Same Day, Cost-Effective Aneuploidy Detection with Agilent Oligonucleotide array CGH and MDA Single Cell Amplification Method

Presenter: Dr. Ali Hellani, Founder, Viafet Genomic Center, Dubai

Wednesday, March 19th, 2014
Content of the presentation

Introducing a new protocol of aCGH on single cell

Advantages of the new protocol compared to Agilent old protocol

Advantages of the new protocol compared to non-Agilent platforms

Eventual applications of the new protocol
Introducing a new protocol of aCGH on single cell
Single cells are collected at day 5 of embryo development.
Rational behind using day 5 biopsy

- Scott et al., 2013 showed that day 3 biopsy lower the pregnancy test
- Trophectoderm biopsy represents much lower mosaicism rate than day 3 Diagnosis of more than single cells makes the testing more accurate
1st Take home message

- **Blastomere (n=101)**
  - Positive Predictive Value (CCS euploid and sustained implantation): 29.2%
  - Negative Predictive Value (CCS aneuploid and failed implantation): 98.1%
- **Trophectoderm (n=131)**
  - Positive Predictive Value (CCS euploid and sustained implantation): 48.2%
  - Negative Predictive Value (CCS aneuploid and failed implantation): 93.5%

P = 0.0016

Note: NS = Not Significant
Pre-implantation Genetic Screening (PGS)

PGS has been suggested and used to improve pregnancy rates for the following indications:
Advanced maternal age
Repeated IVF failure
Repeated miscarriage
Testicular sperm extraction
PGS and chromosomal abnormalities

Although, improvements in IVF outcome after PGS have been observed in multiple case-controlled studies (Munne et al., 2007a; Munne et al. 2007b) its effectiveness in randomized controlled studies is still limited.
Aneuploidy screening

Trisomic and monosomic embryos account for more than 10% of human pregnancies (this percentage might reach up to 50% in advanced maternal age). This percentage is much higher in embryos resulted from IVF cycles.

Aneuploidy is the main cause of recurrent IVF failure (RIF), and recurrent abortion (RA).

2nd Take home message
Aneuploidy screening in embryonic cells

Mastenbroek et al., 2007 reported negative impact of Pre-implantation Genetic Screening (PGS) in Advanced Maternal Age. Many randomized controlled trials (RCT) confirmed this report.

The suggested reasons were:
- Presence of mosaicism in preimplantation embryos, such that a single cell was not representative of the whole embryo.

- Possibility of self-correction of aneuploidies during preimplantation development.
Conversely, some groups argued that most of these studies suffered from:

- Important methodological flaws (Most of the studies were using FISH as the chromosomal aneuploidy screening technique)
- Improper chromosome selection for analysis
- Absence of “no result rescue” in cases of doubtful diagnosis
- High percentage of non-informative embryos after biopsy
- Large and surprising increases in miscarriage rate after PGS
- Minimum number of blastomeres forming the embryo at the time of biopsy
- Harmful biopsy procedure and Culture media selection issues.
Although the benefits of PGS with FISH have been controversial, with optimized embryo culture systems, appropriate patient inclusion criteria, proper biopsy technique and more comprehensive genetic tests, there could still be a place for PGS in an IVF setting.
Aneuploidy screening on embryonic cells is of major importance on the Success of IVF trails if:
- Done properly by choosing the biopsy time
- Done properly by choosing the best tool for the chromosomal abnormality detection (high Resolution testing of the 24 chromosomes)
6:30 hours aCGH protocol on single cell

The new protocol consists of
- Lysing single cells
- Amplifying single cells by MDA
- Purifying and quantification MDA product
- Labeling with CY3-CY5
- Hybridization then reading using CytoGenomics software
Before start lysing, thaw as many RB as needed (one aliquot for 5 different single cell tubes)

When the lysing reaction is over, thaw as many aliquot of phi 29 as needed (one aliquot enough For 5 reactions). Add the content to the RB aliquot, mix by pipetting then add 16 ul of the Solution To the lysed cells. Flick the tube, brief spin then run MDA reaction for 70min on 31C. Inactivate the enzyme by incubating 10min at 65C.
First discovered by Dean et al., 2002 where it was used for very low DNA quantities in clinical samples.

