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Defining the Program of Maternal mRNA Translation During In Vitro Maturation Using a Single Oocyte Reporter Assay

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KEYWORDS:
Oocyte, mRNA, translation, meiotic maturation, time-lapse microscopy, reporter assay, micro-injection

SUMMARY:
This protocol describes a reporter assay to study the regulation of mRNA translation in single oocytes during in vitro maturation.

ABSTRACT:
Events associated with oocyte nuclear maturation have been well described. However, much less is known about the molecular pathways and processes that take place in the cytoplasm in preparation for fertilization and acquisition of totipotency. During oocyte maturation, changes in gene expression depend exclusively on the translation and degradation of maternal messenger RNAs (mRNAs) rather than on transcription. Execution of the translational program, therefore, plays a key role in establishing oocyte developmental competence to sustain embryo development. This paper is part of a focus on defining the program of maternal mRNA translation that takes place during meiotic maturation and at the oocyte-to-zygote transition. In this method paper, a strategy is presented to study the regulation of translation of target mRNAs during in vitro oocyte maturation. Here, a Ypet reporter is fused to the 3' untranslated region (UTR) of the gene of interest and then micro-injected into oocytes together with polyadenylated mRNA encoding for mCherry to control for injected volume. By using time-lapse microscopy to measure reporter accumulation, translation rates are calculated at different transitions during oocyte meiotic maturation. Here, the protocols for oocyte isolation and injection, time-lapse recording, and data analysis have been described, using the Ypet/interleukin-7 (IL-7)-3' UTR reporter as an example.

INTRODUCTION:

A fully-grown mammalian oocyte undergoes rapid changes in preparation for fertilization and acquisition of totipotency. These changes are essential to sustain embryonic development after fertilization. Although the events associated with nuclear maturation are relatively well described, much less is known about the molecular processes and pathways in the oocyte cytoplasm. During the final stages of oocyte maturation, oocytes are transcriptionally silent, and gene expression is entirely dependent on mRNA translation and degradation[1,2]. The synthesis of proteins critical for developmental competence, therefore, relies on a program of timed translation of long-lived mRNAs that have been synthesized earlier during oocyte growth[1,3]. As part of a focus on defining this program of maternal mRNA translation executed during meiotic maturation and at the oocyte-to-zygote transition, this paper presents a strategy to study the activation and repression of the translation of target maternal mRNAs in single oocytes during in vitro meiotic maturation.

In this method, the YPet open reading frame is cloned upstream of the 3' UTR of the transcript of interest. Next, mRNAs encoding this reporter are micro-injected into oocytes together with polyadenylated mRNAs encoding mCherry to control for injected volume. Reporter accumulation is measured during in vitro oocyte meiotic maturation using time-lapse microscopy. The accumulation of yellow fluorescent protein (YFP) and mCherry is recorded in individual oocytes, and YFP signals are corrected by the plateaued level of the co-injected mCherry. After data acquisition, translation rates are calculated for different time intervals during in vitro oocyte meiotic maturation by calculating the slope of the curve obtained by curve-fitting.

This approach provides a tool to experimentally confirm changes in translation of selected endogenous mRNAs. In addition, this method facilitates the characterization of regulatory elements that control translation during oocyte meiotic maturation by manipulating cis-regulatory elements of the 3' UTR of target mRNAs[4–6]. Manipulation of the poly A tail length also allows insight into adenylase/deadenylase activity in oocytes[5]. Mutagenesis of cis-acting elements or RNA immunoprecipitation can be used to study interactions with cognate RNA binding proteins[6,7]. Additionally, this method can be used to identify essential components of the translation program that are critical for oocyte developmental competence by measuring target 3' UTR translation in models associated with decreased oocyte quality [8–10]. This method paper presents a representative experiment where denuded oocytes of 21-Day-old C57/BL6 mice have been micro-injected with a Ypet reporter fused to the 3' UTR of IL-7. The setup and protocol for oocyte injection, time-lapse recording, and data analysis have been described.

PROTOCOL:

The experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco (protocol AN182026).

1. Preparation of media

1.1. Add all components, as described in **Table 1**, to make the basic oocyte collection medium and oocyte maturation medium. For the basic collection medium, set the pH to 7.4. For both

collection and maturation medium, add 3 mg/mL of bovine serum albumin (BSA) and 1 µM cilostamide on the Day of use.

2. Preparation of mRNA encoding for Ypet-3' UTR and mCherry

2.1. Obtain the 3' UTR sequences of the mRNAs of interest.

NOTE: For this study, sequences were previously obtained from mouse oocyte cDNA.

2.2. Design primers to amplify the target 3' UTRs from oocyte cDNA and portions of the pCDNA 3.1 vector containing a Ypet coding sequence, V5-epitope tag, and a T7 promoter.

2.3. Amplify using a high-fidelity DNA polymerase kit. Run the polymerase chain reaction (PCR) products on a gel to check if the fragments have the correct size, cut out the bands, and extract the DNA from the gel using a gel extraction kit according to the manufacturer's instructions.

2.4. Fuse the PCR fragments to a vector using a PCR cloning kit.

NOTE: PCR fragments were incubated on ice for 4 h to facilitate a more efficient recombination process. This is contrary to the manufacturer's instructions, which recommend an incubation time of only 45 min.

2.5. Transfect PCR fragments into competent *5-α Escherichia coli* bacteria.

2.5.1. Add the mix and plasmid to the bacteria, and incubate on ice for 30 min.

2.5.2. Heat shock the mix and plasmid by placing the mixture in a water bath at 42 °C for 45 s, and immediately cool on ice for 3 min.

2.5.3. Add 500 µL of Super Optimal broth with catabolite repression medium and incubate for 1 h at 37 °C.

2.5.4. Spin down at 7000 × *g* for 2 min, and remove most of the supernatant. Resuspend and plate on a Luria broth (LB) agar plate with the appropriate selection antibiotic.

NOTE: Carbenicillin was used in this study.

2.5.5. Incubate overnight at 37 °C. Isolate the colonies by lightly pressing a pipet tip on one of the colonies and placing it in 3 mL of LB medium with 100 µg/mL of carbenicillin. Incubate for 12–24 h at 37 °C.

2.5.6. Extract the DNA of the plasmids using the plasmid DNA isolation kit according to the manufacturer's instructions and confirm the sequences via DNA sequencing.

2.6. For Ypet/3’ UTR:

2.6.1. Produce a linear PCR template for in vitro transcription. Use a forward primer upstream of the Ypet sequence and a reverse primer with 20 additional thymine residues. See **Table 2** for the sequence of Ypet/IL-7 3’ UTR and the sequences of the forward and reverse primers.

NOTE: These additional thymine residues will add oligo(A) to the linear mRNA after in vitro transcription.

2.6.2. In vitro transcribe the PCR product using a T7 transcription kit according to the manufacturer’s instructions.

2.6.3. Purify the resulting complementary RNA (cRNA) using a transcription clean-up kit according to the manufacturer’s instructions.

2.6.4. Elute the purified cRNA in RNAse-free water, measure mRNA concentrations, and evaluate the message integrity by agarose electrophoresis. Store at −80 °C.

2.7. For mCherry:

2.7.1. Produce a linear PCR template for *in vitro* transcription by using a high-fidelity restriction enzyme; use the restriction enzyme mfEI-HF if replicating this example. Digest in a digestion buffer overnight at 37 °C.

2.7.2. Run a gel to purify the sample, and extract the linear DNA using a gel extraction kit according to the manufacturer’s instructions.

2.7.3. In vitro transcribe the PCR product using a T7 transcription kit according to the manufacturer’s instructions.

2.7.4. Polyadenylate the cRNA (150–200 nucleotides) using a poly(A) tailing kit according to the manufacturer’s instructions.

2.7.5. Purify the resulting cRNA using a transcription clean-up kit according to the manufacturer’s instructions.

2.7.6. Elute the purified cRNA in RNAse-free water, measure mRNA concentrations, and evaluate the message integrity by gel electrophoresis. Store at −80 °C.

3. Experimental procedure

NOTE: A schematic overview of oocyte micro-injection and subsequent time-lapse microscopy is given in **Figure 1**.

3.1. Day 1

3.1.1. Intraperitoneally inject 21-Day-old mice with 5 IU of pregnant mare's serum gonadotropin to promote follicle growth to the antral stage[11].

3.2. Day 3

3.2.1. Oocyte collection

3.2.1.1. Sacrifice mice 44–48 h after priming to collect the ovaries, and place them in a plastic Petri dish with basic oocyte collection medium.

3.2.1.2. Carefully open the antral follicles by making a small cut in the follicle wall with a 26 G needle. Isolate intact cumulus-enclosed oocytes (COCs) with several layers of cumulus cells using a mouth-operated glass pipette.

3.2.1.3. Use a smaller pipette (slightly larger than the diameter of the oocyte), and mechanically denude the COCs by repeated pipetting.

NOTE: Alternatively, perform micro-injection on intact COCs.

3.2.1.4. Using a larger pipette, aspirate the denuded oocytes and place them in a Petri dish with maturation medium supplemented with 1 µM of the phosphodiesterase inhibitor, cilostamide, to prevent resumption of meiotic maturation[12]. Place the dish in the incubator for 2 h to let the oocytes recover from the stress induced by isolation of the oocytes from the follicles.

3.2.2. Oocyte micro-injection

3.2.2.1. Prepare the injection needles by placing a 10 cm long borosilicate glass capillary tube in a mechanical puller. For optimal injection, bend the needle tip in a 45° angle using a heated filament.

3.2.2.2. Prepare polystyrene dishes with 20 µL droplets of basic oocyte collection medium, and cover the droplets with light mineral oil.

3.2.2.3. Prepare a reporter mix by adding 12.5 µg/µL Ypet-3' UTR and 12.5 µg/µL mCherry. Prepare a larger volume and make aliquots for future experiments to ensure similar reporter concentrations. Store these aliquots at -80 °C. Upon thawing, first centrifuge the aliquot for 2 min at 20,000 x *g*, and transfer to a new microcentrifuge tube.

NOTE: This centrifugation will prevent the injection needle from getting clogged by potential aggregates in the reporter mix.

