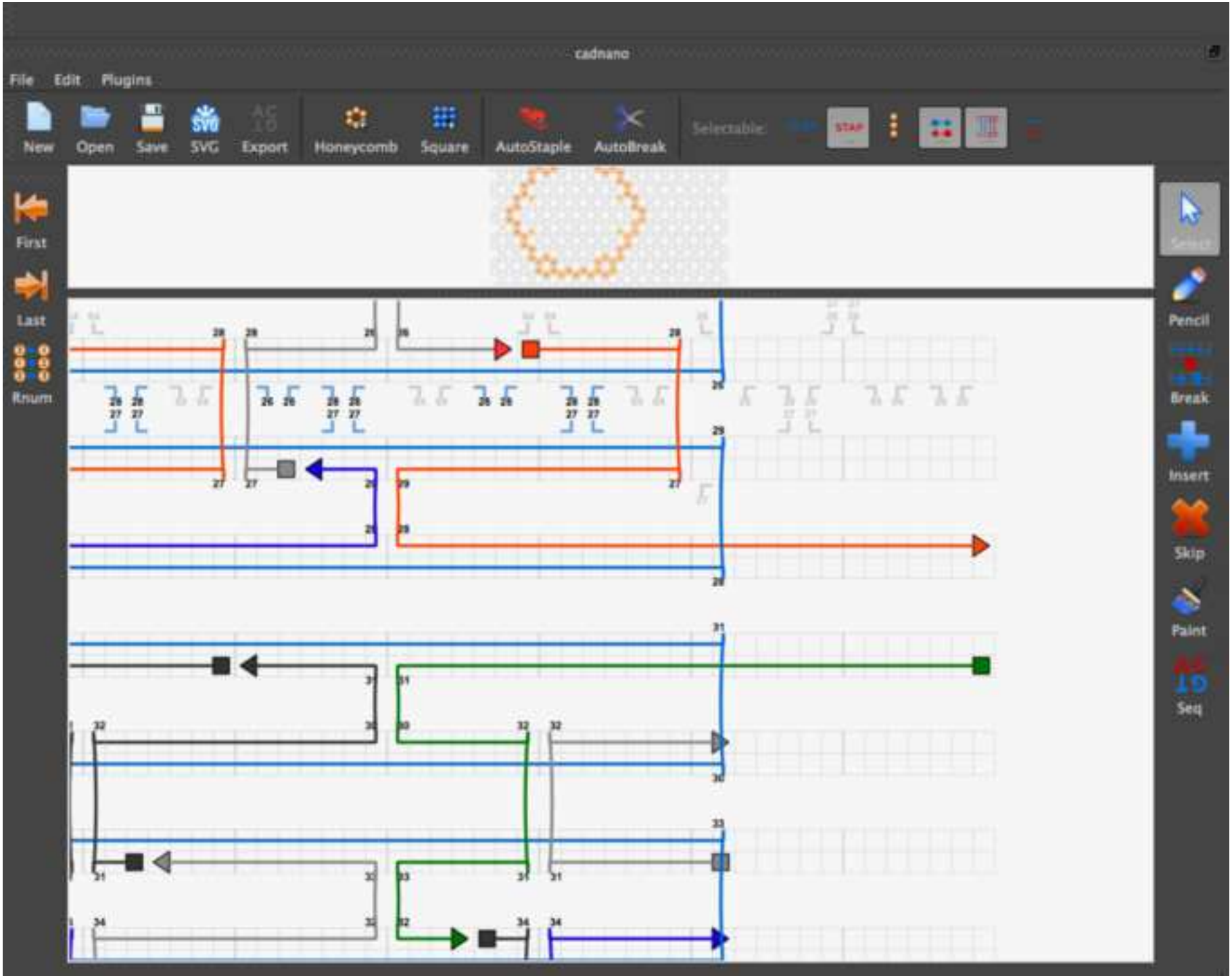
Click here to download high resolution image