In MDA, DNA is amplified isothermally by using Φ29 enzyme.
The MDA product was found to be favored over other PCR-based WGA techniques regarding bias amplification, reproducibility and diagnosis (Hosono et al., 2003)

We were the first (Hellani et al., 2004) to apply MDA in single cell
Multiple displacement amplification (MDA) is a non-PCR based DNA amplification technique. This method can rapidly amplify minute amount of DNA samples to reasonable quantity for genomic analysis. This method has been currently actively used in whole genome Amplification (WGA) and has become a promising method to be applied in single cell genome sequencing and sequencing based genetic studies.
MDA is the golden technique for single cell amplification

Since 2004 MDA became the golden technique used in Single cell

Single cell amplification technique became much easier and standardized

MDA is the success behind the application of aCGH and next generation sequencing on single cell
Single cell lysing

- Lysing protocol used is alkaline based lysing where the reagents are supplemented with the MDA kit (Qiagen)
  - Lysing protocol is the same like the kit insert with slight modifications
    - Cells are loaded in 3ul Global total media and not PBS
    - Lysing master mix is the same
    - Time of lysing is the same
    - Stop solution is the same
    - Cells are kept on cold racks until running the MDA.
      It is recommended to run the samples as soon as the stop solution is added.
Multiple displacement amplification
MDA

Prepare the master mix as described below

- Add 216 ul of DNA free water on the REPLI-g Reaction buffer (yellow cap)
- Vortex, spin and make aliquots of 80ul each (11 aliquots). Store at -70 C for long storage or -20 for short storage (up to 3 months). Label it RB for reaction buffer
- Aliquot the phi29 enzyme as 4.5ul per aliquot (aliquoting is done on -20C cold rack) keep the aliquots at -70C for long storage or -20C for up to 3 months storage
Purification and quantification of the MDA product

After MDA, the product is purified using GE-heathcare PCR purification kit

Follow the insert step, elute your purified DNA with 22ul of buffer type 4

Quantification is done on Nanodrop 2000 or Nanodrop 8000 for high throughput labs

Expected concentration is 80-100 ng/ul. Normal control is expected to yield up 150 ng/ul.

The concentration used in the aCGH is 50ng/ul.
Labelling

Labelling kit components are aliquoted as following:
- Add the content of 10x dNTP to the 5x buffer
- Split the solution into 2x425 ul
- add 85ul of Cy3 to the first half and 85ul of Cy5 to the second half
- aliquot 80ul of Cy3 and Cy5 (6 tubes each)
Labelling step is as following:
- 50ng/ul to be used with 2.5ul Random Priming
- denaturation at 96C for 3 min
- Add 4 ul of Klenow enzyme per aliquot of Cy3
- Add 4ul of Klenow enzyme per aliquot of Cy5
- Mix and add Cy3 (blue) and Cy5 (red) to your samples.
- always run one tube normal female (Cy3) and one tube normal male (Cy5)
- Spin and incubate at 37C 30min followed by 10min at 65C
Labelling (cont)

Mix both Cy3 and Cy5 and purify the labelling product on ProbeQuant G-50 Columns (GE-healthcare).
Mix 15ul of the Cy3/Cy5, 10ul of cot1/BA10x and 25 ul of the 2x hybridization Buffer. Put on PCR machine and run the following protocol: 2min 96C then 15min 37C.
Load the mix on 8x60 gasket, add the 8x60k arrays, assembly and put in The oven for 1:30min.
After hybridization, wash the arrays as per the application protocol.
Scan on 3um, 16bit image than run the CytoGenomics software.
Hybridization

Agilent 8x60K CGH microarray

Table:

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<th>Sample 3</th>
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<tr>
<td>Sample 10</td>
<td>Sample 12</td>
<td>Sample 14</td>
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Data analysis with Agilent CytoGenomics software

Step 1 Import Samples
Data analysis with Agilent CytoGenomics software (cont)

Step 1 Import Samples:
Choose Reference
Step 1 Import Samples: Slide Lay-out

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<th>Array 1_4</th>
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Apply configuration to all same pack images

Finish Cancel
Data analysis with Agilent CytoGenomics software (cont)

Step 2 Describe Samples

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</tbody>
</table>
Data analysis with Agilent CytoGenomics software (cont)

Step 3 Run Analysis

Run summary

View a summary of your samples and the analysis method that will be applied. Assign a job name and description and click Run Analysis.

