PRE-CONGRESS COURSE 2

SIG Embryology
"The human IVF lab in 2008 and beyond"

CONTENTS

PRE-CONGRESS COURSE 2 - PROGRAMME

SIG Embryology

The human IVF lab in 2008 and beyond

Course co-ordinators: E. Van den Abbeel (B), K. Lundin (S), C. Magli (I), D. Royere (F)

Course description: This basic course aims at discussing (1) IVF laboratory safety and quality issues and (2) developments in embryology and IVF technology.

Target audience: Clinical embryologists and clinicians

Programme

09.00 – 09.30	Quality in the IVF lab: how to do? -***P. Kastrop (NL)***
09.30 – 09.45	*Discussion*
09.45 – 10.15	Errors in the IVF lab: how not to do? -***S. Ziebe (DK)***
10.15 – 10.30	*Discussion*
10.30 – 11.00	***Coffee break***
11.00 – 11.30	European cell and tissue directives now implemented: What are the practical implications in the IVF lab? -***J. Van der Elst (B)***
11.30 – 11.45	*Discussion*
11.45 – 12.15	The importance of collection and registration of laboratory data. -***K. Erb (DK)***
12.15 – 12.30	*Discussion*
12.30 – 13.30	***Lunch***
13.30 – 14.00	Automation of embryo selection and production - ***J. Thompson (Aus)***
14.00 – 14.15	*Discussion*
14.15 – 14.45	Non-invasive embryo assessment: proteomic and metabolomic biomarkers - ***P. Nagy (USA)***
14.45 – 15.00	*Discussion*
15.00 – 15.30	***Coffee break***
15.30 – 16.00	An update on embryo culture for human ART: media, performance and safety. - ***T. Pool (USA)***
16.00 – 16.15	*Discussion*
16.15 – 16.45	Safe and efficient vitrification methods for human oocytes: how to do it? -***L. Rienzi (I)***
16.45 – 17.00	*Discussion*
17.00 – 18.00	Special Interest Group Embryology Business meeting

Quality in the IVF lab: how to do?

Peter Kastrop, Ph.D.
Clinical embryologist

University Medical Center Utrecht

ESHRE Barcelona 6 July 2008

Content

- Quality
- Quality management
- Performance indicators
- Standards / Guidelines
- How to do

Quality

Invisible when GOOD, impossible to ignore when BAD

People forget how fast you did a job but they remember how well you did it

Howard W Newton

Quality is never an accident; it is always the result of intelligent efforts

John Ruskin

Quality is never an accident; it is always the result of high intention, sincere effort, intelligent direction and skilful execution; it represents the wise choice of many alternatives

William A. Foster

Quality

ISO definition:

the total sum of properties and characteristics of a product, process, or service which is vital in order to meet the requirements as determined or assumed needs

expectation / trust / satisfaction

Quality in the IVF laboratory

- product / service
- end product / endpoint
- measurable
- manageable
- expectations of the patients

Quality in the IVF laboratory

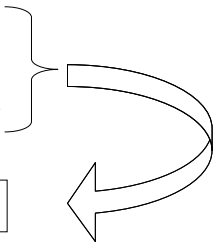
- highest level of patients care
- highest success rates

Quality management

- Quality Control (QC):
  the operational techniques and activities which are carried out in order to meet the quality requirements
- Quality Assurance (QA):
  the total sum of all planned and systematic activities required in order to establish sufficient trust that a product or service meets the quality requirements as determined
  ISO definitions
- Quality Improvement

Quality Improvement

- Risk management
- Quality assessments
- Problem management

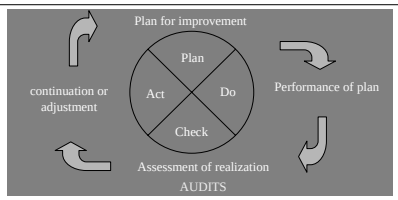


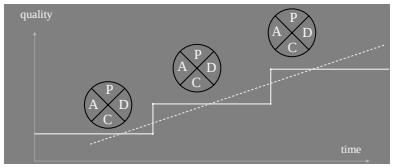
PDCA cycle

Plan – Do – Check- Act

Quality Improvement

PDCA cycle





Risk management

- Risk analysis
  - What are critical steps?
- Corrective activities
  - What to do when something went wrong?
- Preventive activities:
  - What to do to prevent that something goes wrong?

Risk management

Critical steps:

- all actions in which samples are transferred from one dish or tube to another
- all moments where samples of different origin come close to each other
- all transfers of samples to or from physicians or patients

Quality assessments

- Internal audit
- External audit
- Management Review
- Inter professional assessment

Quality assessments

Internal audit

- Quality manager and/or qualified personnel
- Defined and documented standard procedure
- Periodically and systematically
- Non-conformities (deficiencies / deviations)
- Preventive and corrective measures
- Inter / External quality assurance programs

Quality assessments

External audit

- Independent external body
- National / International standard
- Periodically and systematically
- Non-conformities (deficiencies / deviations)
- Preventive and corrective measures
- Internal / External quality assurance programs

> Formal recognition (certification / accreditation)

Quality assessments

Management Review

- Management
- Annually
- Suitability and effectiveness QMS
- Outcome of internal / external assessments
- Non-conformities and complaints
- Preventive and corrective measures
- Performance measures / quality indicators
- Patient satisfaction assessments
- Continuous improvement results

Quality assessments

Inter Professional assessment

- Independent (external) colleagues
- Defined and documented regulation
- Periodically
- Professional competence

Problem management

Problem ⟹ Solve / Correct / Prevent

- Accident
- Complaint
- Defect
- Deficiency
- Deviation
- Error
- Failure
- Incident
- Infection
- Mistake
- Non-conformity

Problem management

- To minimise the number and severity

- To reduce the adverse impact

- To improve quality

Premise: What can we learn from it?

NOT: Who is to blame?

Performance indicators

- Operational
  - Equipment
  - Personnel
  - Methods (IQC and EQA programmes)
- Outcome
  - Methods
  - Programme
  - “Problems”
- Financial

Performance indicators

IVF / ICSI / PGD / IUI / Cryopreservation (embryos / sperm)

Methods	Programme
– Fertilisation rate (2PN, 1PN, 3PN, degen.)	– Oocyte scoring rate
– Damage rate	– Implantation rate
– Cleavage rate	– Biochemical pregnancy rate
– Fragmentation rate	– Clinical pregnancy rate
– Embryo scoring rate	– Multiple rate
– Embryo survival rate	– Abortion rate
– Sperm recovery rate	– Live births
–	–

Standards / Guidelines

- by professional societies
  ESHRE, ACE, AFS
- (Inter)national standards
  ISO, ACHS, CCHSA, CPA, CCKL
- Quality management models
  TQM, EFQM

Standards / Guidelines

- Guidelines for human embryology and andrology laboratories
  - The American Fertility Society , 1992
- Guidelines for good practice in IVF laboratories
  - ESHRE 2000 / 2008?

  http://www.eshre.com
- Accreditation standards and guidelines for IVF laboratories
  - Association of Clinical Embryologists, 1999

  http://www.embryologists.org.uk/downloads/ACCREDITATION_STANDARDS_AND_GUIDELINES.doc

Standards / Guidelines

- ISO 9001: (2000)
  Quality management systems – Requirements
- ISO 17025: (1999)
  General requirements for the competence of testing and calibration laboratories
- ISO 15189: (2007)
  Medical laboratories – Particular requirements for quality and competence

Standards / Guidelines

- Total Quality Management (TQM)
- EFQM Excellence Model

  (European Foundation for Quality Management)

- Strategic way of quality management (philosophy)
- embedded within whole organization
- total commitment of entire organization
- strive of continuous improvement
- continuous scrutiny of all components of the quality system
- assessments and audits (periodically and systematically)

How to do

Quality Control

- detailed written standard procedures
- procedure, safety and policy manuals
- appropriately educated and trained personnel
- correct operation and calibration of instruments
- consistent and proper execution of appropriate techniques and methods
- documentation and record keeping
- system for patient sample collection and management
- system for the appraisal of performance and correction of deficiencies

How to do

Quality Assurance

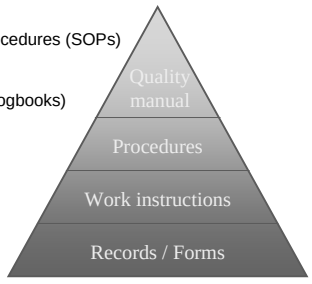
- system for unambiguous patient and patient sample identification
- ongoing method of assessing staff competency in terms of their clinical and clerical skills
- monitoring and evaluation of number and type of accidents, mistakes and deviations
- system for addressing and documenting complaints
- system for the implementation of advances and improvements
- application of apparent laboratory performance indicators

How to do

Documentation

- Quality manual
- Detailed written standard procedures (SOPs)
- Working instructions
- Laboratory forms / records (logbooks)

Organization
↓
Process
↓
Product



How to do

Additional documents

- Job descriptions and curricula vitae
- Training programmes
- (Annual) reports
- Management review

Document control System

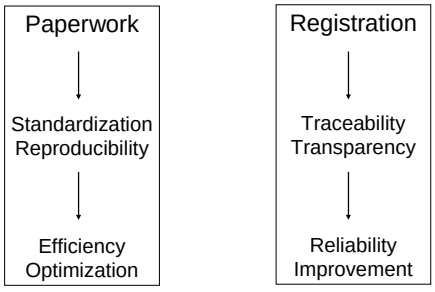
How to do

Requirements

In daily practice:

- separated working places
- recording of every action performed
- verification of critical steps by a second person
- at least 2 persons in the weekend
- minimize unrest
- prevent diminished concentration

How to do



How to do

How to do

Principle:

Say what you do

do what you say

and

show that you do as you say

Errors in the IVF lab:

How not to do?

Søren Ziebe

Errors in the IVF lab:

How not to do!

Søren Ziebe

– *Examples from real life….*

Errors in the IVF lab:

How not to do – anymore!

Søren Ziebe

– *Examples from real life….*

Errors in the IVF lab:

What not to accept – anymore!

Søren Ziebe

– *Examples from real life….*

Errors in the IVF lab:

How not to do?

Søren Ziebe

What is an error?

Don't mix up biological variation and errors just because money and emotions is involved!

This is not an error

- it is just irritating.....

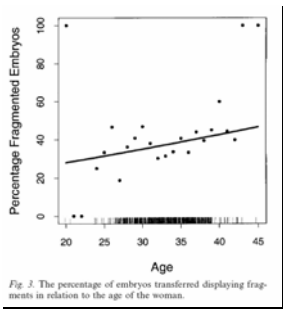


Fig. 3. The percentage of embryos transferred displaying fragments in relation to the age of the woman.

– *Examples from real life….*

This is not an error

- it is just irritating.....

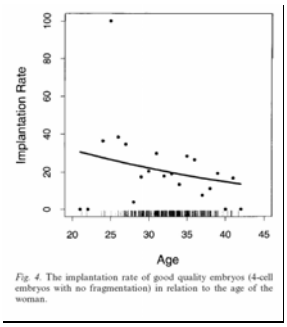


Fig. 4. The implantation rate of good quality embryos (4-cell embryos with no fragmentation) in relation to the age of the woman.