3.2.2.4. Load the injection needle by capillarity with approximately 0.5 µL of reporter mix.

3.2.2.5. Place the holding pipette and loaded injection needle into the holders, and position in the droplet of oocyte collection medium. Aspirate some of the medium into the holding pipette.

3.2.2.6. Open the injection needle by gently tapping it against the holding pipette.

3.2.2.7. Place oocytes in a droplet of basic collection medium, and inject 5–10 pL of the reporter mix.

3.2.2.8. Incubate the oocytes in the maturation medium with 1 µM cilostamide for 16 h to allow the mCherry signal to plateau.

3.2.2.9. Prepare a Petri dish that will be used for time-lapse microscopy (with at least two 20 µL droplets of maturation medium for each injected reporter: one droplet with 1 µM cilostamide for control prophase I-arrested oocytes and one droplet without cilostamide for maturing oocytes. Cover the droplets with light mineral oil and place in incubator.

3.2.3. Time-lapse microscopy

3.2.3.1. After pre-incubation, remove the injected oocytes from the incubator, and wash them four times in maturation medium without cilostamide. Keep some oocytes in maturation medium with 1 µM cilostamide as a prophase I oocyte control group.

3.2.3.2. Transfer the injected oocytes to their respective droplets on the previously prepared time-lapse microscope dish. Cluster the oocytes by using a closed glass pipette (a closed pipette can be prepared by holding the tip in a flame for a few seconds).

NOTE: Clustering the oocytes helps to prevent their movement during the recording.

3.2.3.3. Place the dish under the microscope equipped with a light-emitting diode illumination system and a motorized stage equipped with an environmental chamber maintained at 37 °C and 5% CO_2. To replicate this study, use the following parameters: filter set: dichroic mirror YFP/CFP/mCherry 69008BS; YFP channel (Excitation (Ex): S500/20 × 49057; Emission (EM): D535/30 m 47281), mCherry channel (Ex: 580/25 × 49829; Em: 632/60 m).

3.2.3.4. Enter the appropriate settings for the time-lapse experiment (see **Table of Materials** for the software used in this study): Click on **Apps** | **Multi Dimensional Acquisition**. Select the first tab **Main** and select **timelapse**, **multiple stage positions**, and **multiple wavelengths**.

3.2.3.5. Select the tab **Saving** to enter the location where the experiment should be saved.

3.2.3.6. Select the tab **Timelapse** to enter the number of time points, duration, and time interval.

NOTE: The duration of the time-lapse experiment depends on the animal species studied, as timing of oocyte meiotic maturation differs among species. In this experiment with mouse oocytes, oocytes were recorded every 15 min for 16 h.

3.2.3.7. Select the tab **Stage**. Switch on brightfield and locate the position of the oocytes by opening a new window by selecting **Acquire** | **Acquire** | **Show Live**. Once the oocytes are located, switch back to the **Multi Dimensional Acquisition** window, and press **+** to set the location of the oocytes.

3.2.3.8. Select the tab **Wavelengths**, and set 3 different wavelengths for brightfield (exposure 15 ms), YFP (exposure 150 ms for Ypet/IL7 3' UTR), and mCherry (exposure 75 ms for Ypet/IL7 3' UTR).

NOTE: Adjust the exposure for each Ypet/3' UTR reporter based on the level of reporter accumulation and the injected volume. Ensure that the YFP signal falls in the center of the range of detection at the start of the experiment to prevent underestimation or saturation in case of activation of translation. For mCherry, adjust the exposure for each batch of mCherry as the signal depends on the number of adenine nucleotides that were added during the polyadenylation procedure. Because of the variation in polyadenylation efficiency, use the same batch of mCherry in different experiments for a better comparison between experiments.

3.2.3.9. Start the time lapse experiment by clicking on **Acquire**. See **Figure 2** and the **Supplemental File** for an example of a time-lapse recording in a single oocyte.

3.2.4. Analysis of Ypet-3' UTR translation

3.2.4.1. For this analysis (see **Table of Materials**), perform two region measurements for each oocyte: the oocyte itself—click on **ellipse region** and surround the oocyte—and a small region close to the oocyte to be used for background subtraction—click on **rectangular region**.

NOTE: Ensure that the oocytes do not move out of the selected region during the time-lapse recording. Pay special attention to the placement of the region measurement around polar body extrusion, as this causes movement in the oocyte and may distort the recording.

3.2.4.2. Export the region measurement data to a spreadsheet by clicking first on **Open Log** and then on **Log Data** to export the data.

3.2.4.3. For each individual oocyte and for all measured time-points, subtract the background region measurement from the oocyte region measurement. Do this for YFP and mCherry wavelengths separately.

3.2.4.4. Record the timing of germinal vesicle breakdown (GVBD) and polar body extrusion (PBE) for later reference.

NOTE: Exclude maturing mouse oocytes when GVBD exceeds 2 h.

3.2.4.5. Plot YFP and mCherry expression over time for each oocyte to check for outliers.

NOTE: This should be a smooth curve, if it is not this may indicate that the oocyte moved during the recording.

3.2.4.6. For each individual oocyte, calculate average mCherry expression for the last ten time-points of the recording. For each time-point, divide the YFP expression by the averaged mCherry expression to correct for injected volume to obtain a YFP/mCherry ratio at every time-point for each individual oocyte.

3.2.4.7. Calculate translation rates for a specific time-interval by fitting the slope of the curve by linear regression. Assess the differences in translation rates using statistical inference.

REPRESENTATIVE RESULTS:

Denuded prophase I-arrested oocytes of 21-Day-old C57/BL6 mice were injected with a reporter mix containing mRNA encoding the Ypet reporter fused to the 3' UTR of IL-7 and mRNA encoding mCherry. YFP and mCherry expression was recorded in 39 oocytes, of which 30 were matured, and 9 were arrested in prophase I as a negative control. Three maturing oocytes were excluded for analysis because they either had a delayed GVBD (N=2) or moved in the dish during the recording (N=1). **Figure 3** shows mCherry and YFP expression in prophase I and maturing oocytes. **Figure 4** shows YFP expression of maturing oocytes corrected for plateaued mCherry expression (averaged mCherry expression in the last 10 time-points) to correct for the injected volume. Translation rates of the reporter were measured by curve-fitting (linear regression) the YFP/mCherry values in prophase I and in maturing oocytes during the first 0–2 h or 8–10 h after cilostamide release (**Figure 5A**). The accumulation of the reporter does not follow a linear pattern, as indicated by a significant difference in translation rates between 0–2 h and 8–10 h after cilostamide release ($p<0.0001$; **Figure 5B**). Therefore, these results indicate activation of IL-7 translation during oocyte meiotic maturation.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of the experimental procedure. Oligoadenylated Ypet/3' UTR and polyadenylated mRNA encoding mCherry are micro-injected into denuded oocytes of 21-Day-old C57/BL6 mice. Oocytes are pre-incubated for 16 h in cilostamide containing maturation medium to allow the mCherry signal to reach a plateau. After pre-incubation, a time-lapse recording is started where oocytes are either kept in medium with cilostamide to create a prophase I-arrested control group or released in cilostamide-free medium to mature. Abbreviations: UTR = untranslated region; YFP = yellow fluorescent protein; fw primer = forward primer; rev primer = reverse primer; Ampr = ampicillin resistance; polyA = polyadenyl; oligoA = oligoadenyl; GV = germinal vesicle.

Figure 2: Example of a single oocyte time-lapse recording. Brightfield, YFP, and mCherry recordings of a single oocyte injected with mRNAs encoding Ypet/Interleukin-7 3' UTR and polyadenylated mCherry at prophase I, MI (6 h after cilostamide release), and MII (15 h after cilostamide release). Scale bar = 25 µm. Abbreviations: YFP = yellow fluorescent protein; GV = germinal vesicle; MI = metaphase I; MII = metaphase II.

Figure 3: YFP and mCherry signals recorded by time-lapse microscopy. YFP and mCherry signals of oocytes injected with oligoadenylated Ypet/IL7 3' UTR and polyadenylated mRNA encoding mCherry. Oocytes were either kept in medium with cilostamide to generate a prophase I-arrested control group (N=9) or released in cilostamide-free medium to allow for maturation (N=30). Data are individual oocyte measurements. Abbreviations: IL-7 = interleukin-7; YFP = yellow fluorescent protein.

Figure 4: YFP signal corrected for co-injected mCherry level. YFP signals of prophase I-arrested and maturing oocytes were corrected for injected volume by dividing the YFP signal by the average mCherry signal of the last 10 time-points. Individual YFP/mCherry ratios for (**A**) prophase I-arrested oocytes and (**B**) maturing oocytes and mean ± standard error of the mean YFP/mCherry ratios of (**C**) prophase I-arrested and (**D**) maturating oocytes. Abbreviations: YFP = yellow fluorescent protein; GVBD = germinal vesicle breakdown.

Figure 5: Calculated translation rates at 0–2 h and 8–10 h of maturation. (**A**) Yellow fluorescent protein (YFP) signals of single oocytes corrected for mCherry (YFP/mCherry) at 0–2 h or 8–10 h after cilostamide release and (**B**) translation rates (mean ± standard error of the mean) as calculated by curve-fitting (linear regression) the YFP/mCherry values at 0–2 h and 8–10 h after cilostamide release. Data were analyzed using the unpaired two-tailed t-test. *****$p < 0.0001$.

Figure 6: Example of repression of translation: Oosp2. Re-analyzed data of an experiment where oocytes were injected with Ypet-Oosp2 3' UTR and polyadenylated mRNA encoding mCherry. YFP signals of prophase I-arrested (N=63) and maturing oocytes (N=72) were corrected for injected volume by dividing the YFP signal by the average mCherry signal of the last 10 time-points. YFP and mCherry expression data were obtained using a Xenon Arc lamp, unlike the IL-7 experiment where an LED light source was used. Data represent the mean ± standard error of the mean of individual oocyte measurements and were previously published in Luong et al.[5]. Abbreviations: YFP = yellow fluorescent protein; Oosp2 = oocyte secreted protein 2; UTR = untranslated region; IL-7 = interleukin-7; LED= light-emitting diode; GVBD = germinal vesicle breakdown.