Click here to download high resolution image

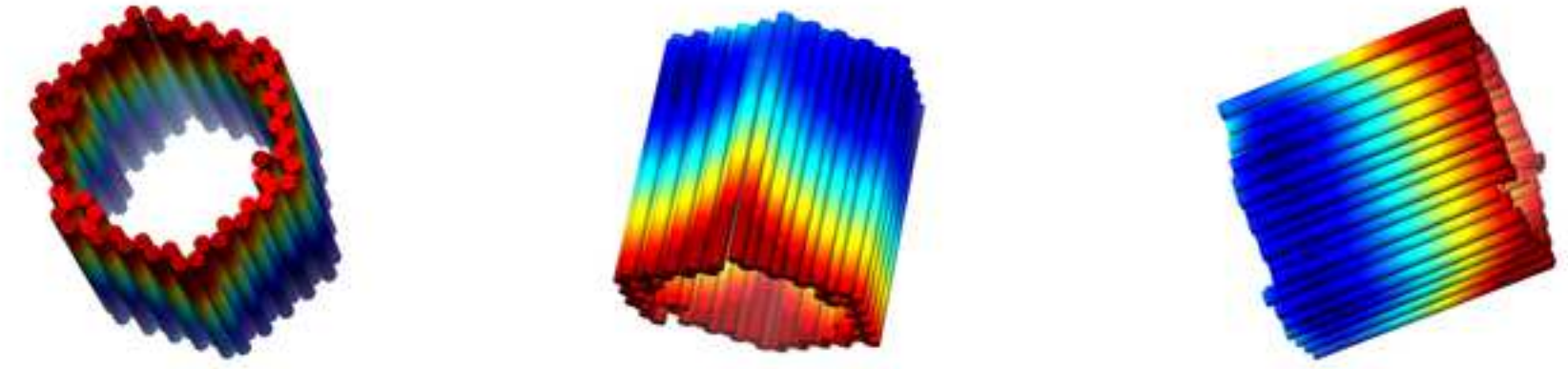


Click here to download high resolution image



Click here to download high resolution image

Click here to download high resolution image



27
Click here to download high resolution image

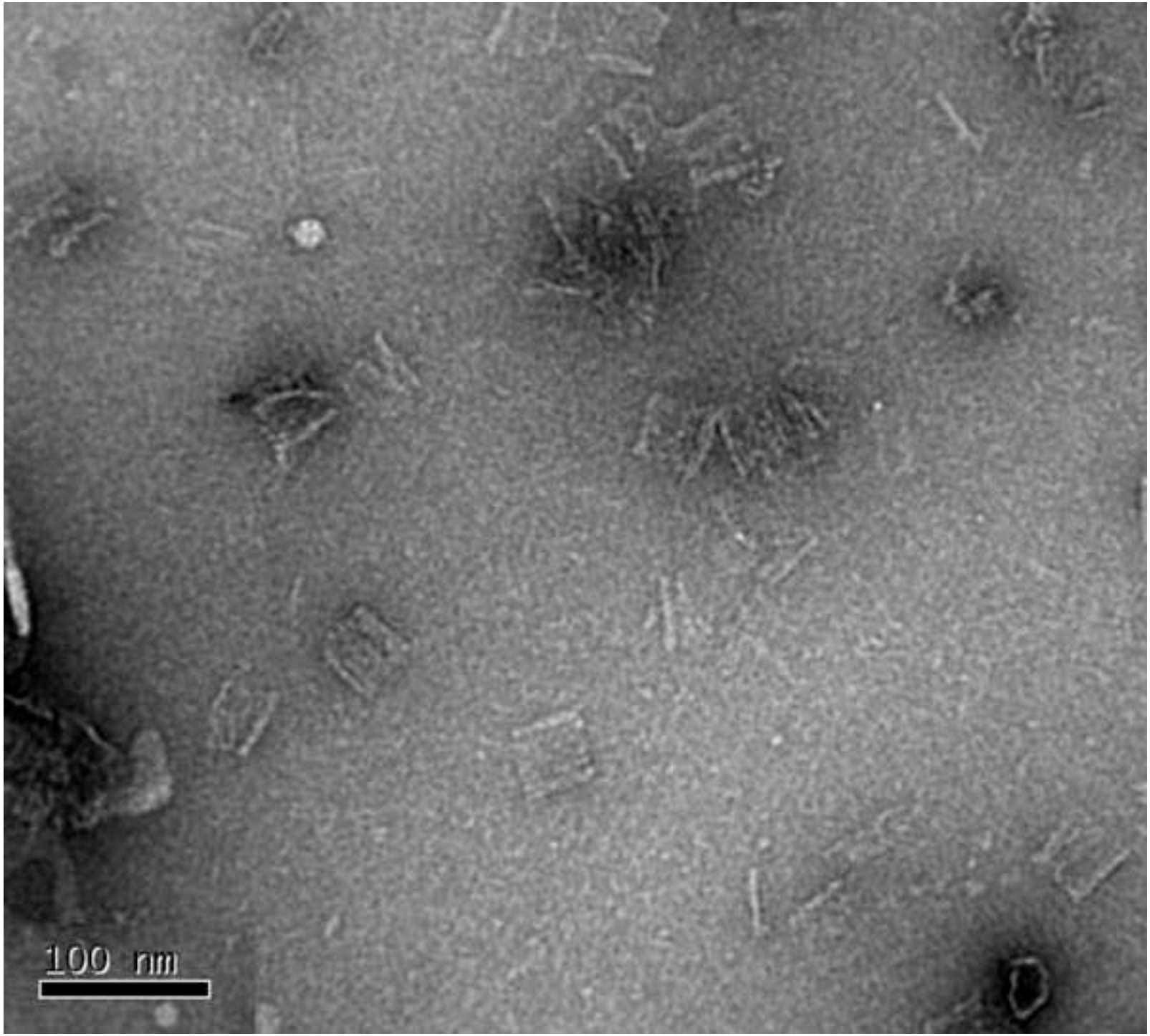


*Table of Reagents/ Materials Used

Click here to download Table of Reagents/ Materials Used: 20121030 JoVE_Materials.xlsx

Name of Reagent/Material	Company	Catalog Number	Comments
Autodesk Maya 2012	Autodesk	http://cadnano.org	A student/academic account needs to be created first (see platform-specific instructions in http://cadnano.org)
caDNAno 2.0 (software)	(Open source)	http://cadnano.org	Software for the design of DNA origami structures
Cando (webpage)	(Open source)	URL: cando-dna-origami.org	Webpage running a simulator of DNA origami shapes

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Designing a bio-responsive robot from DNA origami

Author(s): Eldad Ben-Ishay, Almogit Abu-Horowitz, Ido Bachelet

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name:	Ido Bachelet
Department:	Faculty of Life Sciences & Institute of Nanotechnology and Advanced Materials
Institution:	Bar Ilan University, Israel
Article Title:	Designing a bio-responsive robot from DNA origami
Signature:	
Date:	July 26, 2012

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

October 31st, 2012

To
The Editorial Board
Journal of Visualized Experiments (JoVE)

Re: Author responses to reviewers comments
Manuscript titled "Designing a bio-responsive robot from DNA origami"

Dear Editorial Board Members,

We would first like to apologize for the delay in submitting our revised version of this paper to the Journal of Visualized Experiments. We hope that no inconvenience was caused to the editorial process because of this.