– *Examples from real life….*

This is an error:

– *Examples from real life….*

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– *Examples from real life….*

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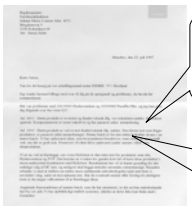


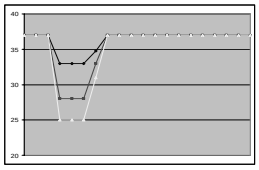
The Hyaluronidase:
*"This product has been re-testet and was found **toxic** according to our toxicity index…."*

The ICSI Oil:
*"This product has been re-testet and was found **toxic** according to our toxicity index…."*

– *Examples from real life….*

The gray zone:

Spindles disassembled after cooling

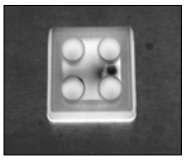


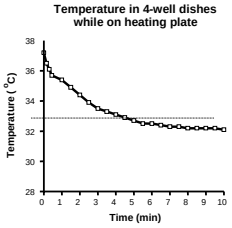
Temperature	Re-assemply
33 degrees	100%
28 degrees	40%
25 degrees	0%

Spindle re-assemply when oocytes were cooled and kept at 33, 28 or 25°C for 10 min

Wang et al. Human Reproduction, Vol. 16, No. 11, 2374-2378, 2001

The gray zone:





Ziebe, Unpublished

Communication

In the old days:
A professional life isolated in the laboratory

Modern ART treatment:
The laboratory are in close contact with the patients

– *Examples from real life….*

Communication to the patients

"Remember – if the sperm sample is more than

60.29 minutes

old you will never have a baby !!!"

– *Examples from real life….*

Consequence….

This car was stopped by the police due to strange driving.....

The couple in the car were trying to produce a sperm sample while driving to the fertility clinic.....

Fik taget sædprøve under bilkørsel

Politiet troede, der var tale om promillekørsel, da de så en bil slingre lidt fra side til side.
Men det var det ikke. Slet ikke!

Da politibilen kom nærmere kunne betjentene konstatere, at der sad en mand bag rattet, og ved siden af en kvinde, der åbenbart havde travlt med gearstangen.
Men det var nu ikke gearet, hun skiftede. Det var nærmest i vejen. For da politibetjente lidt udenfor Førde i Norge, fik stoppet bilen gjorde de... ...store øjne.
Kvinden i vognen havde såmænd taget en sædprøve på manden, hvilket var årsag til hans slingre kørsel. Begge var ædru.
Og forklaringen: Ægteparret, for det var det, var på vej til Førde Centralsygehus, skriver VG Nett, for at aflevere en dugfrisk sædprøve. Og hvad er mere frisk end noget, der netop er leveret ned i glasset.

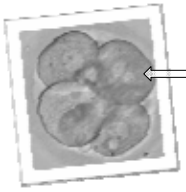
Politifolkene kunne ved selvsyn konstatere, at ægteparret talte sandt, og parret slap for en bøde, men fik dog at vide, at fremover, hvis det blev aktuelt igen, skulle de stoppe bilen, før de tog prøven.
haj@bt.dk

Lagt på www.bt.dk den **30. marts 2006 kl. 17:30**

– *Examples from real life….*

You need to know your biology!

When we are getting desperate....



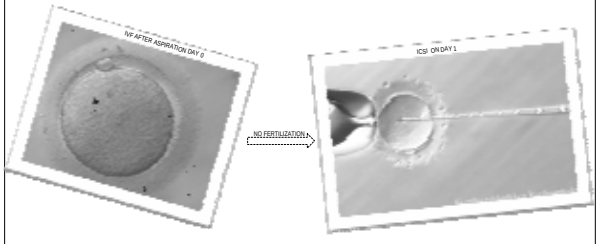
This is <u>not</u> a cell who has duplicated its DNA as part of the cleavage process

– *Examples from real life….*

You need to know your biology!

When we are getting desperate....

Rescue ICSI



– *Examples from real life….*

You need to know your biology!

When we are getting desperate....

Maturing hCG exposed GV and MI oocyte

Table 3 Maturation, fertilization and embryo development

	Control (n = 123)	
	GV	MI
No of oocytes	88	35
Maturation to MII at 4 h (%)	3 (3)	12 (34)
Maturation to MII at 26/48 h (%)[a]	41[b] (47)	25 (71)
Fertilization at 20/26 h (% of ICSI)[b]	27 (66)	20 (80)
Cleaved (% of fertilised)[c]	24/27 (89)	18/20 (90)
4–6 cells 44 h post ICSI[d]	7	7
GQE 44 h post ICSI[d]	1	2
≥ 6 cells 68 h post ICSI[d]	5	1

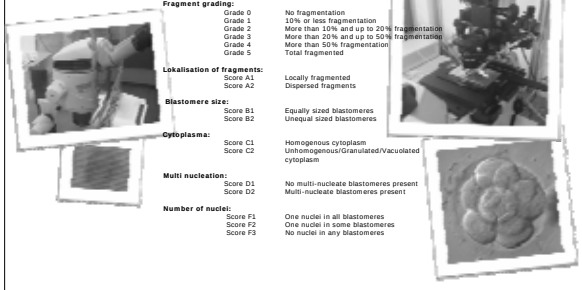
Table 2 Cytogenetic analysis results

	Control (n = 115)	
	GV	MI
Randomised oocytes (minus censored)[d]	83	32
Fixated embryos (> 1 blastomere 68h post ICSI)	24	17
Fixated blastomeres	106	73
Cytogenetically evaluated blastomeres	80	51
Multinucleated blastomeres (% of analysed)	41 (51)	18 (35)[e]
Normal blastomeres (% of analysed)[f]	4 (5)	8 (16)
Overall normal embryos[c]	2	2
Overall abnormal embryos	19	3
Non-classifiable embryos	3	2
Percentage overall normal embryos	8% (2/24)	13% (2/15)
Uniformly normal embryos	2	1

Thus, it is concluded that a majority of hCG exposed oocytes that have not reached MII at oocyte pick-up are defect. This may be the main reason for the poor success rates, and for the lack of positive results from adding FF-MAS, and suggests that FSH/hCG exposed oocytes retrieved in the GV or MI phase should not be used in ART programmes.

– *Examples from real life….*

You can't evaluate embryos in stereo microscopes



Cell number: 0,1,2,3,4,5,6,7,8……

Fragment grading:

Grade 0	No fragmentation
Grade 1	10% or less fragmentation
Grade 2	More than 10% and up to 20% fragmentation
Grade 3	More than 20% and up to 50% fragmentation
Grade 4	More than 50% fragmentation
Grade 5	Total fragmented

Lokalisation of fragments:

Score A1	Locally fragmented
Score A2	Dispersed fragments

Blastomere size:

Score B1	Equally sized blastomeres
Score B2	Unequal sized blastomeres

Cytoplasma:

Score C1	Homogenous cytoplasm
Score C2	Unhomogenous/Granulated/Vacuolated cytoplasm

Multi nucleation:

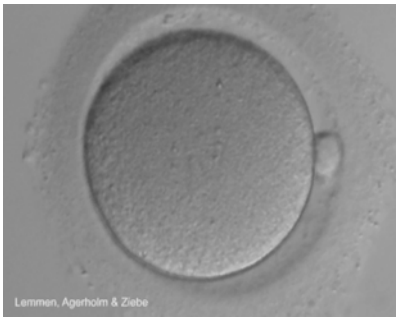
Score D1	No multi-nucleate blastomeres present
Score D2	Multi-nucleate blastomeres present

Number of nuclei:

Score F1	One nuclei in all blastomeres
Score F2	One nuclei in some blastomeres
Score F3	No nuclei in any blastomeres

– *Examples from real life….*

Don't disregard timing



– *Examples from real life….*

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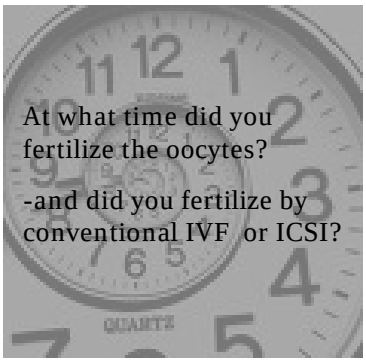

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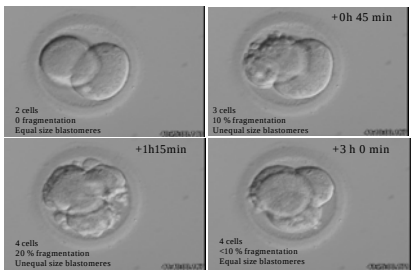

– *Examples from real life….*

Don’t be too focused on details – forgetting the big picture

– *Examples from real life….*

The laboratory is intimately integrated with the rest of the fertility clinic:

A poor laboratory will negatively impact outcome even after an optimal clinical performance

Not even the best laboratory can compensate for a poor clinical performance

An error in the laboratory can therefore be mediated, initiated or closely integrated with the rest of the clinic

Consequence:

An embryologist should choose his/her clinical staff very carefully.....

European Cell and Tissue directives now implemented: What are the practical implications in the IVF lab?

Prof. Josiane Van der Elst, Ph.D.

E A C C

Universitair Ziekenhuis Brussel
Vrije Universiteit Brussel
Centrum voor Reproductieve Geneeskunde

ESHRE 2008 PCC Embryology

Disclosure

There are no commercial relationships or other activities that might be perceived as a potential conflict of interest

2

Learning objectives

- To learn about the origin of the European Cell and Tissue Directives (EUTCD)
- To learn and understand the goal of the EUTCD
- To learn and understand the impact of implementation of EUTCD on IVF laboratory operation
- To learn about communication channels on EUTCD

3

Outline

- EU and EU Cell and Tissue Directives
- Implementation
- Impact for IVF laboratories
- Differences within Europe
- Channels of communication

4

European Union

- The European Union was founded in 1957
- Political treaties define cooperation between Member States
- Treaties result in European Directives
- EU Directives are made by European Institutions
  - → Council of Ministers
  - → European Parliament
  - → European Commission

5

European Treaties and public health

- Treaty of Rome (1957) (EEC)
  - → economic cooperation, no public health issues
- Treaty of Maastricht (1992) (EU)
  - → cooperation on public health: information, education
- Treaty of Amsterdam (1999)
  - → cooperation on public health: protection
  - → provides legal tools to ensure health protection

6

Directive 2004 / 23 / EC

- The European Union Tissues and Cells Directive (EUTCD) is a legal document originating from the European Union's public health programme
- standards of quality and safety for human tissues and cells intended for human applications, in order to ensure a high level of protection of human health
- particularly in order to prevent the transmission of diseases

7

The mother and technical directives

2004/23/EC	of European Parliament and of the Council of 31 March 2004 aka Mother Directive standards of quality and safety for application of human tissues and cells to the human body Into force in EU on 7 April 2006
2006/17/EC	Commission Directive of 8 February 2006 aka Technical Directive 1, EUTD1 donor centered donation, procurement, testing Into force in EU on 1 November 2006
2007/86/EC	Commission Directive of 24 October 2006 aka Technical Directive 2, EUTD2 cell - and - tissue centered coding, processing, preservation, storage and distribution Into force in EU on 1 September 2007

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Outline

- EU and EU Cell and Tissue Directives
- Implementation
- Impact for IVF laboratories
- Differences within Europe
- Channels of communication

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Implementation of EUTCD

- Obligations for Member States
  - → transpose EUTCD in national legislation
  - → install a Competent Authority
    - set up licensing system for tissue establishments
    - organize inspections
    - set up a system for notification of serious adverse reactions/events
    - report back to European Commission

10

Implementation of EUTCD

- Obligations for tissue establishments
  - → 'tissue establishment'
    - a tissue bank or a unit of a hospital or another body where activities of processing, preservation, storage or distribution of human tissues and cells are undertaken.
    - may also be responsible for procurement or testing of tissues and cells

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Implementation of EUTCD

- This Directive should apply to tissues and cells including haematopoietic peripheral blood, umbilical-cord (blood) and bone-marrow stem cells, reproductive cells (eggs, sperm), foetal tissues and cells and adult and embryonic stem cells.
- 'reproductive cells' means all tissues and cells intended to be used for the purpose of assisted reproduction