Figure 7: Effect of micro-injection and fluorescence exposure on timing of GVBD and PBE. Timing of GVBD and PBE of oocytes that were either micro-injected and exposed to fluorescence (injected), or not-injected and not exposed to fluorescence (not-injected). Data are individual oocyte measurements. Data were analyzed using the unpaired two-tailed *t*-test. *****$p<0.001$. Abbreviations: GVBD = germinal vesicle breakdown; PBE= polar body extrusion.

Table 1: Preparation of media. List of components that need to be added to prepare basic oocyte collection medium and oocyte maturation medium.

Table 2: Example of reporter and primer sequences Sequence of YFP/IL7 3'UTR reporter and sequences of forward and reverse primers that were used to produce a linear PCR template for *in vitro* transcription.

Supplemental File: Yellow fluorescent protein (YFP) and mCherry time-lapse recording. YFP and mCherry time-lapse recordings of a single oocyte injected with mRNAs encoding Ypet/Interleukin-7 3' UTR and polyadenylated mCherry. YFP channel (Ex: S500/20 × 49057; Em: D535/30 m 47281), mCherry channel (Ex: 580/25 × 49829; Em: 632/60 m). The oocytes were recorded every 15 min for 16 h (7 frames/second).

DISCUSSION:

The presented method describes a strategy to study activation and repression of translation of target mRNA at different transitions during in vitro oocyte meiotic maturation. IL-7, a cytokine released by the oocyte that may be involved in oocyte-cumulus cell communication[8,13], was chosen for the purpose of describing this method. IL-7 is known to be increasingly translated during oocyte maturation[8] and allows for good visualization of translational activation using this method. If, however, translation occurs at a constant rate throughout the experiment, accumulation of a reporter will follow a linear pattern, and translation rates at the beginning and end of the experiment will be similar. This conclusion can be verified by goodness of fit (R) of a linear regression through the entire recording interval. A repression in reporter translation will present itself as the plateauing of the YFP signal.

An example of repression of 3' UTR translation, can be found in the study of Luong et al.[5], where the authors report repression of Oocyte Secreted Protein 2 (Oosp2) during oocyte meiotic maturation. These data have been re-analyzed and are shown in **Figure 6**. The maturing oocytes show plateauing of the YFP signal, which indicates repression of translation, while the prophase I-arrested control group follows a linear pattern of reporter accumulation, which indicates similar translation rates at the beginning and end of the experiment. When studying repression of translation, it is especially important to include a prophase I-arrested control group to ensure that the plateauing of the YPF signal is not explained by a decrease in oocyte quality due to poor culture conditions, thus confirming the viability of the oocytes. Accumulation of the reporter can be validated by quantitative reverse-transcription PCR using primers for YFP or by western blotting by taking advantage of the V5-epitope tag included in the reporter in this protocol.

Plateauing of the YFP signal may not be solely due to actively regulated repression of translation, but may also be explained by the degradation of the reporter during oocyte meiotic progression. This may mirror destabilization of the endogenous mRNA, which is essential for the transition to embryonic genome expression[14–16]. This possibility can be verified by measuring target gene transcript levels in the prophase I stage vs. metaphase II stage to confirm stability of the mRNAs. When preparing oocyte cDNA, it is preferable to use random hexamer priming over oligo-dT priming. The latter method presents apparent differences in gene transcript levels that may

reflect differences in poly(A) length of these transcripts rather than actual differences in transcript levels[7].

There are several methods to study genome-wide endogenous mRNA translation in mouse oocytes. These methods include polysome profiling[7,17–18] and RiboTag/RNA-Seq[5,19–20]. Ribosome profiling is another method to study genome-wide translation that has been used in yeast, which is another model organism that is used to study meiosis[21,22]. However, these genome-wide analyses to study mRNA translation in the mouse require the use of 150–200 oocytes per sample while the number of oocytes available for analysis is usually limited. The single-oocyte assay described herein to assess translation of 3' UTR of target mRNAs complements genome-wide analyses of translation, as it allows the characterization of elements of the 3' UTR that regulate the translation program during oocyte meiotic maturation[4–6]. In the past, luciferase-based assays were used to assess translation of the 3' UTR of target mRNAs[10,20,23] as it is a very sensitive method that requires only a few oocytes per sample; however, the oocytes need to be lysed to detect the amount of luciferase in a sample.

Others have used a 3' UTR/green fluorescent protein (GFP) reporter to assess GFP accumulation in live mouse oocytes[4]. However, in these experiments, only two time-points were used: the beginning of incubation (prophase I) and the end of the incubation (metaphase II). This greatly diminishes the power of the measurements and increases the possibility of errors. Kinetic data provide accurate measurements based on rates (multiple points) and accurately define the time when translational activation or repression takes place. In addition, repression of translation cannot be assessed at these single time-points, which can lead to an inaccurate conclusion. Therefore, this method was adjusted by applying time-lapse microscopy to assess Ypet/3' UTR reporter accumulation throughout in vitro maturation of mouse oocytes[5,6,9]. By using this strategy, it is possible to study translation at different transitions during oocyte maturation.

There are several important aspects about this method to consider. First, the reporters used include an oligo(A) tail whose omission considerably reduces reporter accumulation. This may be due to both decreased translation as well reporter mRNA destabilization[24,25]. During the pre-incubation in prophase I, translation of an oligo-adenylate probe adjusts to reflect the translation rate of the endogenous mRNA[5]. Second, it is important to realize that an increase in the number of recordings or duration of fluorescence exposure may potentially induce phototoxicity and thereby impair oocyte quality. Although the current experiment adopted 15-min intervals, the sampling rate can be decreased when a high fluorescence exposure must be used in case of low reporter expression.

To limit the amount of phototoxicity, a cold LED light source was used, which requires a shorter excitation[26]. To assess potential phototoxicity effects in the oocytes, the timing of GVBD and PBE was compared in oocytes that were either micro-injected and exposed to fluorescence or not injected and not exposed to fluorescence. The combination of micro-injection and fluorescence exposure was found to slightly delay GVBD ($p<0.001$), while the timing of PBE was similar (**Figure 7**). This method has been used to study translational regulatory elements of the 3' UTR during in vitro oocyte meiotic maturation. Similarly, it may also be used to study functional 5' UTR

elements essential for the regulation of translation or to study translation during in vitro oocyte fertilization.

Although this method has been applied to human and mouse oocytes, it is also applicable to other animal species. Because this reporter assay is performed in single oocytes, it is especially suitable for use in mono-ovulatory species in which the number of oocytes available for analysis is limited. As opposed to using denuded oocytes, another application of this technique is the injection of the reporter into cumulus-enclosed oocytes, as the cumulus cells play a major role in the regulation of the translational program[8,10,27]. However, it should be taken into account that cumulus-enclosed oocytes are more likely to move away from the recording plane during the experiment as compared to denuded oocytes because of the movement of the cumulus cells during COC expansion.

This may result in a larger proportion of oocytes that have to be excluded from analysis. In the future, cell-tracking software may be used that is able to track x, y, and z positions of the oocytes in the droplet, which may solve this issue. In conclusion, the described single oocyte reporter assay represents a strategy to investigate the translational program executed at the oocyte-to-zygote transition. Increased knowledge of this translational program can provide important clues about the molecular regulation of oocyte developmental competence.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

