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Morphometric Protocol for Objective Assessment of Blastocyst Behavior During Vitrification and Warming Steps

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JOVE Editors

Dear Editors,

We are sending you our manuscript, entitled:

Time-lapse Microscopy Based Morphometric Protocol for the Assessment of Blastocyst Behavior During Cryopreservation and After Thawing

We hope you will find it interesting enough for publication in JOVE.

Yours sincerely,

Marjan Taborin,

Borut Kovačič

TITLE:

Morphometric Protocol for the Objective Assessment of Blastocyst Behavior During Vitrification and Warming Steps

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KEYWORDS:

Bioengineering, assisted reproductive techniques, cryobiology, vitrification, blastocyst, bio-imaging, time-lapse, morphometry

SUMMARY:

Here we present a time-lapse morphometric protocol to follow the intensity of blastocyst shrinkage and re-expansion during previtrification interventions and post-warming recovery. The application of the protocol is possible in *in vitro* fertilization laboratories equipped with time-lapse microscopes and is recommended in the development of an optimal blastocyst vitrification method.

ABSTRACT:

This article describes the noninvasive method of blastocyst morphometry based on time-lapse microphotography for the accurate monitoring of a blastocyst's volume changing during individual phases before and after vitrification. The method can be useful in searching for the most optimal timing of blastocyst exposure to different concentrations of cryoprotectants by observing blastocyst shrinkage and re-expansion in different pre- and post-vitrification phases. With this methodology, the blastocyst vitrification protocol can be optimized. For a better demonstration of the usefulness of this morphometric method, two different blastocyst preparation protocols for vitrification are compared; one with using an artificial blastocoel collapsing and one without this intervention before vitrification. Both blastocysts' volume changes are followed by time-lapse microphotography and measured by photo-editing software tools. The measurements are taken every 20 seconds in previtrification phases and every 5 minutes in the post-warming period. The changes of the blastocyst dimensions per time unit are presented graphically in line diagrams. The results show a long equilibration previtrification phase in which the intact blastocyst first shrinks and then slowly refills the blastocoel, entering vitrification with a fluid-filled blastocoel. The artificially collapsed blastocyst remains in its shrunken stage through the entire equilibration phase. During the vitrification phase, it also does not change its volume. Since the blastocyst morphometry shows a constant volume of the artificially collapsed blastocysts during the

previtrification step, it seems that this stage could be shorter. The described protocol provides many additional comparative parameters of blastocyst behavior during and after cryopreservation on the basis of the speed and intensity of the volume changes, the number of partial blastocoel contractions or total blastocyst collapses, and the time to a total blastocoel re-expansion or the time to hatching.

INTRODUCTION:

Cryopreservation of human preimplantation embryos from the *in vitro* fertilization program (IVF) is nowadays a routine practice in most IVF laboratories. The slow embryo freezing method began to be clinically used in 1985, with the introduction of specific cryoprotectants and computer-controlled freezers, which enabled the controlled cooling of embryos down to -7 °C, when ice nucleation (seeding) was induced in the surrounding cryoprotective medium[1]. By continuous cooling, the ice crystals would grow, causing the hyperosmolality of the remaining liquid fraction and, consequently, the dehydration and shrinkage of the embryonic cells. At -30 °C or -80 °C, the embryos would be plunged into the liquid nitrogen for longer storage. What was happening with the embryos or oocytes during cooling could be observed only by specially adapted cryomicroscopes, which helped to improve cryopreservation protocols[2]. The freezing of blastocysts, a higher-volume and more fluid-contained embryonic stage, gave less promising clinical results in those days[3].