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Implementation of EUTCD

- IVF centres are considered as tissue establishments
- Have to fulfill safety and quality criteria
  - → for cell donation, procurement and testing (TD1)
  - → for coding, processing, preservation, storage and distribution of human tissues and cells (TD2)

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Implementation of EUTCD

- Reproductive cells have, due to the specific nature of their application, specific quality and safety characteristics
- 'partner donation' means the donation of reproductive cells between a man and a woman who declare that they have an intimate physical relationship;
- 'direct use' means any procedure where cells are donated and used without any banking;

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Requirements of TD1: donor-centered

Conditions for donation, procurement

→ voluntary donation

→ unpaid donation

→ informed consent

→ unique donor identification

→ safe procurement: use of CE - labelled medical devices, wherever possible

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Requirements of TD1: donor-centered

Conditions for laboratory testing

→ infectious serological testing for partner donation

→ HIV1,2, HBV (sAG and core antibodies), HCV

→ infectious serological testing non-partner donation

→ HIV1,2, HBV (sAG and core antibodies), HCV

→ Syphilis

→ Chlamydia

→ In case of partner donation and direct use laboratory testing may not be necessary

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Requirements of TD 2: Cell - and - tissue centered

- Quality Management System
- Air quality
- Frozen storage
- Critical materials
- Traceability
- Coding
- Notification of adverse reactions and events

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Outline

- EU and EU Cell and Tissue Directives
- Implementation
- Impact for IVF laboratories
- Differences within Europe
- Channels of communication

18

Quality Management System

- There must be a documented quality management system applied to the activities for which accreditation/designation/authorisation or licensing is sought, in accordance with the standards laid down in this Directive
- ISO 9001:2000, ISO 15189 are examples

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Quality Management System

- The personnel in tissue establishments
  - → sufficient number
  - → qualified for the tasks they perform
  - → competency must be evaluated at appropriate intervals
- Opportunity: a new ESHRE initiative is the establishment of a certification system for clinical and senior clinical embryologists. The system aims at certifying both practical and theoretical competence of the laboratory staff

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Air quality

- where tissues or cells are exposed to the environment during processing
- an air quality with particle counts and microbial colony counts equivalent to those of Grade A as defined in the current European Guide to Good Manufacturing Practice (GMP), Annex 1 and Directive 2003/94/EC is required
- with a background environment at least equivalent to GMP Grade D in terms of particles and microbial counts

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Air quality

A less stringent environment may be acceptable where

- A validated microbiological inactivation or terminal sterilisation process is applied
- where it is demonstrated that exposure in a Grade A environment has a detrimental effect on the required properties of the tissue or cell concerned;

22

Air quality

A less stringent environment may be acceptable where

- It is demonstrated that the mode and route of application to the body implies a significantly lower risk of transmitting bacterial or fungal infection to the recipient than with cell and tissue transplantation
- where it is not technically possible to carry out the required process in a Grade A environment (for example, due to requirements for specific equipment in the processing area that is not fully compatible with Grade A)

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Air quality

- but, even in case exceptions are allowed
- an environment must be specified
- it must be demonstrated and documented that the chosen environment achieves the quality and safety required

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Frozen storage

- a system of separate storage must be devised
  - → where HIV 1,2, hepatitis B or hepatitis C test results are positive or unavailable,
  - → or where the donor is known to be a source of infection risk
- storage facilities must be provided that clearly separate
  - → tissues and cells prior to release/in quarantine
  - → from those that are released
  - → from those that are rejected
  - → in order to prevent mix-up and cross-contamination between them

25

Traceability

- Member States shall ensure that all tissues and cells procured, processed, stored or distributed on their territory can be traced from the donor to the recipient and vice versa
- Tissue establishments shall keep the data necessary to ensure traceability at all stages
- Data required for full traceability shall be kept for a minimum of 30 years after clinical use
- Data storage may also be in electronic form

26

Critical materials

- The traceability requirements for tissues and cells, as well as for products and materials coming into contact with these tissues and cells and having an effect on their quality and safety,
- Procurement procedures for tissues and cells
  - → Wherever possible, only CE marked medical devices must be used
- Culture media: CE label?
  - → Cf. France, AFSSAPS: culture media = produits thérapeutiques annexes

27

Coding

- A single European identifying code shall be allocated to all donated material at the tissue establishment
  - → to ensure proper identification of the donor and
  - → the traceability of all donated material and
  - → to provide information on the main characteristics and properties of tissues and cells
- shall not apply to partner donation of reproductive cells

28

Coding

- EC has the duty to develop a unique European code for cells and tissues
- EC ordered a CEN workshop to propose a code
- CEN = European Committee for Standardisation (Comité Européen de Normalisation)
- Three coding systems were compared
  - Italian coding system
  - Spanish coding system
  - ISBT 128

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Notification adverse reactions

TD2- Annex V - Annual notification format - part A

- Transmitted bacterial infection
- Transmitted viral infection
- Transmitted parasitical infection
- Transmitted malignant diseases
- Other disease transmissions
- Other serious reactions

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Notification adverse events

TD2 - Annex V - Annual notification format -part B

- Adverse events which may have affected quality and safety of tissues and cells due to
  - → Tissues and cells defect
  - → Equipment failure
  - → Human error
  - → Other

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Outline

- EU and EU Cell and Tissue Directives
- Implementation
- Impact for IVF laboratories
- Differences within Europe
- Channels of communication

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EU today

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Implementation - questions asked

- Has implementation been done?
- Air quality required?
- Serology “at the time of donation”?

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Ireland (source Tim Dineen)

- Mother directive and technical directives passed into law
- Grade D air quality according to GMP standard, i.e. with the need for pressurized rooms, air-locks etc. Fertility clinics disputing the need for GMP requirements, suggesting that Grade D in terms of microbial monitoring should be sufficient

35

Ireland (source Tim Dineen)

- For IVF/ICSI there will be a baseline serology done
  - → when the couple first present to the clinic,
  - → and then a serology will be done within 30 days of an egg collection
  - → To be done for all egg collections
- For IUI, again all couples will need to have a serology done when they first present to the clinic, and they will subsequently need testing every 6 months

36

Denmark (source Soren Ziebe)

- EUTCD has been implemented
- no specification on air quality except that IVF does not call for the stringent quality
- Serology: at the time of donation: It is stated that the test should be done "prior" to aspiration. In case of oocyte donation it should be done no more than 30 day before. Further, the test is valid for 24 month

37

Norway (source Arne Sunde)

- EUTCD has been implemented
- no specific demand concerning air quality in ART
- Serology: Partner donation – testing

  ART is considered as ONE treatment comprised of a series of interventions. This means that we only need to do serological testing every 12 months and not every time we collect oocytes/semen

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Norway (source Arne Sunde)

- Direct use is defined as a procedure where there is no “storage”. Likely interpretation is that processing of semen prior to IUI is not defined as “storage”
- Major changes in procedures/consumables/equipment must be approved by the authorities prior to implementation in the clinic. As an example, change of culture media is defined as a major change

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Various countries, to be extended

- No implementation in national law so far in Greece, Belgium…
- Only the mother directive (2004/23/EC) has been transposed in Italy
- In Spain the EUTD has been transposed long time ago and the implementation at the level of tissues and cells other than reproductive cells is being undertaken by the national organisation of transplants, together with the Spanish drug agency

No competent authority has been designed for reproductive cells

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Outline

- EU and EU Cell and Tissue Directives
- Implementation
- Impact for IVF laboratories
- Differences within Europe
- Channels of communication

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Channels of communication

- ESHRE's Focus on Reproduction

  ESHRE position paper on EUTCD in Focus on Reproduction, January 2008
- ESHRE's EACC

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EACC: an ESHRE offer you can't refuse

- European Assisted Conception Consortium
- Joint venture between ESHRE and HFEA
- Member state organisation
- Not - for - profit initiative supported by ESHRE
- Established at ESHRE 2005 Copenhagen
- Two annual meetings
  - → one at the Annual ESHRE meeting

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EACC Objectives

- Bring together regulators and IVF professionals from member states
- Communication between member states
- Communication to European Commission
  - →seek how can we work together with the Commission to support implementation of the Directive
  - →present joint position of regulators and practitioners
  - →give expert advice to EC

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EACC Format: Members

All EU member states

Per member state / three members

- → Two practitioners
  - one clinician
  - one embryologist
- → One regulator

Please check ESHRE Website – link to EACC - membership:

check for members of your country

they are your representatives

Non-EU members allowed to join for information

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EACC Format: Executive Committee

→ Five practitioners
- → Anna Veiga (Spain)
- → Ioannis Messinis (Greece)
- → Josiane Van der Elst (Belgium)
- → Cristina Magli (Italy)
- → Arne Sunde (Norway)

→ Three regulators
- → Angela McNAb (chair) (UK)
- → Bernard Loty (Fr)
- → Basil Tarlatzis (Greece)


Josiane Van der Elst


Anna Veiga


Cristina Magli


Ioannis Messinis


Arne Sunde


Basil Tarlatzis


Angela McNab


Bernard Loty

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References

- Link to the directives: http://europa.eu.int/comm/health/ph_threats/human_substance/tissues_en.htm
- Link to EACC: http://www.eshre.com/emc.asp?pageId=678

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THE IMPORTANCE OF COLLECTION AND REGISTRATION OF LABORATORY DATA

KARIN ERB
ODENSE UNIVERSITY HOSPITAL
DENMARK

Eshre Barcelona 2008

- **WHY** collect and register laboratory data?
- **WHAT** should we collect?
- **HOW** to do it?

WHY

- Most large registers and studies focus on:
  - Number of started cycles
    and
  - Pregnancy rates

Everything in between is usually not taken into account - such as the lab processes

Why - Purpose

- Control and administer cycles
- Follow-up and monitor own performance
- Generate data for stakeholders
- Compare key monitoring data with other clinics

Success indicators in IVF lab

Fertilisation rates
Development variables (”GQE”)
Survival cryopreservation
Implantation
Live birth

WHY collect

- Different results from different laboratories
- Different methods/media/setup used

⇒ **Larger data cohorts needed**

Why the differences between studies?

- Patient populations / group sizes
- Embryo / blastocyst morphology and development
- Culture conditions / blastocyst development rates

WHY collect

- Collecting data ensures large-scale information on the influence of:
  - Different aspects of oocyte and embryo morphology
  - Different culture conditions
  - Timing

WHAT to collect

Oocyte info (stimulation regimes)

Fertilisation info
(IVF/ICSI, short time incubation - O/N)

Developmental rates

Embryo morfology (GQE)

Implantation rates

WHAT to collect

Media info (simple, sequential)

Culture conditions (Oxygen presure)

Screening; timing info

ET timing (day 1,2,3,4,5,6,7?)

Freezing / thawing (criteria / survival)

Variables for embryo selection

- Oocyte assessments
- Zygote scoring
- Cleavage rates
- Morphology (fragmentation, cell size)
- Number of nuclei
- Metabolic / genetic status?

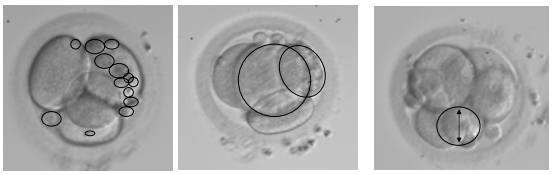
HOW to collect

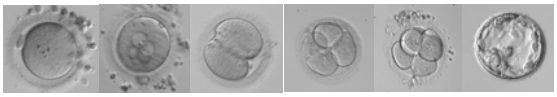
- We need a common system

- Oocyte morphology
- Embryo / blastocyst morphology
- Sperm morphology (?)

- What is a good quality / top quality embryo?

Are we scoring the same things?





How do we score
”good quality embryo”?

Consensus?
Validation?