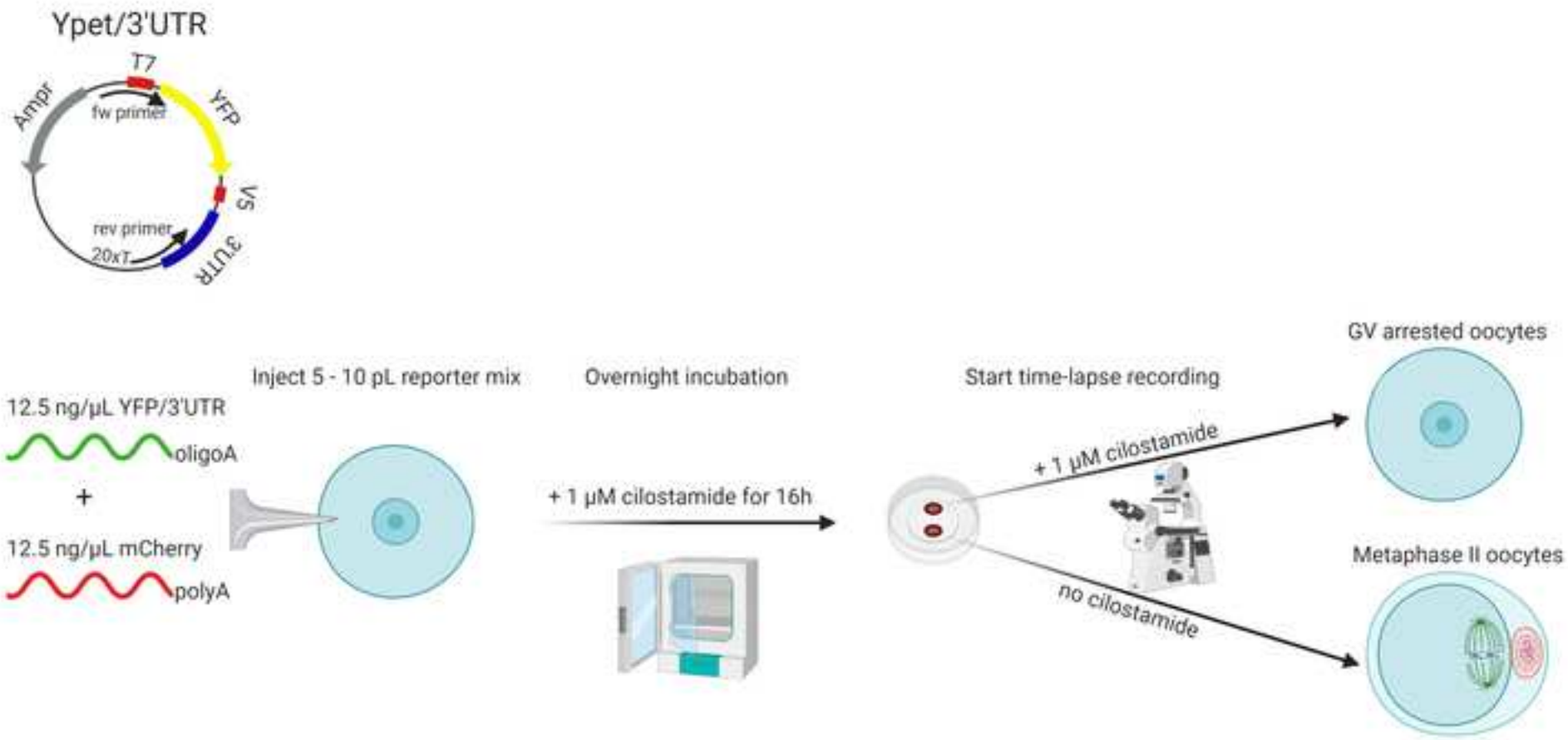


Figure 2

Click here to access/download;Figure;Figure 2 newnew.png

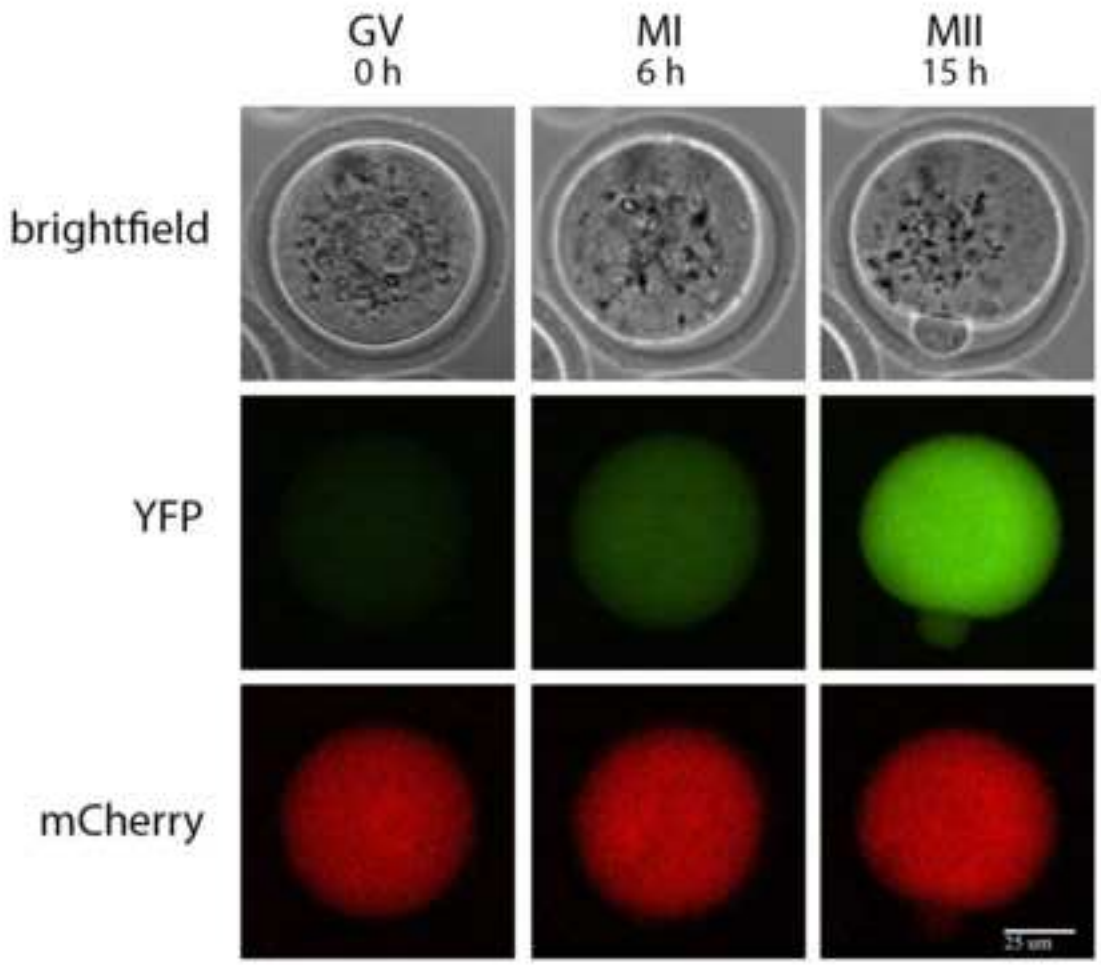


Figure 3

Click here to access/download;Figure;Figure 3 new.jpg