The breakthrough in the cryopreservation of the blastocyst was the introduction of the vitrification method, in which the dehydration of the cells took place before cooling by using high concentrations of cryoprotectants[4]. The removal of the fluid from the blastocoel before vitrification can also be achieved by making a mechanical opening between two trophectoderm cells[5]. Although the immediate blastocyst survival rate after vitrification and warming is above 90%, and the clinical outcome following the transfer of vitrified/warmed blastocyst into the uterine cavity is almost comparable to results after the transfer of fresh embryos, this cryopreservation method has not yet been standardized[6,7]. Vitrification protocols vary according to (a) the type and concentration of the cryoprotectants, (b) the number of previtrification steps, (c) the duration of individual steps, (d) the use of an artificial blastocoel collapsing before vitrification or not, (e) collapsing methods, (f) the blastocyst expansion stage, and (g) the equilibration/vitrification temperature at which the embryos should be vitrified[8]. Since cryoprotectants can be toxic for cells, the blastocyst exposure time to these solutions has to be well defined. However, some cryopreservation media manufacturers allow very flexible protocols.

The interest of scientists is usually focused on studying the blastocyst re-expansion ability with the aim to find new biomarkers with a better prediction of implantation[6,9,10]. How human blastocysts dehydrate at different steps of adding cryoprotectants before vitrification and what happens with blastocysts after vitrification and warming, when cryoprotectants have to be removed from the cells, and how the blastocysts rehydrate and re-expand after warming, is not well described nor understood. The development of a methodology for the objective and quantified monitoring of blastocyst behavior during different cryopreservation steps is, thus, rationale.

With time-lapse microscopes of various manufacturers, it is now possible to monitor the behavior of the blastocyst in pre- and post-vitrification phases. By including additional

computer tools, measurements of their size (morphometry) can also be performed. By measuring the decrease or increase in embryo size at a given time, it is possible to objectify the evaluation of morphodynamics of embryos during dehydration and rehydration.

PROTOCOL:

All methods described here have been approved by the National Medical Ethics Committee on 19 April 2016 (No. 0120–204/2016–2).

1. Set-up of the Microscope Recording System

1.1. Turn off the heating plate of the microscope.

1.2. Before any treatment, take a snapshot of a blastocyst under a camera-equipped inverted microscope. Remember to note the magnification at which the blastocyst was recorded.

2. Selection and Prepreparation of Blastocysts for Vitrification

2.1. Select only fully expanded day-5 or day-6 blastocysts for cryopreservation with at least a few inner cell mass (ICM) cells and cohesive trophectoderm.

2.2. For laser-treated blastocysts with a collapsed blastocoel, subject a blastocyst to a short laser pulse (0.4 - 0.7 ms) with a hole diameter ranging from 4.3 to 9.4 µm, tangentially directed at the zona pellucida and at a junction of two adjacent trophectodermal cells. Allow the blastocyst to fully or partially collapse for 5 min.

2.3. For untreated blastocysts with an intact blastocoel, perform no particular treatment before vitrification.

Note: These blastocysts are considered intact.

3. Preparation of Cryoprotectant and a Petri Dish for the Equilibration Phase

3.1. Use pithe pipette for oocyte denudation (diameter 135 µm) to aseptically fill equilibration media (M-199 HEPES-buffered medium with 7.5% DMSO, 7.5% ethylene glycol, 20% DSS) into the holes of the microdroplet area of a 9-well Petri dish specially designed for time-lapse microscopy and recording.

Note: Oocyte denudation is a process of removing cumulus cells surrounding the oocyte. The use of a pipette for oocyte denudation will help to avoid the creation of air bubbles.

3.2. Overlay the microdroplet area with 30 µL of equilibration media.

4. Transfer of the Blastocyst in Equilibration Solution

4.1. Submerge a laser-treated or intact blastocyst into the equilibration medium to the bottom of the microdroplet area of the microdroplet dish for 10 min at room temperature (equilibration phase).

4.2. Start a 10-min countdown as soon as the blastocyst is transferred to the equilibration medium.

5. Recording of the Blastocyst at the Equilibration Phase

5.1. Transfer the microdroplet dish with the blastocyst to a camera-equipped microscope. Use the same magnification as previously for taking a snapshot of the same blastocyst. Position the microdroplet dish so that the blastocyst is positioned approximately in the center of the recording.

5.2. Start recording with the microscope recording software. Note the countdown time at which the recording is started.

Note: See representative results of recordings of an intact (**Figure 1**, **Video 1**) and a collapsed blastocyst (**Figure 2**, **Video 2**) during the 10-min exposure to an equilibration solution.