Common grading / scoring system?

INTEROBSERVER AND INTRAOBSERVER VARIATION IN DAY 3 EMBRYO GRADING

BAXTER AB, MAYER JF, SHIPLEY SK AND CATHERINO WH
FERTIL STERIL 2006; 86: 1608-1615

Design, Baxter et al.

- 26 embryologists at ASRM in Philadelphia
- 35 embryo videos recorded
  (interobserver variation)
- 7 embryos shown several times
  (intraobserver variation)
- Scale with 5 embryo grades (Veeck)
- Kappa values used for statistics

Results, Baxter et al.

- Interobserver variability (median, range)
  Kappa 0.24 (0.03-0.49) poor
- Intraobserver variability (median, range)
  Kappa 0.69 (0.44-1.00) good

Conclusions, Baxter et al.

We don't always agree

- Only use one embryologist for scoring?
- Use consensus scoring from several embryologists?
- Simplify the scoring system?

INTEROBSERVER AGREEMENT AND INTRAOBSERVER REPRODUCIBILITY OF EMBRYO QUALITY ASSESSMENTS

ARCE JC, ZIEBE, S, LUNDIN K, JANSSENS R, HELMGAARD L AND SÖRENSEN P. HUM REP 2006: 21; 2141-2148

Level of agreement – Top Quality Embryos

	Kappa
Agreement among embryologists	
Central 1 vs Central 2	0.74
Central 1 vs Central 3	0.74
Central 2 vs Central 3	0.72
Consolidated Central vs Local	0.65

Reproducibility (intraobserver)

	28h	44h	68h
Cleavage stage	0.97-0.98	0.85-0.90	0.72-0.83
Blastomere uniformity	NA	0.69-0.80	0.63-0.81
Degree of fragmentation	NA	0.64-0.77	0.68-0.72
Multinucleation	NA	0.53-0.88	0.48-0.66
Cytoplasmic appearance	NA	0.24-0.79	0.65-0.74
Top quality embryos			**0.80-0.81**

Conclusions

- Embryo quality assessments can be associated with high interobserver and intraobserver agreements
- Competence, accuracy and consistency can and should be acquired through continuous training and validation
- For large multicenter studies, a combination of local and central evaluations should provide the most effective and reliable approach to determine embryo quality

"EMBRYO REGISTRATION"

Inge Agerholm[1], Karin Erb[2], Marie Louise Grøndahl[3], Søren Ziebe[4]

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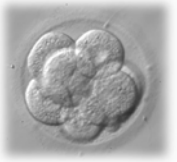


Standardisation
-We need to use the same system

Definitions
- We must use the same definitions for the various parameters

The Danish (Nordic?) system

Register the number of blastomers

Register the degree of fragmentation

The Danish (Nordic?) system

Localisation of fragments:
Score A1 Locally fragmented
Score A2 Dispersed fragment
Blastomere size:
Score B1 Equally sized blastomeres
Score B2 Unequal sized blastomeres
Cytoplasma:
Score C1 Homogenous cytoplasm
Score C2 Unhomogenous/Granulated/Vacuolated

The Danish (Nordic?) system

Number of nuclei:
- Score D1 No multi-nucleate blastomers present
- Score D2 Multi-nucleate blastomers present

Early cleavage
- Score E1 Early cleavage
- Score E2 No Early cleavage

Zona Pellucida variation
- Score Z1 Zona variation
- Score Z2 No zona variation

And so on …

The future – the dream

Common database
- Using the same registration system

Purpose
- Collecting data for national and international reports

- Webservice based data collection

The future – the dream

Stakeholders
- Your own laboratory data
- National Fertility Society
- Eshre – EIM

- Authorities
  - Nathional Medical Agency (EU directive)
  - National Board of Health

- Learning center – improving quality

Training and validation

Internal / external

- Scoring together
- Validation with images / videos

External, e.g. QAP online

- Database with pictures (videos coming?)
- Educational and quality control
- Scoring and comparison with all others or only own clinic
- Questions, such as ”would you transfer/ freeze/ culture to blastocyst…”, or ”rank from best to worst”

Today’s lesson

We have to collect and register laboratory data

Control and administer cycles

Follow-up and monitor our performance

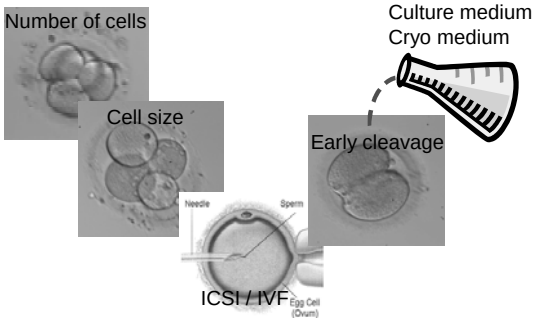
- ***you must know your own system***

Generate data to stakeholders

Compare key monitoring data with other clinics

Goal: To improve outcome

Summary



Automation of embryo selection and production

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Jeremy G Thompson has obtained and currently holds research funding and has consultancy agreements with William A Cook Australia Pty Ltd

Learning objectives

- To define what “automation” in the IVF lab means
- To examine the impact of automation within an IVF lab
- To examine the development and application of technologies for embryo selection
- To examine the development of new embryo production systems

What is meant by “automation”

- Automating activities in the IVF lab that are currently performed by “hand”
  - Removing the “human factor” in a process
- Natural evolution for a technology that is robust and routinely practised

Benefits of automation

- Reliability
  - Does not become distracted
  - Parameters same every time
- Quality
  - Reduced variability
  - Measurable parameters checked
- Improved performance
  - Must be an improvement in performance for automation to be seen as attractive

Will I still have a job?!

- Probable consequence is reducing the need for staff with a *wide range* of skills
- Will allow for increased specialisation
- Will allow more rapid adoption of new techniques that require new training and development

Automation systems

- Analysis of embryos and embryo quality
- Embryo production systems

Automation of embryo quality assessment

Automated visual analysis of embryos

- Adds to or even replaces the subjective visual analysis
- Able to measure the kinetics of embryo development
  - Time lapse videomicroscopy
  - Digital analysis
- Digital imaging and analysis of cell morphology

Digital analysis of embryos

Embryo-guard system

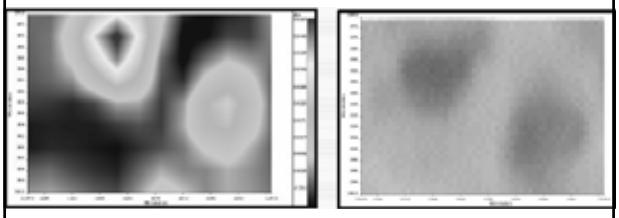
Embryo-guard (www.cryo-imt.com)
(IMT International)

Digital analysis

- Light-penetration
  - Analysis of grey-scale pixelation
  - Analysis of light polarisation
  - Analysis of Infrared spectrum
  - Density & localisation of organelles
- Morphological features
  - Fragmentation
  - Cell number and allocation

New types of microscopy developing

Near Infrared (left) microscopy image of tumour cells (right) – chemical profiling



Baianu et al. http://fs512.fshn.uiuc.edu/Soy2002-Imaging-2.pdf

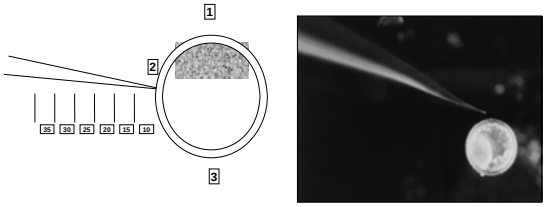
Non-invasive assays for candidate substrates

- **NAD(P)H assays of "spent" medium**
  - **Based on Lowry assays**
  - **Fluorescence**

$$\text{Pyruvate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NADH}$$

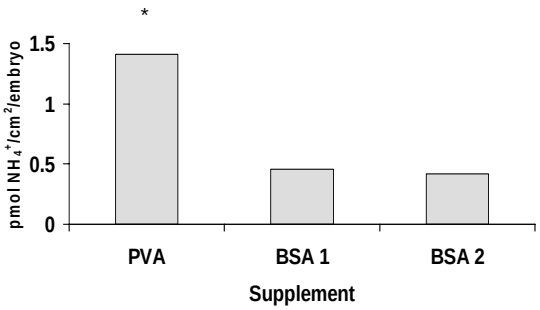
- **HPLC and mass spectrometry of "spent" medium**
  - **Amino acid analysis**
- **Electrode technology – real time measurement of metabolic activity**
  - **Automated scanning electrode – many different ions and compounds**
  - **Polarographic electrode technology - O_2 consumption**

ASET measurement of NH_4^+



Also available for a wide range of ions and compounds, including oxygen & H^+

Ammonium production from bovine blastocysts following culture in different supplements



Biosensors and laminates

- "Smart sensor" technology
- Biofilms sandwiched between impermeable and permeable layers
- Ligand-induced reactions
  - Immunological interactions
  - Receptor-ligand interactions
  - Chemical reactions shifting colour, charge which is measurable

The ‘Omics era is upon us!

- Genomics
  Transcriptomics
  Proteomics
  Metabolomics
- Comparative Genomic Hybridisation is an example of Genomics applied to embryo selection
- Transcriptome microarrays of cumulus cells and embryos are widely used in research
  - Already some application with association between levels of some cumulus cell gene expression and oocyte developmental competence
- Next speaker talking on proteomics and metabolomics

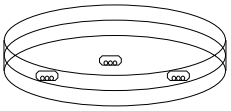
Embryo production/manipulation

Laser Light trapping

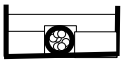
- Particles are “trapped” in the highest energy level of a focussed laser beam
- Possible applications
  - Blastomere removal
  - ICSI
  - Diagnostics on embryos
  - Fertilization

Micro culture systems

Most culture systems are “swimming pools”

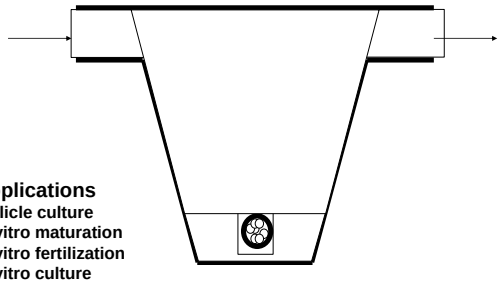


Introduction of microwells, glass oviducts, agar wells



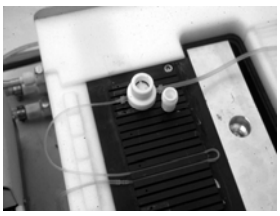
Microperfusion systems

medium in **medium out**



Applications
Follicle culture
In vitro maturation
In vitro fertilization
In vitro culture
Post-hatching development
Preparation for storage

Micro-perfusion prototype in our laboratory (1995)



Advantages/disadvantages

Advantages	Disadvantages
Change components	Dilute paracrine GF's
Add new components	
Oil-free	
Removal of toxins	
Media stability	
Measure effluent	

Adapted from Thompson, Theriogenology 2007

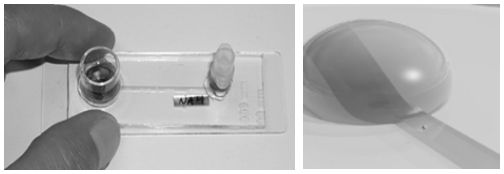
Bovine Day 2 - 7 embryo development in perfusion system

	No. cleaved embryos	Blastocysts (%)	Transferable quality (%)	Cell number
Static	246	44 ± 5	31 ± 4	175 ± 9[a]
Perfusion	131	44 ± 6	19 ± 5	131 ± 12[b]