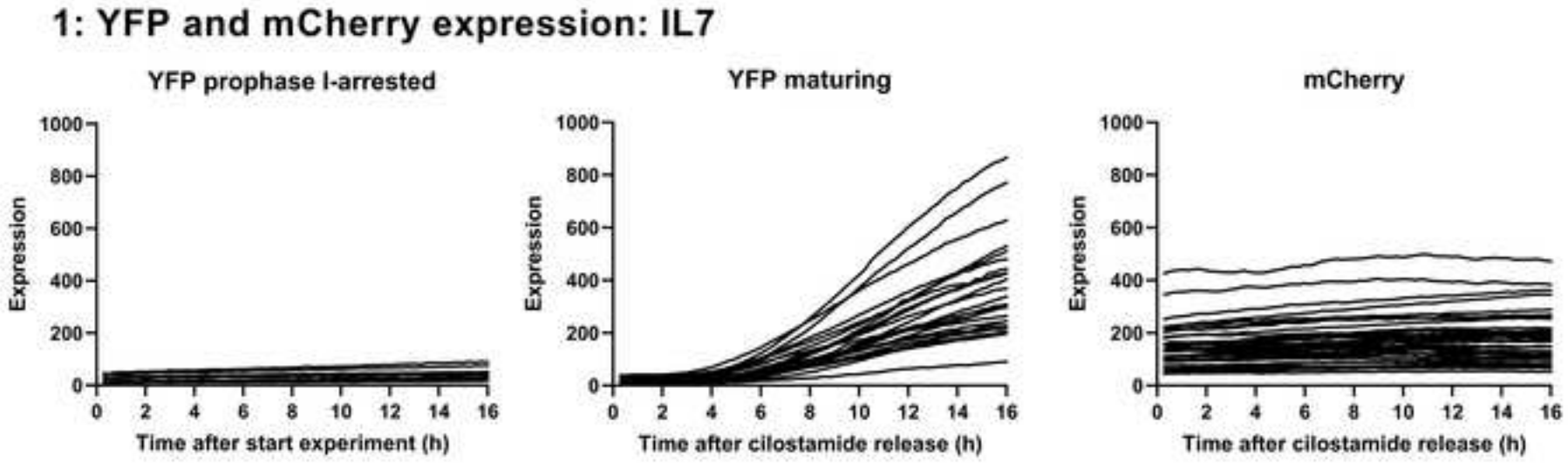


Figure 4

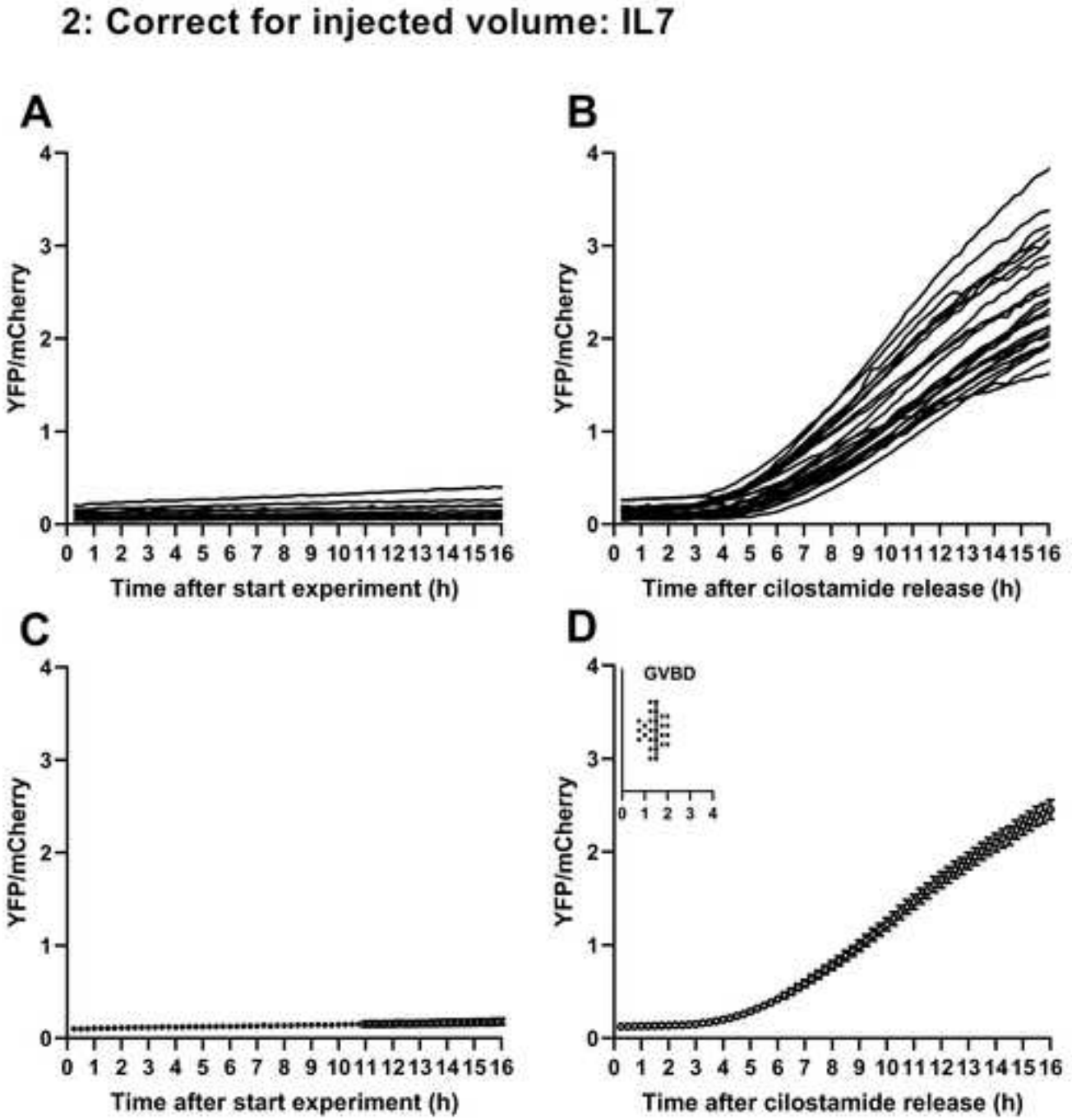


Figure 5

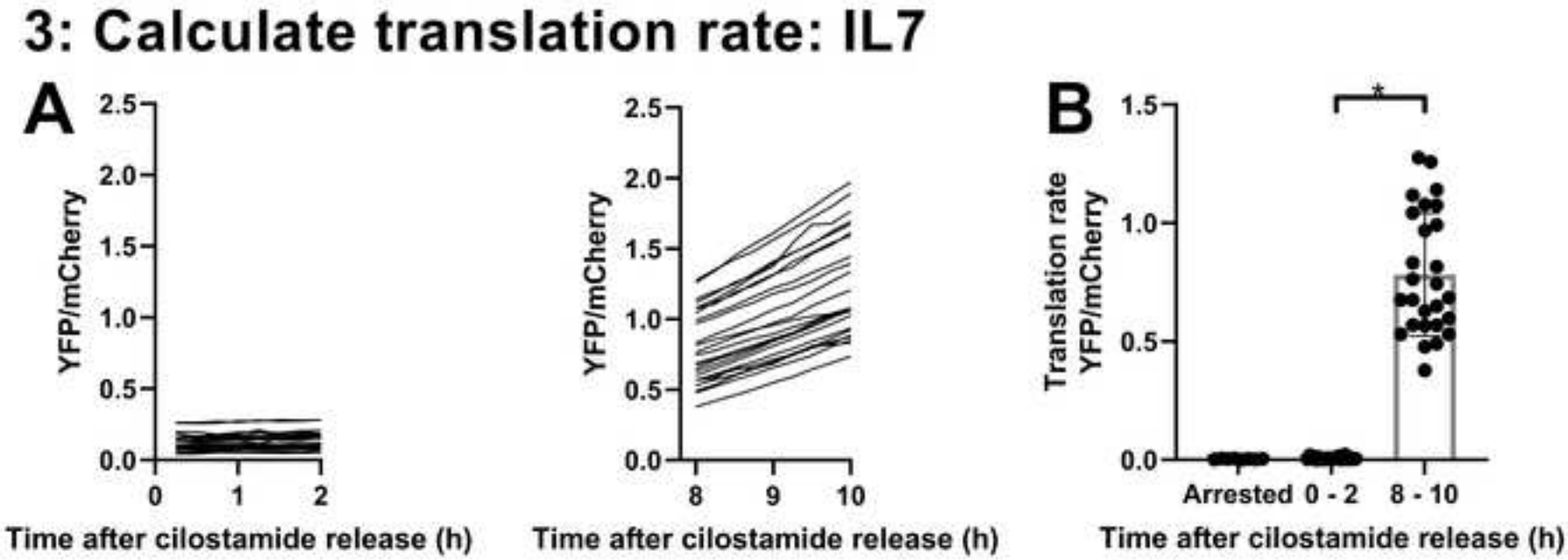


Figure 6

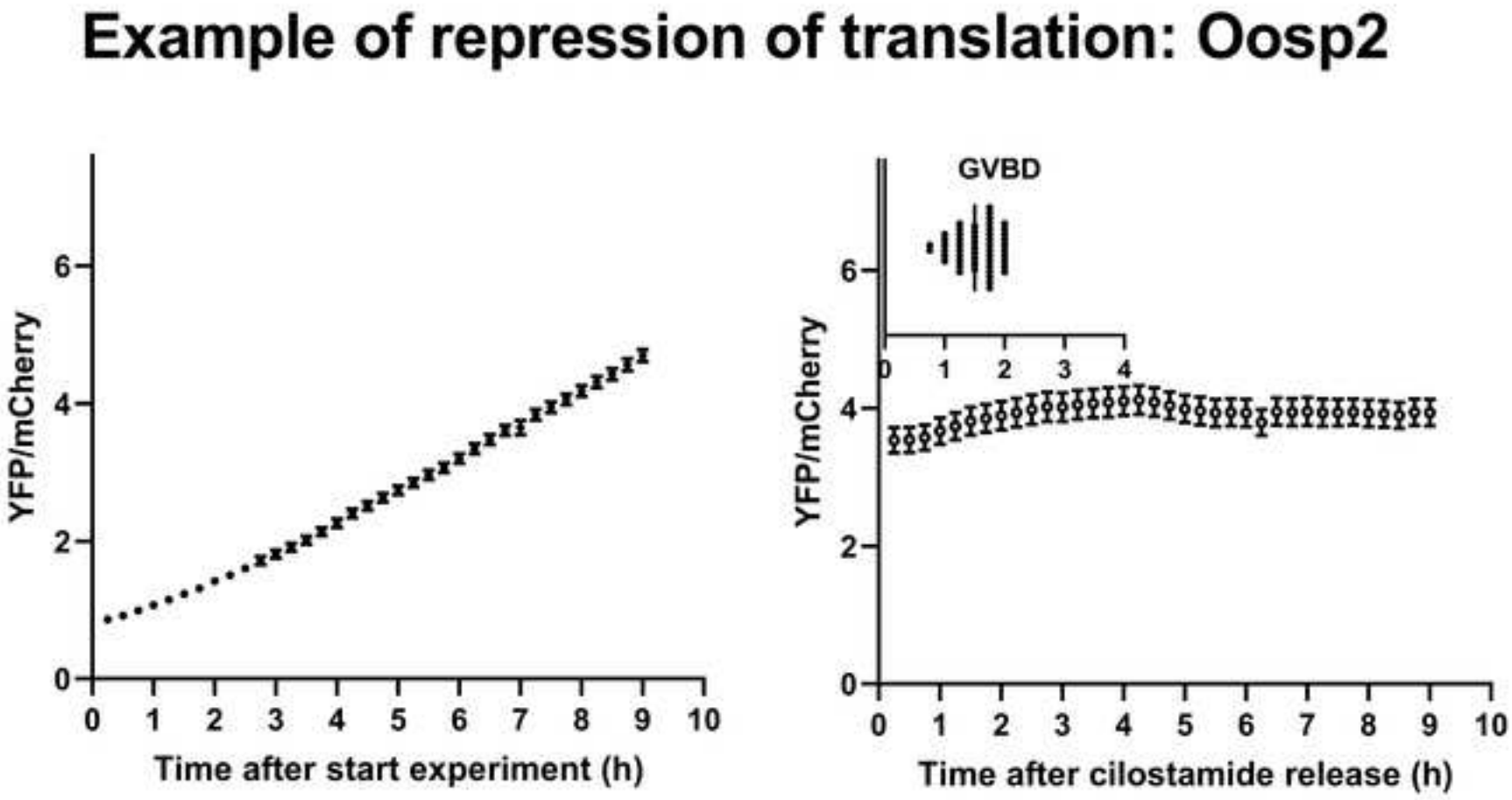
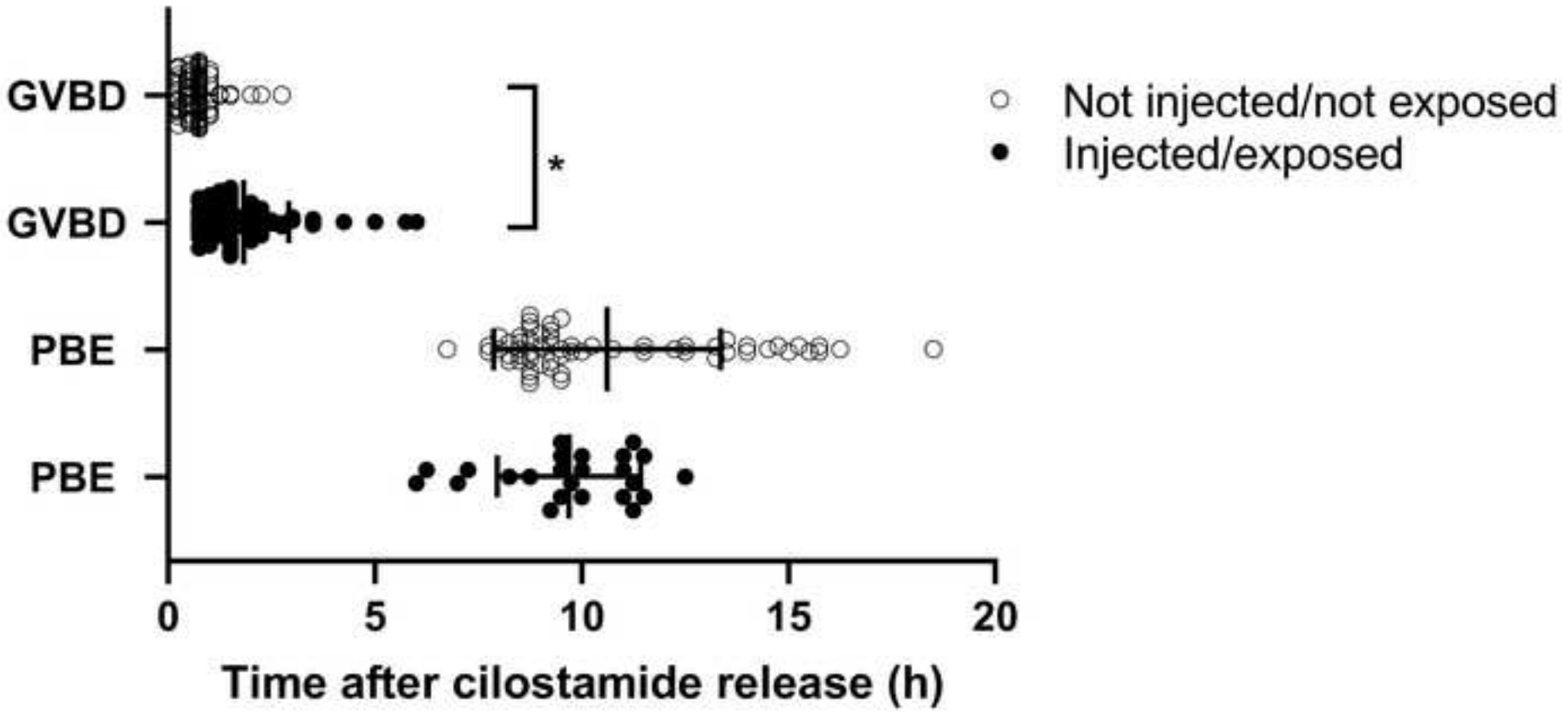


