**Preparation of dsRNA**

Note that all steps should be done with gloves and with RNA free water and solutions.

**PCR gene fragment of interest**

PCR ~500bp fragment from genomic DNA
Resuspend PCR beads in 22 µl of water.
Add 1µl 20 µM forward primer
Add 1µl 20 µM reverse primer
Add 1µl template

PCR (repeat steps 2-4 30x)
1) 94°C 5 min
2) 94°C 1 min
3) 54°C 1 min
4) 72°C 30 sec
5) 72°C 10 min
6) 4°C hold

**Transcription reaction**

Do transcription straight from PCR reaction, no purification required
Use Ambion T7 transcription kit

<table>
<thead>
<tr>
<th>µl</th>
<th>Component</th>
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<tbody>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ATP</td>
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<tr>
<td>2</td>
<td>CTP</td>
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<td>2</td>
<td>GTP</td>
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<tr>
<td>2</td>
<td>UTP</td>
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<tr>
<td>2</td>
<td>10x reaction buffer</td>
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<tr>
<td>2</td>
<td>enzyme mix</td>
</tr>
<tr>
<td>2.5</td>
<td>PCR template (~0.2 µg)</td>
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<tr>
<td>5.5</td>
<td>RNAse free H₂O</td>
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</tbody>
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1) Incubate at 37°C for 5 hours
2) Add 1 µl DNAse, incubate 15 minutes at 37°C
3) Add
   - 0.5M EDTA (0.4 µl)
   - 10 % SDS (0.2 µl)
   - 3 M NaCl (1.6 µl)
4) Put in boiling water and let samples slowly cool down (to anneal RNA strands)
   - to do this, I boil 600 ml of water in a beaker (6 minutes in microwave) and then incubate samples in this on the bench. Takes ~ 3 hours to reach room temperature.
**Purify dsRNA**

1) Transfer RNA to 500 ml tube.
2) Add 20 ml of Phenol/Chloroform/IAA (RNAse free), spin for 1 min @
3) Transfer aqueous layer (top) to new tube an add 20 ml Chloroform, spin for 1 min @
4)Take off aqueous layer (top) and add 2.5 Volumes of Ethanol (RNAse free) and 1/10 volume of NH4Acetate
5) Incubate at -20°C for 30 minutes
6) Spin 15 minutes in cold room, 14000 rpm
7) Wash with 70% Ethanol
6) Dry for 15 minutes and resuspend in 50 µl injection buffer
   For 1 mL of injection buffer:
   240 µl of monobasic sodium phosphate (.2 M)
   260 µl of dibasic sodium phosphate (.2M)
   1.6 µl of KCl (3M)
   500 µl H₂O DEPC
7) Estimate concentration of dsRNA by gel electrophoresis. Aim to get concentration of
   > 5 mg/ml. I usually inject dsRNA at 2-5 mg/ml.

**dsRNA injection into embryos**

Reagents:
RNA (at 5µM)
Embryos
Needles
Coverslips/slides
Filtered H₂O
Halocarbon 27 oil
Halocarbon 700:27 oil (3:1 ratio) for injections

Note: Before getting started, it is best to make sure that your cage of flies is laying very well. The day before I am planning to do the experiment, I flip the cage frequently (every 30 – 60 minutes). This ensures that the flies are used to laying lots of eggs and being flipped often. Also plan on injecting all morning and imaging all afternoon. I would highly recommend not doing anything else the morning of injections.

Experimental setup:

1) Flip the cage first thing in the morning and begin flipping the cage every 30 minutes. This is important. It is best to use apple juice plates with very little yeast to maximize the number of embryos you can recover.
2) Gather H₂O (for control injections), RNA, needles, etc.
3) After you have gathered all reagents and are ready to proceed, wait for the next flip of the cage.
4) Immediately after you have flipped the cage, add oil to the plate and look for blastoderms that have not yet formed pole cells. This is very important. You want to get the youngest embryos possible to maximize the efficiency of your RNA knockdown. I typically aim for 2-4 embryos per slide/injection.

5) Process and orient embryos on glass slide as usual, using embryo glue.

6) Desiccate the embryos using the “desiccation chamber” and Drierite. The timing will vary depending on humidity in the room, outside, etc. A good place to start is 6-7 minutes. It is better, here, to err on the side of under-desiccation or else your embryos will be too wrinkled when imaging. It is also a good idea to do a “practice” desiccation the day before to get an estimate of desiccation time. Identifying a good needle and a proper desiccation time is the most critical part of injections.

   a. Note: During the desiccation step is a good time to load and break needles, preparing them for injection. After breaking the needle, place tip in 3:1 Halocarbon 700:27 oil.

   b. Needle breaking protocol:

      i. Mount embryos with chorions on slide without spacers or coverslip

      ii. Load injection solution into the needle using the extra thin pipette tips (2.5 µl). Make sure solution is as near the tip of the needle as possible, avoid bubbles.

      iii. On injection table: Set pressure to 80 psi and switch to gated injections.

      iv. Lower needle onto embryo so that the long axis of the embryo is parallel to the needle. Compress the embryo slightly.

      v. Open the pressure gate and using the stage controls move the embryo quickly away from the needle. Repeat until bubbles come out of the needle tip.

7) After desiccation, check orientation of embryos again then add 3:1 halocarbon 700:27 oil over embryos on slide.

8) Inject embryo with either H$_2$O/injection buffer or RNA laterally. Note: Typically I try to inject embryos with a 40µm drop diameter (the length of one cell at the end of cellularization). But for RNA injections, you may need to use a bigger drop size for more effective knockdown. A good place to start is to inject until you see clearing in the yolk. Conditions I typically use are 25-30psi with a timing of 30-60ms on “timed”. This will be empirical, but be consistent between embryos. I recommend starting with H$_2$O or control injections first, mainly to optimize desiccation conditions.

9) After injection, remove the oil from the slide by tilting and letting most of the oil run off. Get as much of this oil off as possible because it is more viscous than the typical imaging oil, Halocarbon 27. If you leave injected embryos in too much injection oil, they can start to collapse.

10) Mount the embryos as you normally would, with No. 1.5 spacers, coverslip and Halocarbon 27 oil.

11) Place injected embryos in a safe, dark place (like a drawer) at room temperature.
a. Note: Steps 4-11 should take ~20-25 minutes. Ideally you want to be done with the entire process in time so that you are ready for the next flip of the cage, i.e. every 30 minutes.

12) Typically I try for 6-8 rounds of injections. The first 1-3 will be controls and the rest will be experiments.

13) ~2.5 hours after injection, embryos should be ready to be imaged, but it is best to keep an eye on the slides to see when they may be getting ready. Remember that if you keep the slides at room temperature they will develop a little slower than they would at 25°C.

   a. Note: For example, if you start injections around 9:00am, then you may be ready to start imaging around 11:30am or 12pm. The next injection should be around 9:30am, and imaging at 12pm or 12:30 and so on.