Adapted from McGowan & Thompson, Proc. Aust. Soc. Reprod. Biol. 1997

Microfluidic systems involve creating microenvironments

- improved embryo culture success

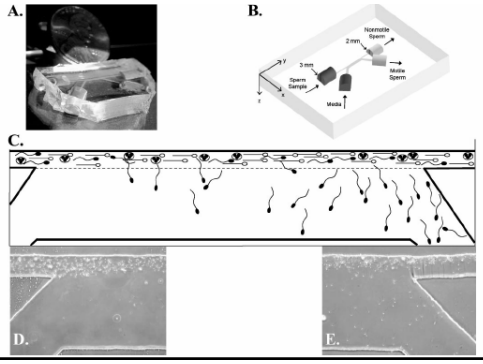


From Vitae LLC www.vitaellc.com

Microfluidics

- Uses the principles of “laminar flow”
- Can move gametes and embryos to different locations in “circuit”
- Can use the principles of “laminar flow” to perform mechanical functions
  - e.g. Cumulus cell and zona pellucida removal already possible
- Represents the next “era” in culture systems
  - Less gamete and embryo handling (including ICSI)
  - Less cellular stress

Microfluidics to separate motile/non motile sperm (Suh et al., J. Androl. 2005)

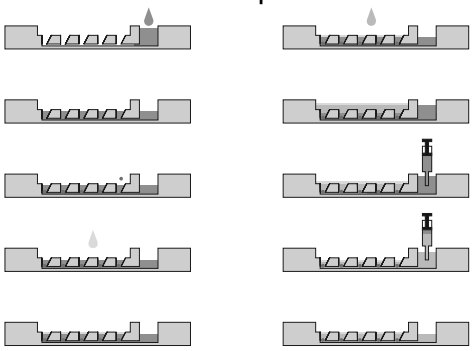


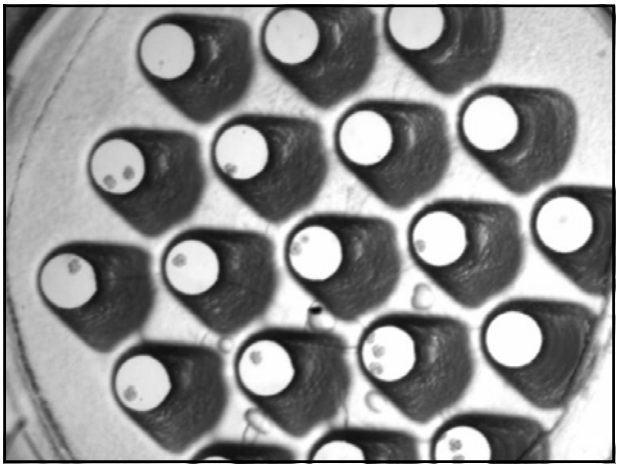
Microfluidic chip – unique addressing

Cook Australia

(http://www.cookgroup.com/profile/med-mfg/aust.html

Operation of micro-chip





Why have microfluidic systems yet to make an impact on IVF?

- Material sciences and performance?
  - Properties of materials, especially plastics and resins not adequate
    - Gas permeability – especially using HCO_3^- -buffer
    - Water permeability – many lithographic materials and plastics are H_2O permeable
    - Both problematic for delivery of a pre-warmed & gassed media
- Clear advantage/improvement in process and/or outcomes?
  - Cost vs benefits

Many thanks to the following for their inspiration:

- **Lindsay McGowan**
  Debbie Berg
  Jim Dunlop
  (AgR New Zealand)
- **Michelle Lane**
  Kara Cashman
  (Uni. Adelaide)
- **David Gardner**
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- **Matt Wheeler**
  (Uni. Illinois)
- **Kim Giliam**
  Jason Spittle
  Andrew Hinsch
  Sean O'Brien
  Mike Junger
  (Cook Australia)
- **Michael Barry**
  (Repromed)

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- McGowan LT, Thompson JG (1997) Perfusion culture of bovine in vitro produced embryos. Proc. Aust. Soc. Reprod. Biol. 28: 24.
- Suh R, Takayama S, Smith GD (2005) Microfluidic applications for andrology. J. Androl. 26: 664-670.
- Thompson JG (2007) Culture without the petri-dish. Theriogenology 67: 16-20.

Non-invasive Embryo Assessment: Proteomic and Metabolomic Biomarkers

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Disclosure: Member of Scientific Advisory Board of Molecular Biometrics

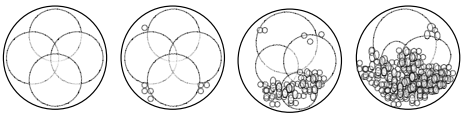
Learning Objectives

- To review the traditional tools of embryo assessment (advantages and limits)
- To review the need for new/improved techniques
- To review alternative, non-invasive embryo assessment techniques
- Future Perspectives / Conclusions

Historic and Current Approaches

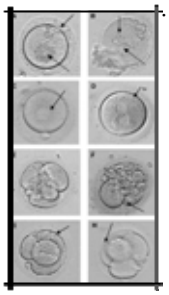
Day 1		Oocyte quality: zona, cytoplasm, PB's 2PN Assessments - Z scoring
Day 2		Early cleavage/ Multinucleation
		
Day 3		Genetic screen (PGD); metabolic evaluation, morphologic evaluation; extended culture decision
Day 4		?
Day 5		Selection blastocyst for transfer, embryo cyopreservation, PGD

Traditional Embryo Development Grading



A	B	C	D
Even Blastomeres	→	→	Uneven Blastomeres
No Fragmentation	→	→	Increased Fragmentation

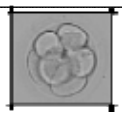
Embryo Score and Blastocyst Development



- Examples of normal and abnormal morphological characteristics of oocytes, zygotes, and embryos. (A) Oocyte with a large perivitelline space (solid arrow) and a dark patch (dashed arrow); (B) oocyte with a vacuole (dashed arrow) and a necrotic patch (solid arrow); (C) oocyte accumulation of smooth endoplasmic reticulum; (D) normal zygote with halo (arrow), central pronuclei and polarized and coalesced nucleoli; (E) embryo with much fragments (out of focus, fragmentation score 1) uneven-sized and irregular-shaped (oval) blastomeres; (F) highly fragmented embryo (fragmentation score 0) and abnormal cytoplasm in one of the blastomeres (clear zone at arrow); (G) four-cell embryo with slightly irregular-shaped (oval) blastomeres, one of which jagged membrane; (H) four-cell embryo with slightly irregular-shaped and uneven sized blastomeres, and a gap between the blastomeres and the zona pellucida, thus classified as "Does not fill the space under the zona."
- Sjöblom. Embryo score and blastocyst development. Fertil Steril 2006.

Day 3 to Day 5 Development

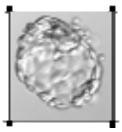
Sibling Embryos



•Uneven Cells
•Fragments
•Multinucleation
•Vacuoles

•Even Cells
•No Fragments

Sibling Embryos



•Poorly Expanded
•Poor Inner Cell Mass
•Thick Zona
•Under Developed Trophectoderm

•Fully Hatched
•Distinct Inner Cell Mass
•Well Developed Trophectoderm

Early Stage Morphological Characteristics

Evaluation of the pronuclear-stage oocyte

1. Number of pronuclei
0 Not fertilized
1 Parthenogenetic activation (or asynchronous development of pronuclei – check later)
2 Normal fertilization (if there is the presence of 2 PBs at the same time)
3+ Abnormal fertilization

2. Size of pronuclei (in case of 2 PN fertilization)
Optimal Normal Size and Equal Size
Suboptimal Larger/Smaller Size and/or Unequal Size

3. Nucleoli (in case of 2 PN fertilization)
Number of nucleoli – in each pronuclei
Size of nucleoli (small or medium or large – in each of the two pronuclei)
Polarization of nucleoli
1, POLARIZED: when all the nucleoli were aligned on the side of the pronucleus near to the other pronucleus
2, non-POLARIZED: when the nucleoli were dispersed (or not completely aligned) in the pronucleus

4. Aspect of Polar Bodies
1, Single non-fragmented Polar Body
2, Single fragmented Polar Body
3, Two Polar Bodies (fragmented or non-fragmented) - the presence of 2 PB is required for normal fertilization

5. Distance between the Polar Bodies (in case of the presence of 2 PB)
Optimal Close
Suboptimal Distant

6. Concentration of cytoplasmic organelles
For each oocyte the % of cytoplasmic organelles concentration/oocyte diameter (%C) is estimated (usually this is between 10% and 20%)

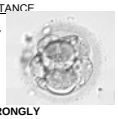
7. Aspect of the zygote cytoplasm
Suboptimal presence of vacuoles
Suboptimal presence of the refractal body

Nagy, RBA

Early Stage Morphological Characteristics

EMBRYO EVALUATION CRITERIA — RELATIVE IMPORTANCE

1. Number of blastomeres — **STRONGLY**



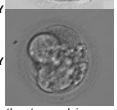
	Day 2	Day 3
Optimal	4-(6) cells	7-8 cells
Medium	3 cells	5-6 cells
Poor	2 cells	2-4 cells

2. Dimension of blastomeres – Day 2 and Day 3 — **MODERATELY/STRONGLY**
Optimal similar or equal size blastomeres
Suboptimal different size blastomeres

3. Proportion of anucleate fragments – Day 2 and Day 3 — **STRONGLY**

Optimal between 0-10%
Good between 10-30%
Medium between 30-50%
Poor more than 50%

4. Quality of the cytoplasm — **MODERATELY**
Optimal: normal appearance
Suboptimal presence of cytoplasm abnormalities (granulated, vacuoles, refractile bodies)

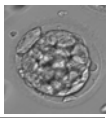


5. Multinucleation of blastomeres — **MODERATELY**
The evaluation of multi-nucleated blastomeres is easier at the 2-4 cells stage.
Optimal Number of cells with a single nucleus
Suboptimal Number of cells with multinucleation - Cells with two equal-sized nuclei
Poor Number of cells with multinucleation - Cells with two unequal sized nuclei or more than two nuclei

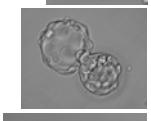
6. Early cell compaction — **MODERATELY**
Cells compaction start after 8-10 cell stage embryo (end of day 3 or day 4)
Optimal Cell compaction is not observed (or not strong) until the end of day 3
Suboptimal Cell compaction is strongly present late day 2 or early day 3

Nagy, RBA

Blastocyst Stage Assessment

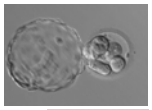


Classification of Blastocyst Development
Stage A - Hatched blastocyst
Stage B - Hatching blastocyst
Stage C - Fully Expanded
- Thin zona p.
- Distinct ICM
- Distinct Trophectoderm

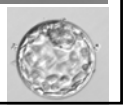


Stage D - A single cavity occupying >50% of the volume of the embryo
- Regular zona p.
- The ICM and trophectoderm may not be clear

Stage E - A distinct single cavity 25- 50% of the volume of the embryo.
- The diameter of the embryo is unchanged.
- Zona is unchanged



Classification of Blastocyst Quality
ICM
Grade 1 - Tightly packed , many cells
Grade 2 - Loosely grouped, several cells
Grade 3 - Very few cells



Trophoblast
Grade 1 - Many cells forming a cohesive epithelium
Grade 2 - Few cells forming a loose epithelium
Grade 3 - Very few cells

Nagy, RBA

Follicle Human Oocyte and Embryo Assessment for ART

Follicular Size

Effect of follicular size on oocyte retrieval, fertilization, cleavage, and embryo quality in in vitro fertilization cycles: a 6-year data collection.
Wittmaack FM, Kreger DO, Blasco L, Tureck RW, Mastroianni L Jr, Lessey BA.