6. Vitrification of the Blastocyst

6.1. Aseptically dispense a 50-µL drop of vitrification solution (M-199 HEPES-buffered medium with 15% DMSO, 15% ethylene glycol, 20% DSS, 0.5 M sucrose) in a Petri dish 2 min before the countdown ends.

6.2. Stop recording when the 10-min countdown ends and quickly transfer the blastocyst to vitrification solution for 30 s at room temperature.

6.3. Gently pipette the blastocyst at least 1x within the vitrification solution.

6.4. Use a vitrification straw for loading the blastocyst. Load, seal, and plunge the vitrification straw into liquid nitrogen (LN) within 80 s, not exceeding 110 s after the initial exposure to the vitrification solution.

7. Video Editing of the Recorded Blastocyst During the Equilibration Phase

7.1. Import a recorded video file into video editing software.

7.2. Move the timeline slider to 20 s. Note the frame number at this time.

Note: This number will be used to decimate unnecessary video frames. Only frames every 20 s will be used.

7.3. Click the **Video** tab, then **Frame rate...**. Under **Frame rate conversion**, check the **Decimate by** field and enter the previously noted frame number. Confirm by clicking **OK**. Click **File** → **Export** → **Image sequence**.

7.4. Choose JPEG as the output format, set the directory to hold the saved sequence of images, and click **OK**.

Note: This will create a directory with up to 30 images of the recorded blastocyst in sequence during the equilibration phase of vitrification.

8. Measurements of the Blastocyst's Cross-sectional Area from Images Created During the Equilibration Phase

8.1. Open the video analysis software. Click the **Window** tab and select **Timeline**.

8.2. In the Timeline pane, click on the film strip sign and choose **Add Media...** to add an image of the blastocyst before the equilibration phase of vitrification—and before the laser intervention, if there was any.

Note: This image will show the blastocyst at its most expanded state and its cross-sectional area will serve as a reference.

8.3. Add the image sequence of the corresponding blastocyst generated previously with video editing software (of the equilibration phase of vitrification).

8.4. Go to **Image** → **Analysis** → **Select Data Points** → **Custom** to open the **Select data points** window.

8.5. Deselect all data points, then select only **Area,** and confirm by clicking **OK**.

8.6. Move the timeline slider in the Timeline window to the first image.

8.7. Click the **Window** tab and choose **Tools**.

Note: This will activate the Tools panel on the left side.

8.8. Choose the **Quick Selection Tool**.

8.9. Position the tool marker inside the blastocyst to touch the edge of the blastocyst. Outline the blastocyst by placing the tool marker on the blastocyst's outer edge, clicking and holding the left mouse button, and dragging the tool marker along the blastocyst's outer edge until the whole blastocyst's cross-sectional area is selected.

Note: Zona pellucida is not a part of the measurement, so it must be excluded (**Figure 5**).

8.10. In the Timeline window, click the **Measurement Log** and press the **Record Measurements** button.

Note: This will record the selected area.

8.11. Return back to the Timeline, right-click on the image, and choose **Deselect**.

8.12. Move the timeline slider to the next image and click on the corresponding tile beneath it.

8.13. Outline the blastocyst in the new image with the Quick Selection Tool. Repeat the measurement.

8.14. Repeat this procedure (steps 8.9 - 8.13) image by image until all the images' cross-sectional areas are measured and recorded.

8.15. In the end, go to the Measurements Log, select all the measurements under the **Area** variable, and export them in a .txt file.

Note: The units of the cross-sectional area are square pixels.

9. Editing of the Data File with the Recorded Blastocyst's Cross-sectional Areas from Images Created During the Equilibration Phase

9.1. Transfer the data of the recorded blastocyst's cross-sectional areas from the .txt file to a spreadsheet editor.

9.2. Use the first cross-sectional area value as reference value 1 and express (transform) all the other values to be a fraction of that reference value.

9.3. Create a new variable Area_r and paste the transcoded area values (except the reference value) under it.

9.4. Next to the Area_r variable, create a Time variable and add the first time value.

Note: The first time value represents the time which passed from the moment the blastocyst was exposed to the equilibration solution to the onset of recording under a camera-equipped microscope.