We have carefully read the comments made by our 4 reviewers, and we must admit that the ultimate selection of reviewers by the Journal was superb. Clearly made by experts wishing to assist us in this task, these comments resulted in significant improvements to the manuscript, and we are deeply grateful to both the reviewers and the journal for giving us this opportunity to learn and improve.

Below is a point-by-point response to the reviewers' comments. We hope you will find the manuscript suitable for publication, and we would welcome as usual any further comment or request for clarification made by the Journal or any of the reviewers.

Best Regards,

Ido Bachelet, Ph.D.

Corresponding Author

Author responses to reviewers' comments

Below is a list of responses to the reviewers' comments on our manuscript titled "Designing a bio-responsive robot from DNA origami".

In their papers, Douglas and Dietz already put forward the foundations of DNA origami design in great detail. Our purpose in the current paper was twofold: a) to provide a highly simplified guide enabling the inexperienced user to rapidly obtain only the technical knowledge, omitting for the time being the thorough theoretical basis, needed to design a DNA origami object; b) to put the emphasis on designing a functional DNA origami device, that responds to biological inputs, rather than a "regular" shape.

As a major conclusion from the reviewers' comments, we have drastically revised the manuscript around two points:

1. Using caDNAno 2.0 from within Maya 2012
2. Demonstrating the design process of a robotic device similar to the one described by Douglas & Bachelet (Science, 2012), as a device already tested and proven functional

Nevertheless we would like to clarify why a toy example designed by caDNAno 1.0 was initially chosen for this paper.