Figure 7



Supplemental File

Click here to access/download

Video or Animated Figure

Supplemental Figure 1.avi

Basic oocyte collection medium

Component
HEPES modified Minimum Essential Medium Eagle
Sodium bicarbonate
Sodium pyruvate
Penicillin/Streptomycin 100x
Ultrapure distilled water (Invitrogen, 10977-015)

Maturation medium

Component
MEM alpha 1x
Sodium pyruvate
Penicillin/Streptomycin 100x

For 500 mL
7.1 g
252 mg
1.15 mL
5 mL
Up to 500 mL

For 500 mL
Up to 500 mL
1.15 mL
5 mL

Table 2

Click here to access/download;Table;Table 2 Example of reporter and primer sequences.xlsx

	Sequence
Ypet/Interleukin-7 3’ UTR	GAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGTTAAGCTTGGTAC CGAGCTCGGATCCACCGGTCGCCACCATGGTGAGCAAAGGCGAAGAGCTGTTCACCGGCGTGGTGCCCATCCTGGTGGAG CTGGACGGCGACGTGAACGGCCACAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGACGCCACCTACGGCAAGCTGACC CTGAAGCTGCTGTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTGGTGACCACCCTGGGCTACGGCGTGCAGTG CTTCGCCCGGTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGAGCGCCATGCCCGAGGGCTACGTGCAGGAGCGGA CCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGGGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGGATC GAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGCCACAAGCTGGAGTACAACTACAACAGCCACAACG TGTACATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCGGCACAACATCGAGGACGGCGGCGT GCAGCTGGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCT ACCAGAGCGCCCTGTTCAAGGACCCCAACGAGAAGCGGGACCACATGGTGCTGCTGGAGTTCCTGACCGCCGCCGGCATC ACCGAGGGCATGAACGAGCTCTATAAGAGATCTTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGC GTACCGGTCATCATCACCATCACCATTGAACAGGACATGTAGTAACAACCTCCAAGAATCTACTGGTTCATATACTTGGAGA GGTTGAAACCCTTCCAGAAGTTCCTGGATGCCTCCTGCTCAAATAAGCCAAGCAGCTGAGAAATCTACAGTGAGGTATGAG ATGATGGACACAGAAATGCAGCTGACTGCTGCCGTCAGCATATACATATAAAGATATATCAACTATACAGATTTTTGTAAT GCAATCATGTCAACTGCAATGCTTTTAAAACCGTTCCAAATGTTTCTAACACTACAAAGTCTACAAAAAGCAAGGCTATGAA GATTCAGAGTCACCACTGTTTTCTTAGCAAAATGATGGTATGGTTAAACATTCATTGGTGAACCACTGGGGGAGTGGAACT GTCCTGTTTTAGACTGGAGATACTGGAGGGCTCACGGTGATGGATAATGCTCTTGAAAACAAGAGTCTATCTTAAAGCAGC AGCAAAAAGAAGCTTAAGGCACTTAAGGCATCAACAAATGTAGTTAAATATGAATGTATAACACATAACTTCAGTAAAGA GCATAGCAGATATTTTTAAATAAAAGTATTTTTAAAGATAGAAATGCACTTATTCCAAAGATACTGAACCTTAGTATTCAGT CGCTTTTGACACTTGTGTATAATAAAGCTTATATAACTGAATTTTCAATTTGAAAAGTATATTTTTAAAAGAATAATATATGC TAGACTTTTAATTAATGTATATGTTTAATTTTGGCATTCTGTCTGTCTCTCTGTCTCTCTCTCTCTCTCTCTCTCTCTACCTAT CTATCTATATATATAATTTTCATATACTACCAATTGCGTACTTTGGATAGTGTCTCTTTTTAACCTAAATGACCTTTATTAACA CTGTCAGGTTCCCTTACTCTCGAGAGTGTTCATTGCTGCACTGTCATTTGATCCCAGTTTTATTGAACACATATCCTTTAACAC ACTCACGTCCAGATTTAGCAGGAGACTAGGACCCTATAACTTTGTTAAGAGAGAAAACACTAATTTCTTGTTTTATAGTAGG GTCTTATTCGTATCTAAGGCAGGCTAGGATTGCAGACATGAGCCAATATGCTTAATTAGAAACATTCTTTTTATGTTAAACT CATGTCTTTTACAAGATGCCTACATATATCCTATGTATATGCCTGTTTAAATCCTTTTTTGTAAGGTCTGCTGTCTTCCTTCAG TTGTAATGGAAAGAAACACTATGTTGTAGAGGCCAAATTTCTGAAAGTGATAAGGGTTTGCTTGTACTGAATTCTCATTCTC CTTGCTTTTTCCAGCCACGTGAGCATCTAGCTATCTATACGCTGGATGTATTTGACCGATGCCTGCTCCACTGGCACATTGCA
Forward primer	GAGAACCCACTGCTTAC
Reverse primer	TTTTTTTTTTTTTTTTTTTTAAAATATTATCCTTTATTTTGCCAAAATC

Name of Material/ Equipment	Company
Preparation of media	
Bovine Serum Albumin Powder Bioxtra	Sigma-Aldrich
Cilostamide	EMD Millipore
MEM alpha	Gibco
Minimum Essential Medium Eagle	Sigma-Aldrich
Penicillin-Streptomycin 100x Solution, Sterile Filtered	Genesee Scientific Corporation (GenClone)
Sodium Bicarbonate	JT-Baker
Sodium Pyruvate	Gibco
Ultrapure distilled water	Invitrogen
Preparation of mRNA encoding YFP/3' UTR and mCherry	
Agarose	Apex Biomedical
Carbenicillin disodium salt	Sigma-Aldrich
Choo-Choo Cloning Kit	McLab
CutSmart Buffer (10x)	New England Biolabs
DNA loading dye (6x)	Thermo Scientific
dNTP Solution	New England Biolabs
DpnI	New England Biolabs
GeneRuler 1 kb DNA ladder	Thermo Fisher
LB Agar Plates with 100 µg/mL Carbenicillin, Teknova	Teknova
LB Medium (Capsules)	MP Biomedicals
MEGAclear Transcription Clean-Up Kit	Life Technologies
MfeI-HF restriction enzyme	New England Biolabs
mMESSAGE mMACHINE T7 Transcription Kit	Invitrogen
Phusion High Fidelity DNA polymerase	New England Biolabs
Poly(A) Tailing kit	Invitrogen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
S.O.C. medium	Thermo Fisher
TAE buffer	Apex Biomedical
Ultrapure Ethidium Bromide Solution	Life Technologies
Oocyte collection	
Aspirator tube assembly for calibrated micro-pipettes	Sigma-Aldrich

Calibrated micro-pipettes	Drummond Scientific Company
PMSG- 5000	Mybiosource
PrecisionGlide Needle 26 G x 1/2	BD
Syringe 1 ml	BD
Oocyte micro-injection	
35 mm Dish | No. 0 Coverslip | 20 mm Glass Diameter | Uncoated	MatTek
Borosilicate glass with filament	Sutter Instrument
Oil for Embryo Culture	Irvine Scientific
Petri Dish	Falcon
Tissue Culture Dish	Falcon
VacuTip Holding Capillary	Eppendorf
Software	
Biorender	BioRender
MetaMorph, version 7.8.13.0	Molecular Devices

Catalog Number	Comments/Description
SIAL-A3311	
231085	
12561-056	
M2645	
25-512	
3506-1	
11360-070	
10977-015	
20-102QD	
C1389-1G	
CCK-20	
B7204	
R0611	
N0447S	
R0176	
SM1333	
L1010	
3002-021	
AM1908	
R3589	
AM1344	
M0530	
AM1350	
27106	
28704	
15544034	
20-193	
15585011	
A5177-5EA	

2-000-025
MBS142665
305111
309659

P35G-0-20-C	For time-lapse microscopy
BF100-78-10	
9305	
351006	For micro-injection
353001	For oocyte incubation
5195000036	

Preparation of Figure 1S
For time-lapse microscopy, analysis of 3' UTR translation

Editorial comments:

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.

This has been checked.

2. Figure 1: Please capitalize the L in volume abbreviations: pL instead of pl.

This has been changed.

3. Figure 2: Please include a scale bar and include a space between the number and the unit: 0 h instead of 0h.

This has been changed.

4. Please sort the Materials Table alphabetically by the name of the material.

This has been changed.

5. Please revise the summary to be a complete sentence.

This has been changed: "This protocol describes a reporter assay to study regulation of mRNA translation in single oocytes during in vitro maturation."

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

This has been changed.

7. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

The Table has been removed. We have now included Table 1 which contains this information.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

This has been changed throughout the manuscript.

9. Please specify the sequences of the mRNA and primers used.

We have included Table 2 which contains this information.

10. Please specify all volumes and concentrations used throughout.

This has been checked.

11. Please revise the highlighting to be under 3 pages to ensure that videography can occur in a single day. This must include a one line space between protocol steps and substeps.

The highlighting has been revised.

Reviewers' comments:

Reviewer #1:

Invited methodology manuscript from Conti lab is trying to adapt the method for visualization of exogenously loaded probe to analyze its translation in time using mammalian oocyte model. The technique is essential and important for general scientific field. Unfortunately, the method is based on the outdated techniques which will be hard or even impossible to compare/implement in another lab or could lead to the faulty biological conclusions. The presented data are in questionable quality. Regrettably, the manuscript is clearly not significant piece of science and requires substantial improvement to serve as a solid source for the field.