Based on this evaluation of a large number of follicles, follicular size is a useful indicator of oocyte recovery, fertilization, and cleavage in IVF cycles. For optimal results, the follicular fluid volume in gonadotropin- and hCG-stimulated cycles should be > 1 mL, which corresponds to a follicle diameter of > 12 mm, and not larger than 7 mL (24 mm).
Fertil Steril. 1994 Dec;62(6):1205-10.

Human Oocyte and Embryo Assessment for ART
Cumulus

Assessment of Cumulus

Assessment of human oocyte developmental competence by cumulus cell morphology and circulating hormone profile
Sato, Chikako; Shimada, Masayuki; Mori, Takahide; Kumasako, Youko; Otsu, Eiko; Watanabe, Hirohiko; Utsunomiya, Takafumi

The highest development-supporting competence was observed not with oocytes in grade A COC harvested from natural cycles, but with oocytes in grade B COC from FSH-primed cycles. Hormonal profiles in patients bearing grade B COC were characterized by moderate response in oestradiol and progesterone production following FSH, with LH/FSH ratio being below 1.0.
Reproductive BioMedicine Online, Volume 14, Number 1, January 2007 , pp. 49-56(8)

Human Oocyte and Embryo Assessment for ART
Oocyte

Morphology of in-vitro matured oocytes: impact on fertility potential and embryo quality
Anne Lis Mikkelsen and Svend Lindenberg
Significantly more embryos of good quality developed after grade I oocytes [54/144 (37.5%)] compared with those from grade II and grade III oocytes (22/120; P = 0.001). The presence of cytoplasmic abnormalities significantly decreased the cleavage rate (P = 0.04) and also the number of good quality embryos (P < 0.001).
Human Reproduction, Vol. 16, No. 8, 1714-1718, August 2001

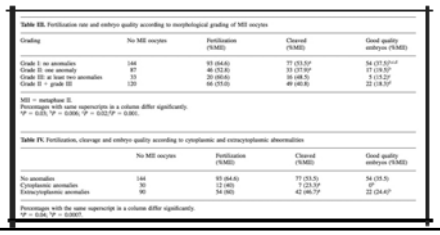


Table III. Fertilization rate and embryo quality according to morphological grading of MII oocytes

Grading	No MII oocytes	Fertilization (%MII)	Cleaved (%MII)	Good quality embryos (%MII)
Grade I: no anomalies	144	93 (64.6)	77 (53.5)[a]	54 (37.5)[a,d]
Grade II: one anomaly	87	46 (52.8)	33 (37.9)[a]	17 (19.5)[a]
Grade III: at least two anomalies	33	20 (60.6)	16 (48.5)	5 (15.2)[a]
Grade II + grade III	120	66 (55.0)	49 (40.8)	22 (18.3)[a]

MII = metaphase II.
Percentages with same superscripts in a column differ significantly.
[a]P < 0.03, [b]P < 0.006, [c]P < 0.02/[d]P < 0.001.

Table IV. Fertilization, cleavage and embryo quality according to cytoplasmic and extracytoplasmic abnormalities

	No MII oocytes	Fertilization (%MII)	Cleaved (%MII)	Good quality embryos (%MII)
No anomalies	144	93 (64.6)	77 (53.5)	54 (35.5)
Cytoplasmic anomalies	30	12 (40)	7 (23.3)[a]	0[a]
Extracytoplasmic anomalies	90	54 (60)	42 (46.7)[a]	22 (24.4)[a]

Percentages with the same superscript in a column differ significantly.
[a]P = 0.04, [b]P = 0.0007.

The Zona Pellucida and Markers of Oocyte and Embryo Viability

Zona P

Significance of the Number of Embryonic Cells and the State of the Zona Pellucida for Hatching of Mouse Blastocysts In Vitro Versus In Vivo

Markus Montag, Britta Koll, Paul Holmes, and Hans van der Ven

Our data show that successful hatching in vitro is dependent on a sufficiently high number of embryonic cells, which enables blastocyst expansion and zona shedding. In vivo, the lower number of embryonic cells detected in zona-free blastocysts indicates that the underlying mechanism of zona escape is different, does not depend on blastocyst expansion, and presumably involves lytic factors from the uterus.

Biology of Reproduction. Article: pp. 1738–1744

Influence of Zona Pellucida Thickness of Human Embryos on Clinical Pregnancy Outcome Following In Vitro Fertilization Treatment

Anette Gabrielsen, Piyush R. Bhatnager, Karsten Petersen and Svend Lindenberg

The degree of zona pellucida thickness variation (ZPTV) of the transferred embryos exhibits a strong correlation with clinical pregnancy outcome following IVF treatment. This potentially reliable indicator of IVF success rate could be used as a criteria for embryo selection during clinical transfers.

Journal of Assisted Reproduction and Genetics

Morphology and Kinetics of Human Pronuclei

Pronuclei

The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology

Jan Tesarik and Ermanno Greco

Clinical pregnancy was achieved in 22 of 44 (50%) treatment cycles in which at least one pattern 0 embryo was transferred, but only in two of 23 (9%) cycles in which only pattern 1–5 embryos were transferred. These data present new evaluation criteria which can be used to predict the developmental fate of human embryos as early as the pronuclear stage, without requiring repeated observations or an exact timing of pronuclear zygote inspection.

Human Reproduction, Vol. 14, No. 5, 1318-1323, May 1999

Human Pronuclei as a Mode of Predicting Viability

Pronuclei

The successful use of pronuclear embryo transfers the day following oocyte retrieval

LA Scott and S Smith

The data show that oocyte quality and pronuclear embryo morphology are related to implantation and that pronuclear embryos can be successfully selected for embryo transfer.

Human Reproduction, Vol 13, 1003-1013

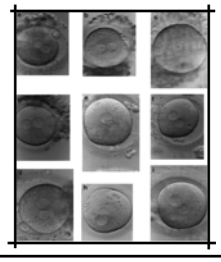


Table III. Implantation and pregnancy rates in patients grouped according to corrected embryo score (CS ≥15; CS ≤14)

	≥15	≤14
Mean CS (SD)	19.7 (3.4)	11.2 (4.1)
Mean age (SD)	32.4 (3.7)	35.3 (3.8)
No. retrievals	48	49
No. of embryo transfers	48	44
No. mature oocytes	480	439
Fertilization rate (SD %)	78 (3.2)	71 (3.8)
No. embryos transferred	175	178
Mean (SD)	3.7 (1.3)	4.6 (2.5)
No. clinical pregnancies (%)	34 (71)	4 (8)
No. ongoing/born (%)	31 (65)	1 (2)
No. implantations (%)	49 (28)	4 (2)
No. ongoing/born (%)	41 (23)	1 (1)
Multiple pregnancies (%)	10/34 (29)	0
Delivery rate/OR (%)	65	0

Pronuclei

Human Pronuclei as a Mode of Predicting Viability

The limited importance of pronuclear scoring of human zygotes
Aidita N. James, Sasha Hennessy, Brett Reggio, Klaus Wiemer, Frederick Larsen and Jacques Cohen
Pronuclear morphology scores (PNMSs) of 1, 2 or 3 do not correlate with live birth rates when assessing unique PNMS embryo transfers. In particular, previously considered poor (type 3) embryos can result in pregnancy with normal live birth rates.
Human Reproduction 2006 21(6):1599-1604

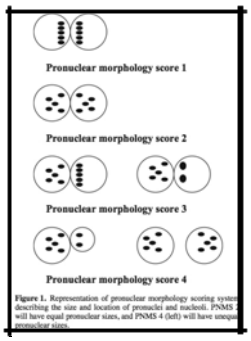


Figure 1. Representation of pronuclear morphology scoring system describing the size and location of pronuclei and nucleoli. PNMS 2 will have equal pronuclear sizes, and PNMS 4 (left) will have unequal pronuclear sizes.

Pronuclei

Human Pronuclei as a Mode of Predicting Viability

TABLE 3

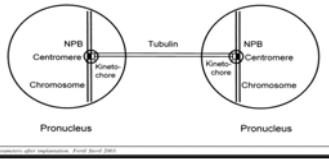
Prospective randomized study correlating embryo quality (combination of cleavage stage and percentage of fragmentation) on day 3 and pronuclear morphology with implantation rates.

Method of embryo selection for ET	Cycles	Age of women (±S.D.)	Fertilization (%) (±S.D.)	Embryos transferred (±S.D.)	Clinical pregnancies (%)	Implantation rate (%)
2 PN morphology	95	36.1 (5.0)	72.5 (23.0)	3.3 (1.7)	22 (23.2)	12.1
Day-3 morphology	98	36.3 (6.0)	77.6 (20.5)	3.6 (1.8)	25 (25.5)	15.1
Day 3 + 2 PN morphology	97	36.1 (5.8)	72.5 (23.4)	3.4 (1.7)	38 (39.2)	21.1

Note: $P<.05$ for clinical pregnancy and implantation rates between group 3 versus group 1 and group 2.
Nagy. Pronuclear parameters after implantation. Fertil Steril 2003.

FIGURE 4

Putative mechanism of pronuclear migration and NPB alignment.



Nagy. Pronuclear parameters after implantation. Fertil Steril 2003.

Nagy ZP, *et al.* *Fertil Steril. 2003 Jul;80(1):67-74.*

Multinucleation

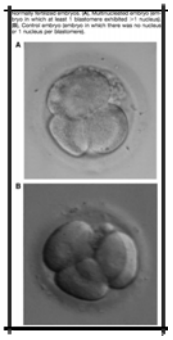
Multinucleation and Mosaicism in the Human Preimplantation Embryo

Multinucleation in normally fertilized embryos is associated with an accelerated ovulation induction response and lower implantation and pregnancy rates in in vitro fertilization-embryo transfer cycles.
Jackson KV, Ginsburg ES, Hornstein MD, Rein MS, Clarke RN
The presence of multinucleated blastomeres in normally fertilized embryos is associated with a more effusive response to gonadotropin therapy and is indicative of a poor developmental outcome and lower clinical pregnancy rates.
Fertil Steril. 1998 Jul;70(1):60-6.

Cycle results in transfers with or without multinucleated embryos.

Variable	Group 1 (Control) [n = 127])	Group 2 (>30% Multinucleated [n = 66])	P value
Embryos transferred	3.26 ± 0.13	3.39 ± 0.19	0.11
All embryos:			
Average cell number	3.55 ± 0.08	2.88 ± 0.14	0.001
Average fragmentation score	1.60 ± 0.07	1.88 ± 0.10	0.05
Transferred embryos:			
Average cell number	3.71 ± 0.08	3.11 ± 0.12	0.001
Average fragmentation score	1.60 ± 0.7	1.88 ± 0.10	0.016
Clinical pregnancy rate	29.1%	9.1%	0.0015
Ongoing/live born pregnancy rate	27.6%	7.5%	0.0012
Implantation rate	14.7%	3.4%	0.0001
Implantation loss rate*	3.3%	12.5%	0.31

* Aborted gestational sacs/no. of total implantations (%).
Note: Data are expressed as means ± SEM unless otherwise indicated.



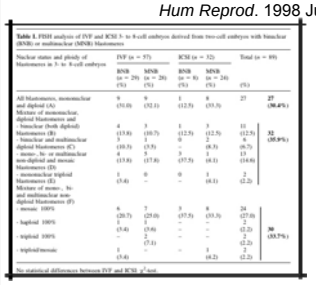
Multinucleation

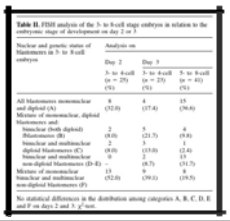
The genetic constitution of multinuclear blastomeres and their derivative daughter blastomeres.

Staessen C, Van Steirteghem A.

These data show that the genetic constitution of bi- or multinuclear blastomeres, and the daughter cells developing from them, are not always abnormal.