On preferring caDNAno 1.0 over caDNAno 2.0 as the interface of choice for untrained beginners

This point recurred in all the reviewers' comments. This point relates to the use of the older caDNAno 1.0 rather than the newer version, caDNAno 2.0, as the interface of choice for beginners. Indeed, caDNAno 2.0 has several features, which constitute significant advancement over caDNAno 1.0.

Nevertheless, our initial choice of caDNAno 1.0 derived from the following points:

1. The caDNAno 1.0 workflow is more 'structured', less flexible and less intuitive (e.g. it does not include automatic crossover generation during initial scaffold drawing, has separate tools for adding staples and erasing them, etc.). As this paper is intended to give the general reader, particularly readers from outside the field of DNA origami with zero training in DNA design, a first hands-on experience in this mission, we felt that these could actually become advantages, enabling the user to carry out each step separately and receive only the relevant explanation for this step. Indeed, using caDNAno 2.0 one might generate the same DNA origami structure significantly faster, but this is not an advantage for a beginner, but rather for the experienced user.
2. The excellent 3D capabilities of Autodesk Maya can be used to generate a 3D

model from a .json file made in both caDNAno 1.0 and 2.0. While caDNAno 2.0 can be operated from within Maya, the caDNAno 1.0 3D viewer is primitive; however, operating caDNAno 2.0 from Maya requires the user to learn at least to some extent the relatively complicated interface of this software, and users without experience in 3D modeling might find this somewhat intimidating.

3. Very clear and didactic video tutorials are available for caDNAno 1.0 but not for caDNAno 2.0.
4. Finally, we collected feedback from untrained users, including high-school students and DIY-bio hobbyists, and these convinced us that caDNAno 1.0 would be the better choice as a beginners' interface for designing DNA origami shapes.

However, after careful consideration of the reviewers' comments we became convinced that re-writing the article to focus on caDNAno 2.0 rather than 1.0 would serve our purpose better.

Limitations of the methods should be discussed and the authors should provide a better explanation in each figure legend.

We added a paragraph describing the technical difficulties of using DNA origami devices as a therapeutic platform. The legends to figures […] have been rewritten in further detail.

When discussing the actual folding of the shape, the authors should cite the review article by Bathe's and Deitz's group: "A primer to scaffolded DNA origami" which appeared in Nature Methods last year. This has details on the folding conditions and goes into greater depth on the physical aspects of DNA origami. They should also explicitly cite the caDNAno tutorials on the caDNAno website.

We thank the reviewers for noting this unfortunate omission. We have added this important paper and properly referenced it.

The present article and video will be a useful tutorial complement to these existing articles but goes into less depth on the basic principles of DNA origami in the interest of walking the viewer/reader through a full applied example.

We agree with this remark. However, we would like to clarify that our idea in proposing this protocol to *JoVE* was to deliver to the reader a highly simplified learning experience, based on a "toy example" of a DNA robot. In contrast to the original caDNAno paper by Douglas and colleagues, which demonstrates caDNAno through a short fragment of a 6-helix nanotube, our emphasis here was to walk the reader through the design of a "live" robot with a logic gate. We encourage the

reader throughout the article to complement this applied know-how with reading the more detailed and theoretical papers by Douglas and Dietz, which make the foundations for the caDNAno design interface.

As stated above, in writing this paper we collected responses from untrained users, who were given this manuscript and asked to follow the instructions step by step. Our impression was that the manuscript provided a sufficient technical understanding of caDNAno to enable reconstruction of our toy robot in less than 30 minutes after starting from a zero knowledge point, which was our purpose here.

The screenshots are from the first version of caDNAno, which is now considered a legacy version by some users. They should give links to the v2 and v1 software in the text and explain the differences (which are minor for our purposes here).

The manuscript was changed accordingly, see p. 1 of this letter.

In discussion the creation of the basic helix geometry (left panel), the authors could note that for a circular scaffold path, the natural arrangement of helices divides the structure into groups of two helices. Then "internal" crossovers are made within the groups of two and "external" crossovers are made between them. This allows the approximation of many shapes using two-helix building blocks. The authors could explain that this constraint is relaxed for a linear as opposed to circular scaffold.