The reviewer argues that our contribution is not a "significant piece of science". We respectfully disagree. In the past, luciferase-based assays were used to assess translation (Franciosi et al., 2016; Piqué et al.¸2008; Yang et al.¸2017). Although this is a very sensitive method which requires only a few oocytes per sample but still requires averaging behaviors of different cells; conversely time lapse allows measurement in a single oocyte and therefore analysis of different members of a population; More over with luciferase assays, the oocytes need to be lysed to detect the enzyme activity. We have adapted this well-established method by applying time-lapse microscopy to generate kinetic data, as is described in the current manuscript. These kinetic data provides accurate measurements and can be used to define the time when translational activation or repression takes place. This enables precise investigation of changes in translation at different transitions during oocyte maturation. We consider this is a significant improvement over the previously established technique.

Please see also our response to the other comments below.

Please, see my comments below.

* The movie presented here should be ,,cherry on the cake,,. Unfortunately, it is not. Appropriate description of the channels and time points is missing. Additional visualization of major protein and organelle (e.g. histone, spindle) and BF would be beneficial here for the reader. Authors somehow set up 16hrs to obtain mCherry plateau (L189) however movie shows visible increase of mCherry signal during maturation. Sadly this is in contradiction to proposed mCherry plateau and quantification in the Fig.3. Moreover movie related to Fig.6 is not presented.

The reviewer asks for a description of the channels and time points. We have added this to the Figure legend as follows:

"YFP and mCherry time-lapse recordings of a single oocyte injected with mRNAs encoding Ypet/Interleukin 7 3'UTR and polyadenylated mCherry. YFP channel (Ex: S500/20 × 49057; Em: D535/30 m 47281), mCherry channel (Ex: 580/25 × 49829; Em: 632/60 m). The oocytes were recorded every 15 minutes for 16 hours (7 frames/second)."

In order to visualise major protein and organelles the oocytes need to be fixed to perform immunofluorescence. Using the current protocol, reporter accumulation is measured during in vitro oocyte meiotic maturation using time-lapse microscopy, which requires live oocytes. It is therefore not possible to visualise the protein and organelles simultaneously. In addition, we also believe this is beyond the scope of the current protocol as the aim of this protocol is to study mRNA translation of specific targets and not to visualize proteins and organelles.

The reviewer mentions that there is a visible increase of mCherry signal during maturation. As shown in Figure 3, there is a slight increase in mCherry signal from the start to the end of the experiment (183 vs. 145, for the average signal at the end of the experiment and beginning of experiment, respectively). However, we use mCherry as an injection control by using the average signal of the last 10 time-points of the experiment, when mCherry signal is very stable (183.5 vs. 183.4, for the average signal at the last time-point vs. average signal at 10 time-points before end of experiment, respectively). We therefore believe that this small increase in mCherry signal in the beginning of the experiment is not affecting our measurements.

"Major flaw here is significant contradiction between Fig.3 (YFP expression) and Fig.4. How the data were ,,Corrected,,? The "volume" here is measured absolutely improperly (extracted from published literature). Presented methodology approach creates unique microinjection pipette which in combination with "balance pressure" will result in different injection volume which is impossible to repeat by the presented method. To administrate precise injection volume different approach is required (e.g. PMID: 15575609; PMID: 19085140). My additional concern is about the measurement of cRNA concentration (see comments below).

It is true that the exact amount of injected reporter mix is difficult to be determined. This is the reason why we correct for injected volume by dividing the YFP signal for each time-point by the amount of plateaued mCherry signal (average of last 10 time-points). We also show that this correction reduces some of the variability among oocytes (Figures 3 and 4). The same injection system has been used extensively throughout literature (e.g. Dumont et al 2007; Freimer et al., 2018; Gui et al., 2005; Pirino et al., 2009)

Please see below for our response to the reviewer's concern about the measurement of cRNA concentration.

* Method should implement appropriate control to extract conclusion from proposed method. E.g. how experimentally modified 3'UTR translationally behaves in comparison to studied motive?

The aim of the presented manuscript is to explain the protocol and to show some representative results. This is the reason we show accumulation of Ypet/IL7 3'UTR, as IL7 is known to be increasingly translated during oocyte maturation and allows for good visualization of translational activation using this method. In addition, we show an example of repression of translation using Ypet/Oosp2 3'UTR. Indeed, as also mentioned in the Introduction of the manuscript, the described method can also be used to study cis-regulatory elements of the 3'UTR of target mRNAs (e.g. Dai et al., 2019; Luong et al., 2020; Yang et al., 2020a). However, we did not include examples of these results as we consider them to be outside the scope of the current methodological manuscript.

Specific comments:
* Fig.1. Scheme of the Ypet/3UTR construct should be presented. Depiction of polar body is strange.

We thank the reviewer for this suggestion. A scheme of the Ypet/3'UTR construct has been added to Figure 1.

With regard to the polar body, we have used BioRender to create Figure 1 and unfortunately we can not modify the depiction of the polar body. We have included a reference to BioRender in the Figure legend to make this more clear.

* Fig.3. In the Figure legend authors state that "to generate a prophase I-arrested control group" the oocytes were kept in medium with cilostamide, but the heading of the X axis in the "YFP prophase I-arrested" graph claims "Time after cilostamide release (h)".

We have changed the x-axis label in Figures 4 and 6 to "Time after start experiment (h)".

Fig.4 and Fig.6. Similar problem as in Fig.3. The data of both Maturing and Prophase I-arrested groups are plotted in each graph but there is only "Time after cilostamide release (h)" X axis heading.

We have changed the x-axis label in Figures 4 and 6 to "Time after start experiment (h)".

* Fig.4. Each time point needs to be correlated (both channels).

We assume the reviewer means corrected instead of correlated. As explained in the manuscript, we calculate average mCherry expression in the last ten time-points of the recording. Then, for each time-point, we divide the YFP expression by the averaged mCherry expression. To clarify this further, we have adjusted Figure 4 which now shows the YFP/mCherry ratio at each time-point for each individual oocyte as well as the mean±SEM of all oocytes at each time-point.

* Fig.6. Hard to believe to the conclusion presented here based on this experimental approach. It is known that GVs in arrested oocytes clearly show increasing expression of cRNA (e.g. H2b or Tubulin; without experimental polyA or motive addition). Moreover maturing pattern of the construct clearly correlate with mCherry plateau presented in Fig.3. Instead, oocytes should be injected directly in the specific stage where expression will be monitored.

We are not completely sure what the reviewer is asking. We will shortly explain our method again. In the presented protocol we use a Ypet/3'UTR to measure accumulation of the reporter and we use a polyadenylated reporter without a 3'UTR which is plateauing (as explained previously, also see Figure 3) and is used as an injection control. This is an approach that has been extensively used in literature which make use of luciferase to study reporter translation (e.g. Arumagum et al., 2010; Eliscovich et al., 2008; Franciosi et al., 2016; Piqué et al., 2006 and 2008; Yang et al.¸2017). In these studies, polyadenylated Renilla is used as an injection control. We have modified this well-established technique by applying time-lapse microscopy to generate kinetic data in live oocytes during oocyte meiotic maturation. This kinetic data provides accurate measurements and can be used to define the time when translational activation or repression takes place. Since, the exact time is not known before performing the experiment, we injected the oocytes in the GV stage.

* Figs. 3-6. Any experienced investigator noticed variability of fluorescence after injection of oocytes, so I am curious how authors obtain so beautiful data (correlated) in Fig.4 & 6 without variability (error bars)? The data in Fig.3 & 5 are contradictory to presentation of data in Fig.4 & 6.

It is true that there is variability among oocytes. The presented method allows for the study of this variability as we measure individual oocytes. This in contrast to other techniques such as Western Blotting which require a large number of oocytes in each sample. The variability of fluorescence after oocyte injection can be seen in Figure 3. Part of this variability is due to variation in the amount of reporter that was injected and part is due to biological variability. In Figure 4 and 6 we show the corrected YFP signal (YFP/mCherry at plateau), which reduces variability as we remove variation in the amount of reporter that was injected. The biological

variability is still present and can be seen in Figure 4 and 6 by the error bars (SEM). To visualise the error bars more clearly, we have reduced the size of the dots in Figures 4 and 6.

* L127 To relay on the cRNA concentration measured by the NanoDrop is inappropriate. Precise cRNA concertation should be measured accurately by fluorescence spectrometer. Thus concentration mentioned in the paper is most likely far from reality which is also supported by YFP expression profile in YFP maturing oocytes in Fig.3. Thus, adaptation/confirmation of the state of presented method by another lab will be very. difficult

We agree that a fluorescence spectrometer can more accurately measure RNA concentration in samples with a low RNA concentration. However, we measure RNA concentration in our undiluted reporter. In the case of IL7, the RNA concentration was 220 ng/µL which falls well within the detection range of NanoDrop (2 to 3750 ng/µL according to manufacturer's website). Almost every molecular lab has access to a NanoDrop and will be able to measure the RNA concentration of the reporter.

* L186 How authors came to conclusion about injected volume 5-10pl? How this is connected to the L273 "to correct for the injected volume"?

The 5-10 pL is an estimation based on calculations that have been done in literature using a similar system (Kline et al., 2009). We have previously explained how we corrected for injected volume, please see our response above.

* L189 mCherry signal to plateau? Even if I overlook visible differences in mCherry fluorescence at the beginning and end of the presented movie (see above) the 16hr in vitro GV block is detrimental for meiotic/developmental competence. The method should utilize up to date passively loaded probes that are nontoxic to living cells and are available dyes in a range of fluorescent channels to match instrument lamp/lasers/filters to accommodate additional probe(s). Moreover, microinjection of additional cRNA overloads translational machinery which will interfere with protein expression and cell physiology.

As explained previously, there is indeed a small increase in mCherry signal between the beginning and end of the experiment. However, mCherry signal is very stable towards the end of the experiment. As we used the last 10 time-points of the recording for the correction of the YFP signal, we do not expect that this small increase in mCherry influences our results.

