Chimera Production by 8 cell injection

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There are a number of options for generating chimeras from ES cells but the principle method by far remains blastocyst injection. The result of a blastocyst injection will be chimeric animals with variable ES cell contribution. It would be hoped that ES cell contribution is sufficient to enable germline transmission to result and with transmission rates sufficient to enable heterozygote offspring to be obtained from 1st litters so that both the time and the numbers of mice generated to achieve that milestone are low.

It remains a challenge to achieve good and reliable results particularly with C57BL6 ES cells where greater variation in outcomes is likely.

Alternatives to blastocysts injection are the aggregation techniques (1), either involving diploid or tetraploid embryos, a smaller amount of work has assessed the injection of 8 cell, 4 cell or morulla stage embryos.

The aggregation techniques have the advantage that microinjection is not required. It is of interest that whereas blastocyst injection has frequently been successful with the use of 129 ES cells injected into inbred blastocysts, aggregation that employs morulla stage embryos routinely requires that hybrid or outbred embryos are used. The use of inbred embryos results in reduced birth rates and can lead to the birth of chimeras with very high ES cells contributions with an increased incidence of runts or early death.

In the case of tetraploid aggregations, the host embryo is not able to significantly contribute to the embryo and so this method is used to produce completely ES cell derived mice, mice that can be termed F0 and bred as heterozygote mice. Inbred ES cell lines are less able to generate these ES cell derived mice and so in a routine setting this method employs the use of hybrid background ES cell lines, for example 129 x C57BL6. These cells are more robust, in the tetraploid system leading to reliable generation of healthy ES cell derived mice.

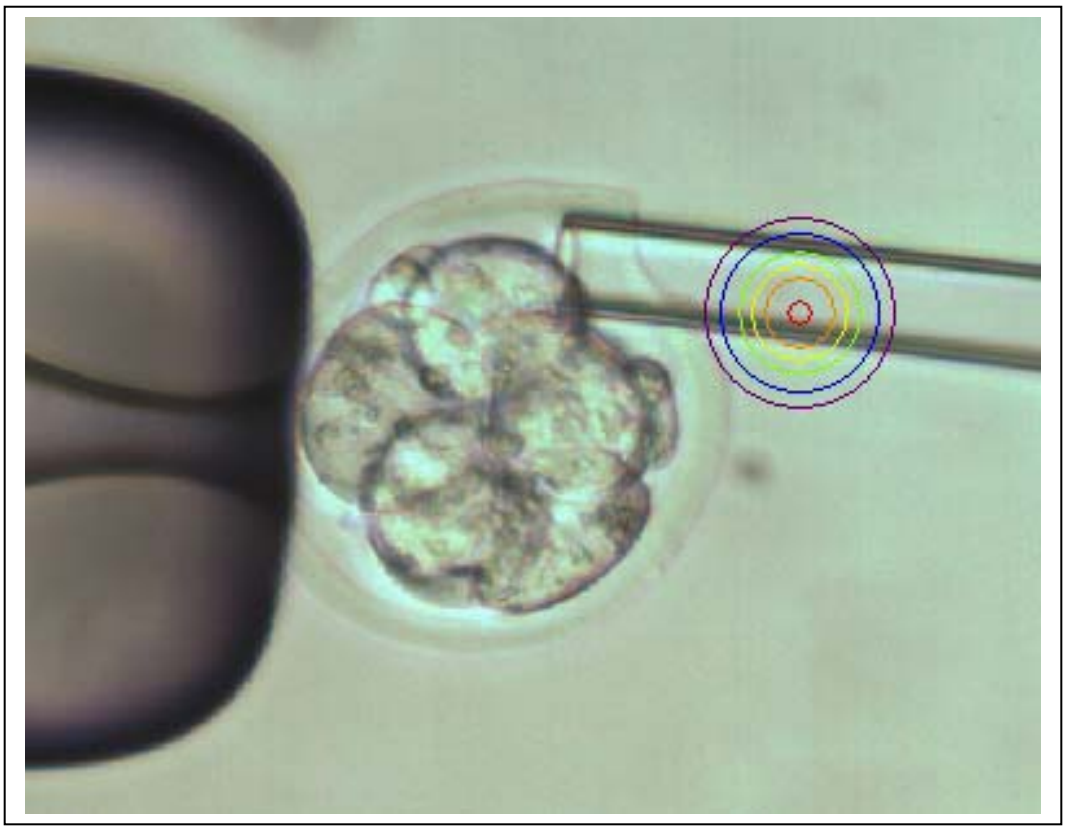
Interest in the injection of pre blastocyst stage embryos was reawakened in 2007 with a publication in 2007 from the US company Regeneron (2). There methodology generated fully ES cell derived mice by an easier, more practical means from a variety of ES cell backgrounds. There had been publication outlining results of such injections (3,4,5,6,7). In summary, there was the possibility that contribution could be skewed to the ES cell over the host but that some care was required in the injection of earlier stage embryos for reasonable birth rates to result.

The Regeneron paper made use of the XYClone laser to hole the zona pellucida and further work in response to this publication has shown successful generation of ES cell derived mice through the use of peizo injection (7) or through the use of standard bevelled needles (8).

In my own laboratory we have generated ES cell derived mice using the laser and bevelled needles with similar success. We do find the XYClone laser to be the easiest means to carry out an 8 cell injection with minimal disturbance to the embryo. The laser is also used in our blastocyst injection sessions, where the use of a blunt ended needle in combination with the XYClone laser enables even the most overexpanded (hatched) or underexpanded blastocyst to be injected with ease and success.