We thank the reviewers for this comment. We have added this explanation in the appropriate place.

Some of the writing could be cleaned up a bit. For instance in the short abstract the phrase "subsequently relayed to a desired effect" could be removed. Likewise the sentence "We are very much hopeful that people will find warm uses that we can think about" in the discussion seems unnecessary. In general making the writing somewhat shorter and tighter could improve the flow but is not necessary.

We have revised the manuscript to exclude these and similar statements.

The paper demonstrates the creation of a 'toy' example which roughly approximates the shape from the referenced Science paper. This alternative requires additional steps to generate an appropriately short scaffold strand, and even recommends leaving in 4000+ bases of unwanted scaffold. Did the authors actually make this smaller device? Have functional assays been used to demonstrate it works? Why not just show how to construct the previously published device?

The manuscript has been changed accordingly, see p. 1 of this letter.

Finally, to answer the reviewer's important question: we have indeed tested two schemes of folding small origami shapes:

1. Computationally fragmented scaffold. In this method, the shape is designed as usual, a scaffold is chosen, and an .svg file is exported to Adobe Illustrator to view the part of the scaffold sequence that is actually used. This sequence is then split to fragments in Illustrator, making sure splitting is carried out to ensure optimal overlapping between staples and scaffold fragments.
2. A digested ssDNA as a custom scaffold. Here we simply choose a restriction fragment of any available ssDNA, which corresponds with our desired scaffold length, and paste the digested sequence as a custom sequence in caDNAno.

Both of these were used to fold a small, roughly cubic DNA origami object of ~0.6 Kb total length, and were successful in some of the attempts based on gel electrophoresis results.

It seems the authors are suggesting some preparation steps that might not work very well. Specifically, there are published protocols for generating scaffold, folding, and then purifying a full-size, M13-based origami. But is it actually so easy to purify a 1kb-origmi from a 4kb unused phiX174 scaffold fragment, as the authors suggest? They claim that the fragment is 'irrelevant', but unless they have tested this, it is not obviously true.

We fully agree with this point, and the manuscript has been revised accordingly.

Gel electrophoresis, and microscopy (TEM or AFM) are widely used methods for characterization of DNA origami. While the authors present several nice screenshots of caDNAno and CanDo, they do not offer any example experimental assays (with proper controls) to help the reader check whether or not they are successfully following the presented protocols. If the authors could include a gel and some AFM or TEM images of the described shape, it would potentially address my concerns described in #1 and #2.

Interpreting gel or AFM/TEM images of DNA origami shapes has been described. We felt it would be better to strip the current article from discussing interpretations, for the sake of making it focused entirely on the design aspect, while referring the reader to several of numerous examples of how DNA origami objects should appear on a gel or micrograph.

Still, we fully agree with this point in general, and would like to propose a follow-up paper focusing on both examining the folding by gel and AFM/TEM, and some bioassays testing the function of the device on living cells.

If it were believed that the current article would not be of value without this discussion and data, we would then propose to rewrite it using a device we already built and tested, for which such data exists.

In Step 1.1: Suggests to download the software from cadnano.org. When visiting the site, it's possible to easily download version 2 of the software, but what is shown here appears to actually have been downloaded from here: http://cadnano.org/legacy

The manuscript has been revised accordingly, see p. 1 of this letter.

One of my main concerns is that the article focuses on the use of an older (now obsolete) version of caDNAno which is no longer available for download at the website and has been superseded by a more user-friendly (i.e. now featuring an undo function) version with increased usability and display possibilities (i.e. the integration into Autodesk Maya). Although not entirely different from the version described in this manuscript, the workflow as well as the steps to create a DNA origami structures are somewhat different. I think - although this might mean an extensive change - that the authors should rewrite the affected parts of the manuscript (protocol and figures) with focus on the current version of caDNAno.

The manuscript has been revised accordingly, see p. 1 of this letter.

Figure 1a and 1b should be switched. 1a should show the closed state and 1b the open. At the moment this is mixed up.

The manuscript has been revised drastically and the entire figure layout has changed and re-checked for consistency.

Page 4, section 1.4: "Note that helices no. 25 and 0 connect ?" At this point in the design process, there is no helix no. 25 yet, neither in the helix side-view (panel 1) nor in the diagram view (panel 2).