It is true that a prolonged GV block may be detrimental for oocyte developmental competence. However, the same approach has been extensively used throughout literature as there is unfortunately no way around it.

The reviewer suggests to use passively loaded probes. It is true that these probes can passively enter the cell and do therefore not require oocyte micro-injection. Therefore, these passively diffusing dyes cannot be used to correct for the volume of injection. Passively loaded probes can also not be used to study translation rates in live oocytes during oocyte meiotic maturation, since their accumulation in the oocytes does not depend on mRNA to protein translation.

We agree that there is a possibility that we overload the translational machinery if we inject too much of the reporter. This is the reason why we only inject a very small amount of the reporter (625 – 1050 x 10E-6 ng). This amount is similar to what is commonly injected in mouse oocytes (e.g. Chen et al., 2011; Dumont et al., 2007; Gui et al., 2005).

* L276-278 and L287-89 Presented approach does not support presented conclusion since cilostamide and mCherry translation might artificially influence IL7 expression. Authors simply do not think about the recovery of cell from long inhibitor treatment.

Please see our response above. In addition to this, we have confirmed that the translation of the IL7 mRNA increases during oocyte maturation using ribosome loading and by measuring the IL7 protein accumulation in the oocyte medium (Cakmak et al., 2016).

* L311-312 The methods of polysome profiling described in the cited papers (Chen et al., 2011; Mašek et al., 2011) do not relate to the method of the genome-wide endogenous mRNA translation study in oocytes. Authors should mention here PMID: 32070012 instead.

We respectfully disagree with the reviewer, as polysome profiling is a widely accepted method to study genome-wide endogenous mRNA translation (as reviewed by Larsson et al., 2013). We have added a reference to this review to the discussion of our manuscript.

* L342 Phototoxicity? What is the exposure time (L224 mention only BF exposure time)? Authors most likely used inappropriate setup which significantly promote GVBD delay. Delay of GVBD could be indication of damage of the cell by inappropriate experimental setup.

We have added YFP and mCherry exposure time to the protocol. We used 150 ms for YFP and 75 ms for mCherry. It is true that there is a delay of GVBD which may be due to the stress induced by micro-injection or fluorescence exposure during the experiment. This is one of the limitations of the technique, as we also explained in the Discussion of our manuscript. However, we can assume that this delay in GVBD is not due to overloading of the translational machinery because it is known that GVBD in the mouse is not dependent on protein translation (Han et al., 2017).

* L360-363 Inappropriate approach suggested, absence of widely available and well know techniques to properly undertake experiments (e.g. cell traction software, specialized plastic chambers, matrix culture -alginate).

The main issue with analysis of cumulus-enclosed oocytes is that the oocytes will not only move in x and/or y position, but may also move in z-position (up or down in the droplet) because the cumulus cells will expand. This may result in an increased number of oocytes that need to be excluded from analysis as mentioned in our manuscript. In the future, cell-tracking software may be used that is able to track x, y and z positions of the oocytes in the droplet, which may solve this issue. We have mentioned this possibility in the discussion.

* L369-370 Idea is not supported by the method presented here.

This sentence has been removed from the manuscript.

* Instead of citing relevant papers authors clearly fall in to self-citation mode.

We apologize if we give the impression that we are referring only to papers of our lab. We have added additional references where required.

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Reviewer #2:

Manuscript Summary:

In the study "Defining the program of maternal mRNA translation during in vitro maturation using a single oocyte reporter assay", Costermans et al demonstrated the details of their protocol for measuring mRNA translation, which is based on microinjection of in vitro-transcribed reporter mRNAs and microscopic analysis of reporter fluorescence. Translational control of maternal mRNAs is crucial for promoting meiosis and embryonic development in almost all animals but is still difficult to be analyzed due to limitations in techniques. This protocol is valuable for the analysis of translational control of mRNAs in oocyte meiosis. I have only several comments for minor points as follows.

We thank the reviewer for the positive evaluation of our manuscript.

Minor Concerns:
- Throughout the manuscript, the fluorescent protein named YPet was used for measuring the translation of mRNA of interest. To clarify the advantage of using YPet, explanations for YPet or the reason why the authors selected this protein should be included.

We have used a Ypet reporter because our current microscope system allows measurement of YFP signal (filterset and settings for YFP). However, it is also possible to use GFP or any other fluorochrome couple with excitation and emission wavelengths that are far enough apart from each other. We have included a schematic representation of the Ypet plasmid in Figure 1.

- This reviewer is not sure how the authors prepared the template DNA for making in vitro-transcribed mRNAs. For YPet-3'UTR reporter mRNA, the authors described as "Use a forward primer upstream of the Ypet sequence and a reverse primer with 20 additional tymine residues (p.4, lines 118-119)". However, to transcribe them by using mMESSAGE mMACHINE T7 Transcription Kit, the sequence of T7 promoter should be included upstream of YPet sequence. In addition, examples of forward and reverse primers would be useful for readers to recapitulate the experiment. As in the case of mCherry template, authors might use a linear plasmid DNA, not a linear "PCR" template (p.4, lines 130-131).

We added a schematic overview of the Ypet plasmid that was used. In addition, we added Table 2 which contains the primer sequences that were used.

- Before microinjection, authors incubated oocytes isolated from ovaries for 2 hours (p.5, line 165). Explanation of the reason should be included.

This 2 hour recovery period is important for oocytes to recover from stress from oocyte isolation. This has been added to the protocol of the manuscript:

"Place the dish in the incubator for 2 hours to let the oocytes recover from the stress induced by isolation of the oocytes from the follicles."

Reviewer #3:
This is a clearly written and complete description of a technique that will be useful to many workers in the field. My only suggestion is optionally) define/explain Ypet at the beginning of the manuscript. It becomes evident later on, so this is a very minot issue.

For the authors' information, most of the figures were not visible in the pdf - they were replaced by solid black boxes. However, the tif files available for download were correct.

We thank the reviewer for the positive evaluation of our manuscript. To explain Ypet at the beginning of the manuscript, we have added a schematic representation of the Ypet plasmid in Figure 1.

Reviewer #4:
Manuscript Summary:
This manuscript describes a method to track translation rates in oocytes as they undergo meiotic maturation. The time-lapse assessment of fluorescence signal provides power lacking in other methods. The protocol is well written with details needed to perform the technique in the reader's lab. Additional uses of the technique has also been described.

Major Concerns:
It was difficult to understand the appropriate interpretation of the data generated. There are points that need clarification for appropriate interpretation. First, I assume that the data in Fig 4 represents the fluorescence detected for IL7, which is actively translated during maturation while Fig 6, you show an example of translational repression. In figure 4 prophase I shows little accumulation of fluorescence but there is linear accumulation of fluorescence in prophase I controls in Fig. 6.

The assumption of the reviewer is correct, Figure 4 represents fluorescence detected for IL7, while Figure 6 shows fluorescence detected for Oosp2, which we included as an example of translational repression. To clarify this issue, we have added IL7 or Oosp2 to the headings of Figures 3, 4, 5 and 6.

It is also not clear how fluorescence levels for constant translation and repressed translation differ. I would suggest a schematic to demonstrate what the different outcomes might be and how to interpret.

The reviewer mentions that it is not clear how fluorescence levels for constant translation and repressed translation differ. This has been clarified in the manuscript by adding the following sentences to the Discussion:

"These data have been re-analyzed and are shown in Figure 6. The maturating oocytes show plateauing of the YFP signal which indicates repression of translation, while the prophase I-arrested control group follows a linear pattern of reporter accumulation which indicates similar translation rates at the beginning and end of the experiment."

A second point is with regards to Time-lapse microscopy; specifically part 3.8. You indicate that exposure for Ypet and mCherry should be adjusted based on the the specific 3'UTR used and batch of reporter, respectively. How specifically is this empirically determined. End point at MII? Measures at the beginning and end of IVM? This would be helpful information to ensure proper imaging.

We determine appropriate exposure time by making sure the YFP signal falls in the center of the range of detection at the start of the experiment (GV). This prevents underestimation of low signals and saturation of high signals in case of extensive activation of translation. This additional information has been added to the protocol of the manuscript:

"The YFP signal should fall in the center of the range of detection at the start of the experiment to prevent underestimation or saturation in case of activation of translation."

Minor Concerns:
There are a few additional minor points:
1. Figures 3-7 show up as black boxes in the pdf and are still very dark when the tif is downloaded. Furthermore, when I tried to open the supplementary video, QuickTime could not play it.

We are sorry to hear that the reviewer was experiencing issues with opening the images and video. We have uploaded the images in jpeg and hope this will solve the problem.

2. Define Ypet as this is not necessarily a commonly known vector. Why is this used instead of GFP. In the discussion you indicate that others used GFP but not timelapse. Is there a reason that you did not perform timelapse imaging with GFP.

We have included a schematic representation of the Ypet plasmid in Figure 1. We have used a Ypet reporter because our current microscope system allows measurement of YFP signal (filterset and settings for YFP). However, it is also possible to use GFP or any other fluorochrome couple with excitation and emission wavelengths that are far enough apart from each other.

3. Line 102 you describe the use of Choo-Choo Cloning kit. You state the instructions were followed except PCR fragments were incubated on ice for 4 hours. What point of the protocol does this occur. Prior to ligation? Instead of a shorter incubation? A little more information would be helpful. Expecially given that the Topo TA cloning was described in detail (lines 104-110) but the description of cloning was sparse.

This has been clarified in the protocol of the manuscript:
"PCR fragments were incubated on ice for 4 hours to facilitate a more efficient recombination process. This is contrary to the manufacturer's instructions which recommend an incubation time of only 45 minutes."

4. Is it possible to perform IVF after the imaging during IVM. This would be a great way to prove that oocytes are developmentally competent in the case of abnormal regulation of translation.

It is true that the translation pattern can be studied during in vitro fertilization. This has been added to the Discussion of the manuscript:

"In our laboratory, we use the described method to study translational regulatory elements of the 3'UTR during in vitro oocyte meiotic maturation. Similarly, this method may also be used to study functional 5'UTR elements essential for regulation of translation or to study translation during in vitro oocyte fertilization."