The 8 cell embryo injection is done in a similar way to blastocyst injection. The media and cells for example can be exactly as would be used with blastocyst injection. The differences are that the embryo is injected at I-o clock (on the ‘embryo clockface’ rather than at 3-o-clock. This avoids the

injection pipette touching the blastomeres. The ES cells can be dropped towards the back of the embryo, away from the hole in the zona. Normally the injections are a little quicker than blastocyst injection.



Normally we inject fewer cells, 4 rather than 10 – 15 as with our blastocyst injections. We use embryos from superovulated C57BL6 mice rather than naturally mated, these are from mice housed on a 5am (on) 7pm (off) light cycle, with injection times at midday. Staging of the embryos may be important, we see a lot of variation from session to session in the staging of embryos collected. While it has been a preference to inject pre compaction 8 cell embryos this has not always been possible. The 8 cell Injection of compacted and non compacted embryos generated under this regime have produced good results but we can't yet ascribe a level of significance to the relative success rate.

We inject 60 – 100 8 cell embryos rather than 36 – 48 blastocysts.

Injected embryos are cultured overnight in KSOM (supplemented with 10%FCS) before transfer into the uterus of a day 2.5dpc foster mouse or into the oviduct of a 0.5 dpc foster mouse.

18 – 20 blastocysts are transferred, bilaterally, into each foster mouse.

Initially 3 parental, early passage 129 ES cell lines of established track record were injected. All resulted in either no births or very low birth numbers. These were either wild type or in a single case resulted in 2 rather poor (non transmitting) chimeras. This was at odds with the results outlined in the Regeneron publication and may support the argument that some ES cell lines are significantly more successful in 8 cell injection than others.

We next injected a hybrid (129 x B6) parental line and immediately obtained fully agouti, male offspring. This prompted us to carry out a fuller comparison with blastocyst injection using the constant influx of targeted clones

A summary of the injection of targeted ES cell clones generated from a hybrid ES cell line, by 8 cell or blastocyst microinjection into C57BL6J embryos

Background	Transferred	Born (%)	Chimera (%)	Male (%)	% Mean contribution
129B6 (B/C)	3936	675 (17)	156 (23)	140 (90)	89
129B6 (8 cell)	4613	350 (8)	62 (18)	57 (92)	99

The results show that the 8 cell method is an effective means to generate chimeras. Perhaps more strikingly the table shows the advantage of the use of hybrid ES cells. Even in blastocyst injection the cells produce offspring with a contribution that is skewed to the ES cells so that, in comparision to a variety of 129 ES cell lines the proportion of 100% coat colour chimeras is far higher. The 8 cell method can build on the skew of the hybrid ES cell contribution so that the proportion of 100% coat colour chimeras is even higher. The other point to note is that the greater contributions are at the expense of birth rate. The ES cells we employ are generated in house and cultured according to our own protocols and would accept that culture may not yet be as optimal as is achieved in other laboratories.

We do however have results from 8 cell injection which are a practical means for routine chimera generation. We have found that all resulting 100% chimeric male mice transmit at the expected rate (for an F0 or heterozygote mouse) of 50%, so that even a birth of 1 such mouse can be sufficient. The agouti offspring are now routinely ear snipped and confirmation of genotype at this stage taken as confirmation. In side by side comparison we have found that the clones that produce agouti offspring from 8 cell injection are the same clones that will generate good chimeras and will readily transmit. Clones that aren't up to the job in blastocyst injection are as unlikely to be successful in 8 cell injection.

While the results above, achieved with hybrid ES cells, represent successful 8 cell injection the case for 8 cell injection isn't as strong as it might be. Blastocyst injection too reliably generates good numbers of agouti, 100% chimeras from the same clones.
We are also generating chimeras from C57Bl6 ES cells and in this case the results highlight a clearer advantage to 8 cell injection.

A summary of the injection of targeted ES cell clones generated from a C57BL6(N) ES cell line, by 8 cell or blastocyst microinjection

Background	Transferred	Born (%)	Chimera (%)	Male (%)	% Mean contribution
C57BL6 (B/C)	1012	222 (22)	40 (18)	38 (95)	48 (Balb/c)
C57BL6 (8 cell)	989	141 (14)	21 (15)	14 (67)	68 (CD1)

As in the Regeneron publication we found that the injection of outbred or hybrid embryos results in chimeric as well as F0 mice being born. This is in contrast to the injection of inbred embryos where the birth of F0 mice is far more likely.

For routine 8 cell injection we chose to use readily obtainable CD1 8 cell embryos. In blastocyst injection C57BL6 ES cells unlike hybrid ES cells are unlikely to produce high contributions to the chimeras. Here a skew to ES cell contribution from the 8 cell injection is highly advantageous for increasing the extent and likelihood of germline transmission. Strikingly we have generated completely black mice from the injection of CD1 (albino) embryos that readily transmitted. Again birth rates have suffered but unlike aggregation offspring with such high contributions such offspring have been robust and healthy with no incidence of runt animals. We are currently comparing injection outcome across a large number of albino C57BL6 8 cell embryo injection with the expectation that the incidence of F0 animals will be higher but possibly with further reduced birth rate. Tailoring the choice of method to the cell line and host embryo seems important for each cell line rather than the choice of host embryo being determined solely by the genetic background of the ES cell line.

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