We thank the reviewer for pointing this out. This sentence was included by mistake and was properly removed.

Page 5, section 3. I think that the mechanism of creating locks for the desired device is not explained well enough. Readers might be confused by the fact that the staple strands designed to lock the device are not connected to anything during the design process. I think it would be important to mention, that this actually binds to the second part of the shell that makes up the complete device (as depicted in the closed state in Figure 1).

We have included a specific explanation for this potentially confusing part. We also included a reference to Fig. 1 which clarifies the role and context of the gate as a part of the complete device.

Page 6, section 4.2: It should read "repeat step 4.1" not 2.2.

This error was properly corrected.

Point 5.1 needs clarification. Can one use a circular scaffold? If yes, is the start and end point created in this step important?

A break needs to be inserted into the scaffold strand so a sequence can be assigned to it. In the actual reaction, the scaffold strand (e.g. M13mp18 ssDNA) is in fact circular, and usually does not need to be linearized although this depends on the specific shape folded (see Rothemund, 2006). We agree with this point, and clarified the manuscript accordingly.

Page 7, section 6.1: Can this assignment be done automatically by leaving the helper scaffold strands on the respective helices in place and assigning a different sequence to these?

Yes it could; however, because both the gates and the loading strands are important for function, they are not usually automatically assigned. The gates should be specific DNA sequences that respond to the desired cue (e.g. aptamers that bind a target protein, or a DNA restriction site that is cleaved in the presence of the proper nuclease, etc.). Loading sites can be automatically assigned, but these sequences need to hybridize with the strands covalently linked with the cargo molecules; since usually the same strand is used for different devices, the loading sites will have the same sequence and therefore should not be automatically assigned.

Page 8, section 6.2.1: I would change "biological cue" to "biological input". This should also be changed in the abstract (page 2). Also "profile of cues" should be changed to "sequence of inputs" maybe?

'Cue' has been changed to 'input' throughout; 'profile of cues' was changed to 'signature of inputs' – we hope that this satisfies this requirement and would gladly consider alternatives.

Page 9, section 8: The part "? that the relevant scaffold fragment makes up less than half of the total material ?" might be obvious for researchers in the field but less intuitive for "newcomers". This part would benefit from a one or two-sentence explanation what this exactly means.

We thank the reviewer for raising this point, and we have included an appropriate

explanation of this heuristic.

Page 11, description of figure 22: The third sentence should be rephrased for better clarity.

The sentence was rephrased as requested.

Page 11, description of figure 23: RMS deflections in nm don't make sense here as there is no scale bar with absolute values. I would delete the "nm" part.

We agree but preferred to keep the 'nm' and manually add a color scale bar for RMS deflections.

In the discussion (page 12): What do the authors mean with "? scientific devices from DNA origami"

This has been clarified in the manuscript. Our meaning was that in addition to therapeutic tools, one could design DNA origami research device, e.g. a device that places two proteins in a specific spatial configuration upon receiving a biological input (such that the device is inducible), enabling to record the output resulting from this artificial placement of a protein-protein pair.

Again: cues should be changed to inputs

'Cue' has been changed to 'input' throughout

"Alternatively, restriction enzyme was already" seems to be incomplete

This typo was corrected and the sentence is now complete.

The remote control approach is an interesting aspect. Could this also be achieved by a user-supplied synthetic biological input signal? How would that work in a living organism?

In fact the interesting possibility of generating remote control interfaces for DNA origami devices based on synthetic biology is a topic of study in our lab. Some of the ideas include programming the device to respond to a remotely inducible input such as a hormone or a neurotransmitter. A more advanced possibility is to use an inducible fragment of DNA as a 'walk-in' gate, meaning the devices are deployed gateless, and once the gate is synthetized by a user input, it attaches to the device in the proper position and functionalizes it. We actually added a short discussion on user interfaces with origami devices as this point is closely related to the purpose of this article.

"We are very much hopeful that people will find warm uses that we can think about". This sentence should be rephrased

As mentioned above, this sentence has been rephrased and the entire manuscript has been revised throughout for such unnecessary additions.

Last sentence: "? which can be replaced by any user because the are all compatible" should be rephrased.

This sentence has been erased.