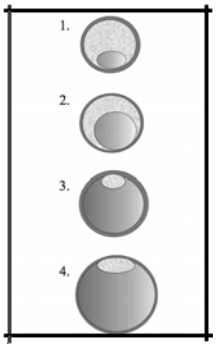
Hum Reprod. 1998 Jun;*13(6):1625-31.*





D5 Morphology

Analysis of Blastocyst Morphology



Assessment of embryo viability: the ability to select a single embryo for transfer—a review

D.K. Gardner, and D. Sakkas

Placenta (2003), 24, S5–S12

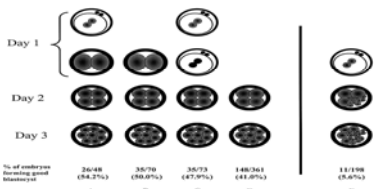
"Synthesis"

Cumulative Scoring and Embryo Liability

Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development

E. Neuber1, P. Rinaudo2, J.R. Trimarchi3 and D. Sakkas1

***Human Reproduction*, Vol. 18, No. 6, 1307-1312, June 2003**



Neuber, E. et al. Hum. Reprod. 2003 18:1307-1312; doi:10.1093/humrep/deg269

HUMAN REPRODUCTION

Current IVF Outcomes
The Need for New/Improved Techniques

Fresh Embryos From Non-Donor Oocytes

	<35	35-37	38-40	41-42
Number of cycles	37,168	21,336	18,174	8,631
Percentage of cycles resulting in live births	38.8	30.6	20.6	10.9
Percentage of cycles with elective single embryo transfer	3.3	1.9	0.7	0.3
Implantation rate	30.8	23.5	15	8.2
Average number of embryos transferred	2.3	2.5	2.9	3.2
Percentage of live births with twins	32.3	27.7	21.4	15.5
Percentage of live births with triplets or more	2	1.9	1.4	0.6

https://www.sartcorsonline.com/rptCSR_PublicMultYear.aspx?ClinicPKID=0 -2006

Estimated IVF Birth Efficiency

- 3-6% of Follicles
- 6-12% of Oocytes
- 10-20% of Embryos (D3)
- 15-40% of Blastocysts

Complications of Multiple Pregnancies

- The incidence of cerebral palsy is increased
  - 8-fold in twins and
  - 47-fold in triplets
- Infant deaths (birth to 1 year) are increased
  - 11.2/1000 live births for singletons
  - 66.4/1000 live births for twins
  - 190.4/1000 live births for triplets and higher order gestations

•Keith et al. Int J Fertil Womens Med 2000;45:206–14.
•U.S. 1998. DHHS Pub No. (PHS) 90–50212.

24

What is Expected from the Alternative Tests?

- Non-invasive (culture medium / fluid / sem. plasma)
- “Easy” (ease of use)
- “Simple” (no need for special knowledge / training)
- Rapid (should take only minutes)
- Cheap (both the instrument and “reagents”)
- Reliable / reproducible function
- Fit to the IVF Lab
- Does not interrupt standard routine (dish / culture)
- Provide more useful results than morphology

Does this currently exist? NO

Possible Targets to Use for Alternative Embryo Assessment Approaches

Morphology	- Birefringence (SpindleView) *
Metabolic Activity	- Pyruvate/Glucose uptake - Amino acids * - Oxygen consumption (Respirometry) *
Constituents	- Genome - Transcriptome - Proteome * - Metabolome
Secreted Factors	- PAF - HLAg * - “Secretome” *

Polscope / SpindleView

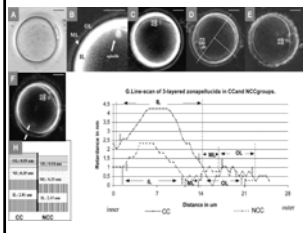


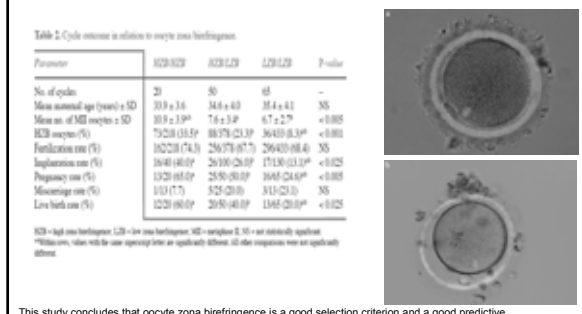
Table II. Mean retardance magnitude and thickness of the individual zona layers as assessed by Polscope microscopy in oocytes contributing to conception cycle (CC) and non-conception cycle (NCC) groups

	CC (transferred)	NCC (transferred)
No. of patients	23	40
No. of oocytes	65	101
Zona inner layer (mean ± SD)		
Retardance (nm)	2.81 ± 0.60[a]	2.15 ± 0.41
Thickness (μm)	11.25 ± 1.44[a]	9.36 ± 1.74
Zona middle layer (mean ± SD)		
Retardance (nm)	0.35 ± 0.08	0.35 ± 0.07
Thickness (μm)	3.92 ± 0.76	3.66 ± 0.65
Zona outer layer (mean ± SD)		
Retardance (nm)	0.55 ± 0.18	0.55 ± 0.14
Thickness (μm)	4.80 ± 1.40	5.55 ± 1.03
Zona total thickness (mean ± SD) (μm)	19.87 ± 1.92[b]	18.58 ± 1.82

[a,b]Significantly different from the NCC group: [a]$P \leq 0.001$, [b]$P \leq 0.01$ (t-test).

Shen, Y. et al. Hum. Reprod. 2005 20:1596-1606

Polscope / SpindleView



This study concludes that oocyte zona birefringence is a good selection criterion and a good predictive criterion for embryo implantation potential.

Montag et al., 2007 RBMonlin

Polscope / SpindleView

Light retardance by human oocyte spindle is positively related to pronuclear score after ICSI

Shen Y, Stalf T, Mehnert C, De Santis L, Cino I, Tinneberg HR, Eichenlaub-Ritter U.

The study suggests that quantitative evaluation of mean retardance of light by the oocyte spindle predicts oocyte health, is related to PN score of the embryo and may be especially useful to assess oocyte quality in countries with legal restrictions to select after fertilization.

Reprod Biomed Online. 2006 Jun;12(6):737-51.

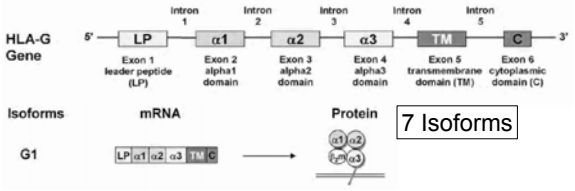
De Santis; Rienzi; Keefe: Spindle and Fertilization / Embryo Development

Evaluation of metaphase II spindle length, retardance and its relationship to embryo quality on day 3 and day 5

T. H. Taylor, T. Elliott, S. A. Gitlin, S. Jones-Colon, H. I. Kort, Z. P. Nagy. Spindle length, as measured by the Oosight, correlates to embryo quality on day 3 and blastocyst quality on day 5. Fertility and Sterility, Volume 86, Issue 3, Pages S115-S116

Assessment of Soluble Human Leukocyte Antigen G in Human Embryos

Detection of soluble HLA-G in embryo culture medium has been correlated to pregnancy success in 12 studies, but three studies were not able to detect sHLA-G



- HLA-G expression has been correlated with an increased rate of cleavage division.
- Soluble HLA-G may alter cytokine expression on maternal cells at the site of implantation as well as induce apoptosis in CD8+ maternal T cells to allow successful implantation (ability of the embryo to modulate the maternal immune response and successfully implant into the uterus)

Warner et al., 2008

Assessment of Soluble Human Leukocyte Antigen G in Human Embryos

Soluble HLA-G and Pregnancy Success
Carol M. Warner, Paula W. Lampton, Judith A. Newmark, and Jacques
RBMonline; in press

HLA-G Detected in Culture Supernatant: Fuzzi et al.; Roussev et al.; Sher; Noci et al.; Yie et al.; Sher et al.; Sher et al.; Criscuoli et al.; Desai; Fisch et al.; Rebmann et al.; Rizzo et al.

HLA-G NOT Detected in Culture Supernatant: van Lierop et al.; Sageshima et al.; Sageshima et al.

To be standardized: **- Culture methods**

- Sampling methods

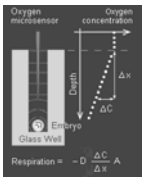
- Detection methods

There are many questions that remain to be answered before one would have full confidence in using sHLA-G as a selection marker for embryos

Embryo Respiration Unisense

Embryo Respirometry – A novel technique to improve embryo selection in IVF procedures by measuring respiration rates of individual embryos

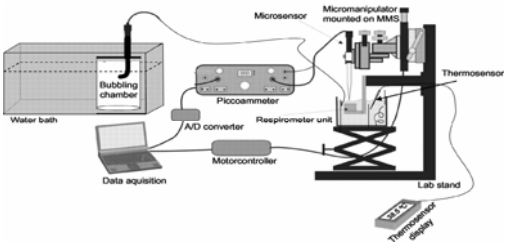
Henrik Callesen, Lars Hauer Larsen, Lars Damgaard, Ana Sofia Lopes, Torben Greve and Niels Birger Ramsing.
Oral presentation at The XVI Nordic IVF Meeting., January 3-6 2005 in Åre, Sweden.



Improved embryo selection using embryo respirometry
Henrik Callesen, Danish Institute of Agricultural Sciences, DK-8830 Tjele, Denmark
Oral presentation, ESHRE June 19-22, 2005, Copenhagen.

Lopes et al., 2007; Scott et al., 2008

Embryo Respiration Unisense

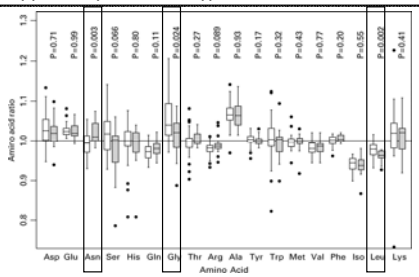


Respiratory category	Pregnant	Non-pregnant
High (>1.10 nl/h)	25% ($n = 1$)	75% ($n = 3$)
Medium (0.78–1.10 nl/h)	100% ($n = 13$)	0% ($n = 0$)
Low (<0.78 nl/h)	48% ($n = 11$)	52% ($n = 12$)

Respiration rates of individual bovine *in vitro*-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. Lopes et al., 2005

Amino Acid Uptake

Box plots of the 18 individual amino acids showing the medians (thicker lines), inter-quartile ranges (boxes), ranges (whiskers; excluding outliers) and outlying observations (open circles) for amino acid appearance in the culture medium



Brison, D.R. et al. Hum. Reprod. 2004 19:2319-2324;

Technique: High performance liquid chromatography.
Results: The turnover of three amino acids, Asn, Gly and Leu, was significantly correlated with a clinical pregnancy and live birth.
Conclusions: Non-invasive assay of amino acid turnover has the potential to improve significantly the prospective selection of the most viable embryos, or single embryo, for replacement in an IVF cycle.

Amino Acid Uptake Novocellus

Immediate goal: 30% increase in IVF success rates.