9.5. Calculate each next consecutive time value by adding 20 s to the previous time value.

Note: This is the time interval used at decimating unnecessary video frames created during the recording.

10. Warming of the Blastocyst

10.1. Prepare media for warming.

10.1.1. A day before warming the blastocyst, aseptically dispense three 150-µL drops of recovery medium in a 4-well dish. Also, aseptically dispense recovery medium in a microdroplet 9-well dish specially designed for time-lapse microscopy and recording. Cover the recovery medium with paraffin oil and preincubate it at 37 °C with 6% CO_2 and 5% O_2.

Note: Recovery medium is a cultivation medium for blastocyst-stage embryos with added human serum albumin (HSA). The HSA in the recovery medium should be 12 mg/mL. To fill the holes in the 9-well dish, use a pipette for oocyte denudation to avoid the creation of air bubbles, then overlay the microdroplet area with 30 µL of recovery medium.

10.1.2. On the day of warming the blastocyst, aseptically dispense 500 µL of thawing solution (TS) in a single well of a 4-well dish and allow it to warm to 37 °C in an incubator without CO^2.

10.1.3. At room temperature, aseptically dispense one 50-µL drop of dilution solution (DS) and two 50-µL drops of washing solution (WS) in a sterile Petri dish. Cover the DS and the WS1 and WS2 drops with paraffin oil.

Note: Instead of a Petri dish, a 4-well dish can also be used.

10.2. Warm the blastocyst.

10.2.1. Identify the HSV straw with the vitrified blastocyst to be removed from LN storage and quickly transfer the straw to an LN-filled portable reservoir in preparation for the warming procedure.

10.2.2. Lift the straw with forceps, just enough to expose the colored handling rod. Use a self-adjusting wire stripper to cut the straw at the height of the colored handling rod.

10.2.3. Grab the handling rod and extract it from the straw with a swift, yet controlled movement, and immediately plunge the curved spatula of the handling rod into the 37 °C TS.

10.2.4. Gently swirl the handling rod to detach the blastocyst and leave it for a total of 1 min in TS.

10.2.5. At room temperature, transfer the blastocyst consecutively to DS, WS1, and WS2 for 4 min in each medium.

10.2.6. After the warming procedure, transfer the blastocyst to a 4-well dish with recovery medium and wash it in all three drops by gently pipetting it.

Note: The washing is carried out in approximately 1 min.

10.2.7. Transfer the blastocyst to the time-lapse 9-well dish with recovery medium. Place the 9-well dish under a time-lapse camera in an incubator (at 37 °C with 6% CO^2 and 5% O^2).

Note: Thre warming procedure, which continues with placing the 9-well dish under a time-lapse camera in an incubator, lasts approximately 17 min.

11. Time-lapse Recording of the Blastocyst Re-expansion During Recovery Post-warming

11.1. Run time-lapse recording software.

11.2. Choose a recording camera.

11.3. Press the **Live mode** button.

11.4. Place the mouse cursor on the image and use the scroll button to enlarge it. Click and hold the left mouse button and move the cursor to place the well with the blastocyst in the middle of the screen.

11.5. Under **Focusing**, use the up-down green arrows to focus the recording plane of the blastocyst. Under **Light intensity**, set the light intensity.

11.6. Click the **Microscope parameters** button to set the Exposure time and Gamma.

11.7. Press **Close live mode**.

11.8. Press the **Start project** button and enter the project data, select the culture dish type (3 x 3 or 4 x 4) and uncheck all positions except the one to be recorded.

11.9. Set the capture timing to take a picture every 5 min.

11.10. Start recording by pressing the **Approve** button. Record at least 150 min.

11.11. Stop recording by pressing the **Stop project** button.

Note: See the representative results of the recording of an intact (**Figure 3**) and a collapsed blastocyst (**Figure 4**) during the post-warming recovery period.