Job Name: Job_14Mar2014_17.49.29

Job Description: Job_14Mar2014_17.49.29
Please write the job description here.

Selected Sample(s): 3417-E3_Vs_Female_252192423525_1_1_A
3417-E3_Vs_Male_252192423525_1_1_A
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3417-E6_Vs_Male_252192423525_1_4_A
3419-E14_Vs_Female_252192423525_1_4_B

Analysis Method: Single Cell Analysis Method - CGH v1

[Run Analysis button highlighted]
Cells are loaded

Lysing and MDA (1:45 hours)

Checking MDA product and quantification (30min)

Labelling, purification, and hybridization (2:30 hours)

Washing, scanning, data analysis and releasing reports (1 hour)

Total of 5:45 hours and the result is released
Per batch of 14 samples
Chromosome 19 loss in day 5 blastocysts hybridized for 2 hours analyzed against the male reference sample (blue) and the female reference sample (pink) identified by Agilent CytoGenomics software 2.9 (beta)
Chromosome 21 gain in day 5 blastocysts hybridized for 6 hours analyzed against the male reference sample (blue) and the female reference sample (pink) identified by Agilent CytoGenomics software 2.9 (beta)
Agilent platform is useful for single cell analysis because:

- Reproducible with no inter-experiment variation
- Software (Cytogenomics) is easy to use with many features that help Releasing and tracking the result
- The result is achieved after 5:45 hours (per 14 embryos) using very high resolution slides (8x60k)
Content of the presentation

Introducing a new protocol of aCGH on single cell

Advantages of the new protocol compared to Agilent old protocol

Advantages of the new protocol compared to non-Agilent platforms

Eventual applications of the new protocol
Advantage of the new protocol compared to Agilent old protocol

- The new protocol is much shorter
  - Important for time-sensitive applications
  - Enables laboratories to run analysis on single cells within the same day
  - Enables laboratories to use 5 day embryos while avoiding embryo freezing

- The new protocol uses MDA
  - Opens up the possible applications to use Next Generation Sequencing in single cell analysis
Introducing a new protocol of aCGH on single cell

Advantages of the new protocol compared to Agilent old protocol

Advantages of the new protocol compared to non-Agilent platforms

Eventual applications of the new protocol
Advantages of the new protocol compared to non-Agilent platforms

- Other platform uses BAC-ARRAY with significantly lower resolution
- New Agilent protocol is cost effective especially with the running of 14 samples per run
- Shorter running time (half the time needed to achieve much more samples)
Content of the presentation

Introducing a new protocol of aCGH on single cell

Advantages of the new protocol compared to Agilent old protocol

Advantages of the new protocol compared to non-Agilent platforms

Eventual applications of the new protocol
Potential applicability of the new protocol
Aneuploidy screening

Future PGS/PGD (Pre-implantation Genetic Screening/Diagnosis) applications
- Aneuploidy screening for 5 day embryos
- Studies are showing that day 5 biopsy are the safest with the best outcome for the embryos.
- Most importantly the new protocol allows the clinics to transfer the embryos diagnosed on day 5 the same day avoiding the hassle of freezing and transferring embryos after a second frozen embryo transfer cycle

4th Take home message
Potential applicability of the new protocol
Next Generation Sequencing (NGS)

Single cell analysis by NGS

There is a drastic increase in NGS applications. Analysis of single cells is the next target for NGS.

Agilent new protocol presented here can be the protocol of choice where all the other protocols failed.

5th Take home message
Take home messages

1st take home message: Day 5 biopsy is better than day 3
2nd take home message: Aneuploidy is the main cause of
    - Recurrent abortion
    - Recurrent IVF failures
3rd take home message: Testing of the whole set of chromosomes using high resolution microarray testing
4th take home message: biopsy and transfer the same day is of extreme high importance for many reasons: more success, less hassle for the laboratories, clinics and essentially patients
5th take home message: when you choose a protocol, always choose the one that keep doors widely open for improvement.

What ever protocol that take into consideration these 5 criteria is the best For the field of PGS/PGD
Special thanks

I would like to thank Agilent team especially:
Alessandro Borsatti
Yann Filaudeau
Iman Kishawi
Anniek De Witte
Andreas Polten
Thank you