12. Video Editing of the Recorded Blastocyst During the Re-expansion Post-warming

12.1. Go to the project folder and locate recorded images that are roughly 80 KB in size.

12.2. Create another folder and transfer these images into it. Rename the images in numbers according to the time created. Import them into the video editing software as an image sequence.

12.3. Go to **Video tab** → **Filter** → **Add** and select **Null transform** filter.

12.4. Click the **Cropping** button on the right side and crop the image, leaving visible only the field with the recorded blastocyst. Confirm with **OK** and again with **OK**.

12.5. Click **File** → **Export** → **Image sequence**. Choose JPEG as the output format, set the directory to hold the saved sequence of images, and click **OK**.

Note: This will create resized (cropped) images prepared for further measurements in the video analysis software.

13. Setting of a Measurement Scale in Video Analysis Software for the Images Created with the Time-lapse Recording Software

13.1. Run the Analyzer of the time-lapse recording software.

13.2. Open the project with the recorded blastocyst.

13.3. Click the **Analyze** button on the field with the recorded blastocyst.

Note: A new Analyze window will open. Click on the **Measurement** button to show the measurement tool.

13.4. Use the measurement tool to measure the width of the whole image.

13.5. Right-click on the image and save it. In the properties, check the image's width in pixels.

13.6. Divide the image's actual width with its width in pixels.

Note: This calculates the actual length of a single pixel in this image.

13.7. Run the video analysis software.

13.8. Click the **Window** tab and select **Timeline**.

13.9. In the Timeline pane, click on the film strip sign and choose **Add Media...** to add the image sequence of the post-warm re-expanding blastocyst.

13.10. Click **Image** → **Analysis** → **Set Measurement Scale** → **Custom** to open the Measurement Scale window. For Pixel length, enter 1; for Logical length, enter the previously calculated actual length of a pixel (step 13.6); for Logical units, enter µm. Save the preset.

14. Measurements of the Blastocyst's Cross-sectional Area from Images Created During the Re-expansion Post-warm

14.1. Once the Measurement scale is set and the image sequence imported, measure the blastocyst's cross-sectional areas the same way as described in step 8, with the only difference being that the measured cross-sectional areas in these measurements have units in μm^2.

15. Editing of the Data File with the Recorded Blastocyst's Cross-sectional Areas from Images Created during the Re-expansion Post-warm

15.1. Transfer the data of the recorded blastocyst's cross-sectional areas from the .txt file to a spreadsheet editor.

15.2. Create a Time variable next to the Area variable and add the first time value.

Note: In this case, the first time value is set to 0 minutes, which is the onset of the time-lapse recording. Each next consecutive time value is calculated by adding 5 minutes to the previous time value. Five minutes is the time interval used between two consecutive time-lapse recordings.

16. Creation of a Line Diagram

16.1. Import the spreadsheet data file in statistical analysis software for creating a time plot of the blastocyst's cross-sectional area changes in time during the equilibration phase of the vitrification or re-expansion post-warming.

REPRESENTATIVE RESULTS:

In a demonstration, we showed blastocyst morphodynamics in only one previtrification and one post-warming phase. A difference in the blastocyst's volume at the end of the equilibration phase and at the beginning of recovering in culture medium showed the intensity of embryo shrinkage, which is, in fact, the intensity of embryo preservation against ice crystallization.

As it can be noticed from **Figure 1** and **Video 1**, the intact blastocyst did not collapse completely in equilibration solution. The blastocoel contracted only partially, but within 10 minutes, it slowly reached the re-expansion size of 70%. Contrary to this, the artificially collapsed blastocyst completely emptied the blastocoel immediately after laser treatment, but in equilibration solution, its volume did not change any more (**Figure 2**, **Video 2**). The presentation of blastocoel re-expansion in recovery medium (**Figures 3** and **4**) with microphotographs and with line diagrams shows different patterns of blastocoel growth. A display of a single measurement of the cross-sectional area of the blastocyst is shown in **Figure 5**.

In vitrification medium with a higher concentration of cryoprotectants, intact blastocysts intensively shrunk once again, while the collapsed blastocyst's volume remained almost unchanged. From a graphical presentation, by using the protocol presented here, it is evident that the intact blastocyst undergoes a stepwise reduction of the blastocoel (**Figure 6**), once in the equilibration solution and once in the vitrification medium, while the collapsed blastocyst reached a shrunken stage at the beginning of the intervention with a laser (**Figure 7**). This poses the question whether 10 minutes of equilibration phase is really necessary for collapsed blastocysts, or whether this period could be shortened.

The presentation of blastocoel re-expansion can be linear or interrupted with several bigger or smaller contractions (**Figure 8**).

FIGURE AND VIDEO LEGENDS:

Figure 1: Time-lapse microphotography of the changes of an intact blastocyst during exposure to equilibration solution. Single images were recorded every 20 seconds. The scale bar is 100 µm.

Figure 2: Time-lapse microphotography of the changes of an artificially collapsed blastocyst

during exposure to equilibration solution. Single images were recorded every 20 seconds. The scale bar is 100 µm.

Figure 3: Time-lapse microphotography of the changes of an intact blastocyst during recovery post-warming. Single images were recorded every 5 minutes. The scale bar is 100 µm.

Figure 4: Time-lapse microphotography of the changes of a collapsed blastocyst during recovery post-warming. Single images were recorded every 5 minutes. The scale bar is 100 µm.

Figure 5: Display of a single measurement of the cross-sectional area of a blastocyst. The blastocyst is carefully encircled with the appropriate selection tool in video analysis software. The zona pellucida is always excluded from the measurement. The scale bar is 100 µm.

Figure 6: Representation of the changes in the cross-sectional area of an intact blastocyst. These panels show the representation of changes in the cross-sectional area of an intact blastocyst during exposure to equilibration solution (**A**) at vitrification and (**B**) during recovery post-warming. The units of the cross-sectional area are relative to the initial blastocyst's cross-sectional area before vitrification. The dashed line connecting panels **A** and **B** is imaginary, representing the changes during vitrification, cooling to -196 °C, and warming to 37 °C.

Figure 7: Representation of the changes in the cross-sectional area of a collapsed blastocyst. These panels show the representation of changes in the cross-sectional area of (**A**) a collapsed blastocyst during exposure to equilibration solution (**B**) at vitrification and (**C**) during recovery post-warming. The units of the cross-sectional area are relative to the initial blastocyst's cross-sectional area before collapsing and vitrification. The dashed line at panel **A** is imaginary and represents the starting and ending point during the blastocyst collapse. Also the imaginary dashed line between panels **B** and **C**, represents the changes during vitrification, cooling to -196 °C, and warming to 37 °C. Only the solid lines in panels **B** and **C** are the result of actual measurements of the cross-sectional area during the equilibration and recovery process.

Figure 8: Graphical presentation of different patterns of blastocyst recovery after warming. The scale bar is 100 µm.

Video 1: Time-lapse video of the changes of an intact blastocyst during exposure to equilibration solution. The video represents the changes, which last almost 10 minutes in real-time.

Video 2: Time-lapse video of the changes of a collapsed blastocyst during exposure to equilibration solution. The video represents the changes, which last almost 10 minutes in real-time.

DISCUSSION:

The protocol for the observation of blastocyst morphodynamics during and after cryopreservation can also be carried out by using similar instruments and software tools from other manufacturers. Time-lapse systems adjusted for embryology allow the continuous

monitoring of embryo development. The purpose of this work was to introduce the quantification of blastocyst behavior during the preparation of blastocysts for vitrification and after their warming. This was done by the objective measurement of changes in the morphology on a timescale. Obtained results of these measurements can be mathematically analyzed and compared with other results. Among the parameters that can be followed by the described protocol are changes in the size of the blastocyst, its inner cell mass, the blastocoel, or the zona pellucida within a certain period of time. The size can be displayed as the surface area in the largest blastocyst cross-section, the blastocyst circumference or diameter, and, after calculation, even as its volume.

In previous studies using time-lapse systems, blastocoel expansion velocity measurements were made only on fresh embryos[11]. In vitrified/warmed blastocysts, only the blastocysts' sizes were measured immediately after warming and before the embryo transfer, or the time period was analyzed at which warmed blastocysts re-expanded to the zona pellucida[9,10]. More detailed blastocyst re-expansion dynamics were analyzed only in our previous study[8]. In this study, the morphometric protocol has already been used to track the speed and pattern of blastocyst re-expansions after warming. Although these blastocyst growth biomarkers have shown not to have a high predictive value for implantation, they can be used for the comparison of blastocyst recovery potential after vitrification and warming with different cryopreservation media and protocols. Namely, the greater contractions of the blastocyst during blastocoel re-expansion suggest the weakness of the trophectodermal layer that could be caused during cryopreservation, and its inability to withstand the pressure made by the fluid-filled blastocoel.

The described morphometric protocol can provide many additional comparative analyses of blastocyst behavior, not only after cryopreservation, by measuring the speed and intensity of the changes in volume, the number of partial blastocoel contractions or total blastocyst collapses, the time to total blastocoel re-expansion, or the time to hatching. Moreover, it can also be used during previtrification phases for determining the most optimal timing of blastocyst exposure to different concentrations of cryoprotectants, as was presented in the results. Vanderzwalmen *et al.* showed on mice oocytes that vitrification can also be successful if intracellular osmotic pressure does not reach an equilibrium with external cryoprotectant solution[12]. The only problematic phase for the recording of blastocysts during preservation is the period in vitrification solution due to limited time; the embryo has to be exposed to a higher concentration of cryoprotectants and packed into the straw in less than 90 seconds. For these measurements, it would be recommended to use only blastocysts that are donated for research. Nevertheless, clinically available blastocysts can be recorded and measured again immediately after warming, with the aim to observe how they behave in dilution and washing solutions. Immediately after a blastocyst transfer from the washing solution to the recovery medium, blastocysts tend to float off the bottom. This can represent a difficulty in keeping the embryo in the same focal plane during recording. To solve this problem, it is recommended to prepare a dish with flattened drops of medium. A similar problem has been observed by Vanderzwalmen *et al.*[12].

In further research, it would be useful to explore whether the length of exposure of artificially collapsed blastocysts to cryoprotectants could be reduced, consequently minimizing the toxic effect of these chemicals.

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The authors have nothing to disclose.

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Figure

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Video 1

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Video 1_equilibration_intact_blastocyst.mp4

Video 2

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Video or Animated Figure

Video 2_equilibration_collapsed_blastocyst.mp4

Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5

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Figure 6

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Figure 7

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Figure 8

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Table of Materials

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Name of Material/ Equipment	Company
Inverted microscope Eclipse TE2000-U	Nikon, Japan
Saturn 5 Laser System	Research Instruments, Origio, Denmark
Digital camera DC1	Research Instruments, Origio, Denmark
Digital camera DC2	Research Instruments, Origio, Denmark
Cronus 3.7	Research Instruments, Origio, Denmark
Incubator with 6% CO2, 5% O2	Binder, Germany
Primo Vision microscope	Vitrolife, Sweden
Primo Vision Capture software	Vitrolife, Sweden
Adobe Photoshop CS6 Extended software	Adobe Systems Incorporated, USA
VirtualDub	Avery Lee
Microsoft Office Excell	Microsoft, USA
PrimoVision culture dish	Vitrolife, Sweden
G2-plus medium	Vitrolife, Sweden
Human Serum Albumins	Vitrolife, Sweden
Paraffin oil	Vitrolife, Sweden
Equilibration solution medium	Irvine Scientific, Ireland
Vitrification solution medium	Irvine Scientific, Ireland
Thawing solution medium	Irvine Scientific, Ireland
Dilution solution medium	Irvine Scientific, Ireland
Washing solution medium	Irvine Scientific, Ireland
HSV Vitrification straws	CryoBio System, France
Liquid nitrogen	/
Cryo vessel Biosafe 120 MD β	Cryotherm, Germany
Cryo tank	Cryotherm, Germany
Forceps	/
Scisors	/
Pippete for blastocyst manipulation	Gynetics, Belgium
Pipette for oocyte denudation	Vitromed, Germany
Pipettor EZ-Grip	Research Instruments
Digital interval timer Assistent	Glaswarenfabrik Karl Hecht
IBM SPSS Statistics 21	IBM, USA
Self adjusting wire stripper	Knipex, Germany

Catalog Number	Comments/Description
/	
/	
/	
/	
/	microscope recording software
/	
16600	
16608	time-lapse recording software
/	video analysis software
/	video editing software
/	spreadsheet editor
16604	
10132	cultivation medium for blastocyst stage embryos
10064	
10029	
90131	
90132	
90134	
90135	
90136	
025246, 025249, 025250, 025248	
229286	
/	
/	
ID275/10	diameter 275 µm
V-DEN-135	diameter 135 µm
7-72-2802	
41977010	
/	statistical analysis software
1262180	

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Author(s): Marjan Taborin[1], Borut Kovačič[1]

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The manuscript is corrected according to editor's and reviewers' comments:

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Changes to be made by the Author(s):

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./

Done.

2. Please revise the title to be more concise.

Done.

3. 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 …"

Done.

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

At the end of Introduction one paragraph was added.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations./

Done.

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Done.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.)./

Done.

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Done.

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Done.

10. Please include single-line spaces between all paragraphs, headings, steps, etc./

Done.

11. After you have made all the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol. There is a 10-page limit for the Protocol. Please revise the protocol section to meet this page limit./

Done.

12. 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.

13. Please number the figures in order of appearance./

Done.

14. Figures: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend./

Done.

15. Figures 6 and 7: Please include a space between numbers and their units (e.g., -196 °C, 37 °C)./

Done.

16. Figure 8: Please provide the unit for Time in the x-axis. Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible./

Done.

17. Please upload the videos separately as “Animated/Video Figure” to your Editorial Manager account in the form of an .mov, .mp4, .m4v file.

Done.

18. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Well written.

Major Concerns:

none

Minor Concerns:

It will be of value to compare good quality and poor quality embryos.

By using described method, the behaviour of good- and poor quality embryos can be compared as well.

Reviewer #2:

Manuscript Summary:

Thank you for giving me the opportunity to review this very interesting manuscript of Taborin & Kovačič. This paper not only provides detailed information of blastocyst behavior during vitrification but also during the warming process. It is of particular interest since the authors used a single cryoprotectant to work with. They raised the question whether the rather long equilibration process is required at all which would be a major step towards facilitating this technique. Thus, it is of definite interest for the reader (method is reproducible also with instruments of different providers) and should be published after minor corrections have been made.

Major Concerns:

No

Minor Concerns:

Title: the authors may reconsider the term "thawing". Thawing indicates that ice crystal formation had taken place which one wants to avoid during vitrification. Much rather, the term "warming" should be used throughout the text. In the discussion the authors did so./

Done. Lines 2, 81, 200, 201, 202, 211, 219, 234, 241, 262, 288, 296, 302, 311, 353, 359, 396, 401 in the first version.

Introduction: Line 53: this process is called "seeding".

Correction done.

Line 60: please clarify that this statement is valid only in older studies. E.g. by simply adding the words "in these days" at the end of the sentence.

Done.

Differences in vitrification protocols: what is the difference between d. and e. Should the "carriers" be mentioned (maybe not part of the Vit protocol).

The authors believe that (d) referes to whether the method is used or not used at all and (e) referes to variations of the method when it is used.

Line 106&207: the inexperienced reader won´t know what a "pipette for denudation" is (since denudation process is not mentioned at all)/

Oocyte denudation explained. Added diameter of pipette for oocyte denudation.

Mat&Met: this section cannot be reviewed in great detail since this reviewer uses a different time-lapse incubator. However, the protocols are very detailed and it should be easy to work with them!

Line 171: it should read "zona pellucida"/

Done.

Lines 234-239: how long does it take in minutes from the removal from lN2 to the first frame of the video?/

Information added as a Note in 10.2.7.

Discussion: Vanderzwalmen et al (2013) published an interesting experiment which should be discussed (Human Reproduction, Volume 28, Issue 8, 1 August 2013, Pages 2101-2110)

Done.