Ultimate goal - 50% increase in Single Embryo Transfer rates to match those with two or more embryos replaced

Need to use proprietary media

Culture in micro volumes

Proteomics

A proteomic analysis of mammalian preimplantation embryonic development

Mandy G Katz-Jaffe, Donald W Linck, William B Schoolcraft and David K Gardner

Colorado Center for Reproductive Medicine, 799 E Hampden Ave, Suite 520, Englewood, Colorado 80113, USA
Correspondence should be addressed to M Katz-Jaffe; Email: mkatz-jaffe@colocrm.com

Analysis of protein expression (secretome) by human and mouse preimplantation embryos

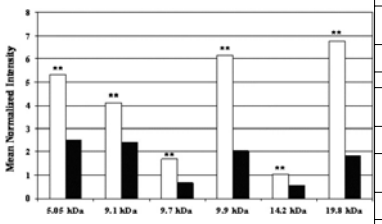
Mandy G. Katz-Jaffe, Ph.D., William B. Schoolcraft, M.D., and David K. Gardner, Ph.D.
Colorado Center for Reproductive Medicine, Englewood, Colorado

Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability

Mandy G. Katz-Jaffe, Ph.D., David K. Gardner, Ph.D., and William B. Schoolcraft, M.D.
Colorado Center for Reproductive Medicine, Englewood, Colorado

Proteomics

Techniques: Anion Exchange Chromatography / time-of-flight mass spectrometry
Results: Several up-regulated and down-regulated proteins were detected in embryos
Conclusions: Protein expression profiles relate to morphology

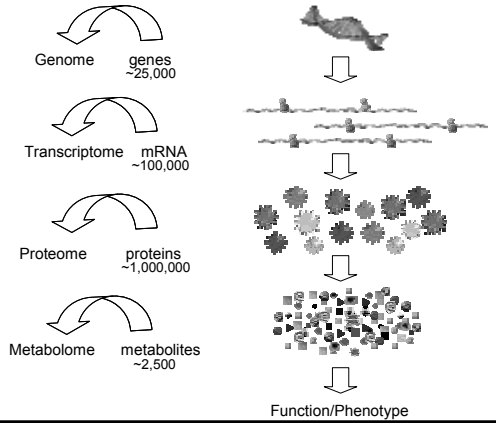


Open bars, degenerating embryos; *solid bars*, developing blastocysts.

Katz-Jaffe. Human embryo proteome. Fertil Steril 2006

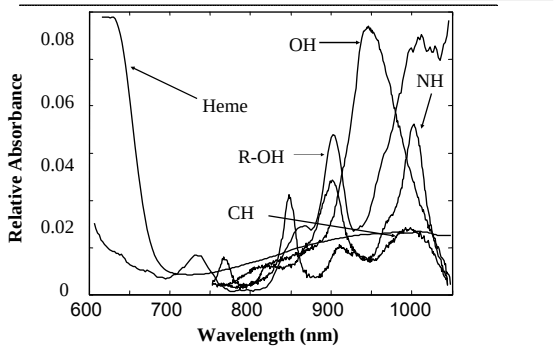
m/z (kDa)	*P*	Potential ID candidates
9.7	0.0009	Heparin-binding EGF-like growth factor precursor (HB-EGF) (MW = 9,729.32)
14.2	0.008	Cystatin-9-like precursor (MW = 14,239.95)
9.9	0.008	CART (MW = 9,941.53)
		NADH-ubiquinone oxireductase (MW = 9,965.71)
9.1	0.004	Beta-catenin-interacting protein 1 (MW = 9,170.35)
		Cytochrome c oxidase subunit VIIIa 3, (MW = 9,174.71)
19.8	0.01	Caspase-1 precursor (MW = 19,843.81)
5	0.01	Inhibitor of growth protein 1 ING1-like tumor suppressor protein, X-linked (MW = 5,076.86 Da)

Flowchart of "Omics" - Metabolomics

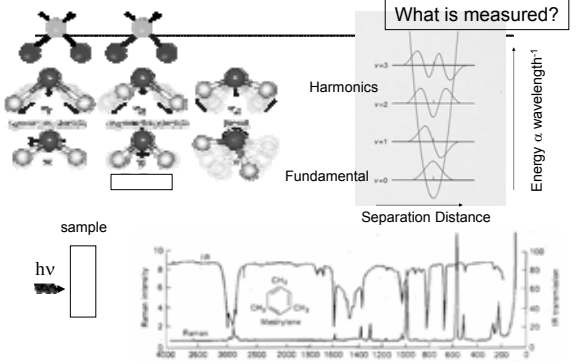


Biomarker Spectral Signatures
(by NIR)

What is measured?



Basis of Vibrational Biospectroscopy



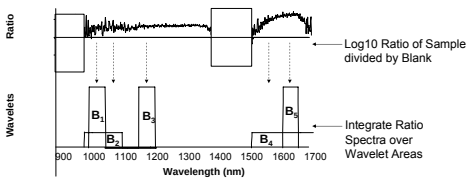
What is Measured?

- Clinically
  - How the embryo modifies its environment
- Biologically
  - Changes in concentrations of:

Functional Groups	Constituents
•CH	•Albumin
•NH	•Lactate
•OH	•Pyruvate
•SH	•Glutamate
•C=C	•Glucose

41

Generation of Embryo Assessment Score

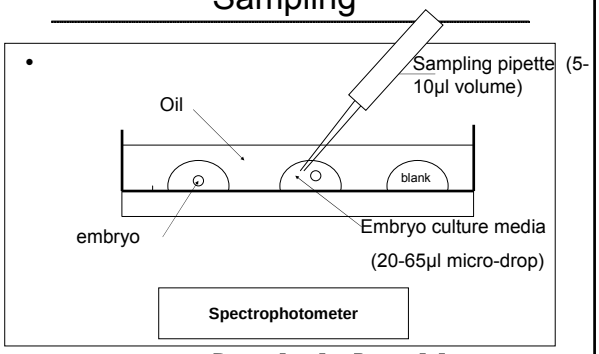


$0.35 + 0.081B_5 - 0.061B_4 - 0.023B_3 - 0.0015B_2 + 0.0026B_1$

ViaTest-E™ Score

42

Non-invasive Embryo Selection Sampling



Reproductive Potential: 9 1

Multicenter, Multinational Study Design Single Embryo Transfer

- Standard IVF procedure
- Single embryo culture
- Morphology assessment
- Single embryo transfer: D2, D3, D5
- Media sample plus controls (7µl)
- NIR spectral analysis; Bioinformatics
- Pregnancy confirmed by FCA (Fetal Cardiac Activity)
- 677 samples from 1768 patients analyzed

Accuracy

- A measure of a test's ability to correctly identify positive and negative FCA pregnancy from a complete IVF patient population.
- The accuracy of a test can be determined by calculating:

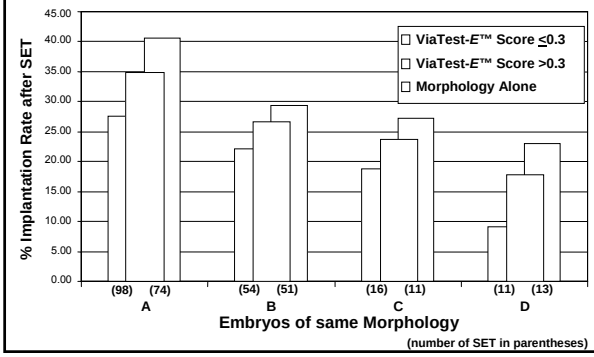
$$\frac{TP + TN}{TP + FN + TN + FP}$$

- where TP = true positive
- TN = true negative
- FP = false positive
- FN = false negative

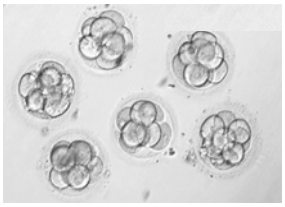
Accuracy of ViaTest-*E*™ vs. Morphology

Day of Transfer	% Accuracy	
	Morphology	**ViaTest-*E*™**
Day 2	31.9	71.3
Day 3	55.0	74.0
Day 5	48.3	79.2
Global Average	45.1	74.8

SET Implantation Rates of Day 3 Embryos Comparing the Same Morphology Grade and a ViaTest-E™ Score of ≤ or > than 0.3



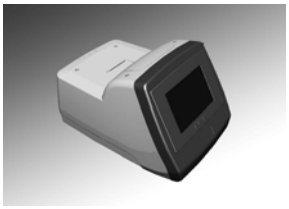
…so the challenge remains



Objective: **Singleton ongoing pregnancy**

How to select the best Embryo???

Where will we end up……?



VS

Conclusions

- The current “traditional” evaluation methods have limited capability to accurately and reliably assess gamete / embryo viability, and developmental potential.

- There is a clear need to improve ART efficiency – higher pregnancy rate and lower (or no) risk for multiple gestation.

- To achieve these goals, a new, reliable (alternative or additional) embryo / gamete assessment technology is required.

Conclusions

- This new system should be a non-invasive, simple, rapid, reliable “on site” system that can fit easily in any laboratory and in any routine.

- Currently, there are a number of techniques under development / investigation that in the future may become useful tools to help to assess gamete / embryo potential / viability.

- These systems have to be evaluated in a prospective, randomized (blinded if possible) fashion to demonstrate their benefit.

An Update on Embryo Culture for Human Art: Media, Performance and Safety

Thomas B. Pool, Ph.D.
Fertility Center of San Antonio
San Antonio, Texas USA

Commercial Relationships

- **Irvine Scientific, Inc.**
- **Incept Biosystems, Inc.**

Learning Objectives

Following this presentation, attendees should be able to:

1. **Identify the nutritional strategies employed in contemporary human embryo culture media.**
2. **Describe the basis of concerns for potential health hazards introduced by embryo culture.**
3. **List the cautions to be taken in evaluating data from animal and human studies with respect to the introduction of potential health hazards.**
4. **Discuss the rate-limiting aspects of contemporary embryo culture and new approaches to alleviating culture-induced stress.**

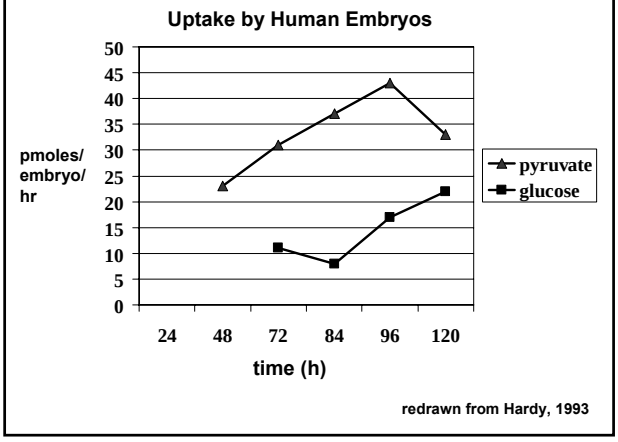
Somatic Cell Media Used for Human IVF and Embryo Culture

Year	*Investigator*	*Medium*	*Use*
1880	Ringer	salt soln	amphib. heart
1907	Tyrode	salt soln	intestine
1943	Earle	salt soln	somatic
1950	Morgan et al.	Med 199	somatic
1956	Eagle	MEM	somatic
1963	Ham	F-10	somatic

Dawn of Specific Media for Human Embryo Culture

Menezo *et al.*, 1984

Quinn *et al.*, 1985

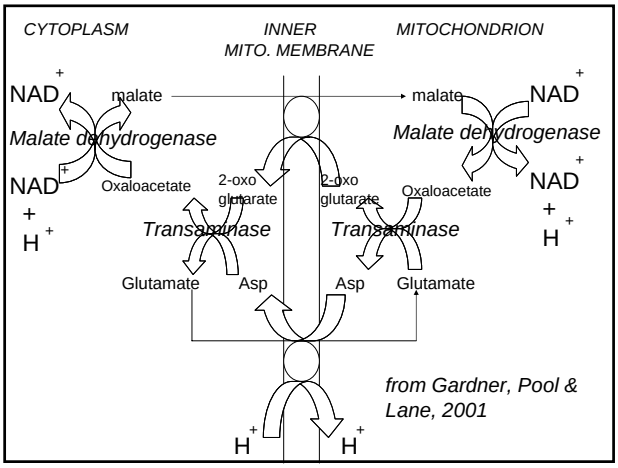


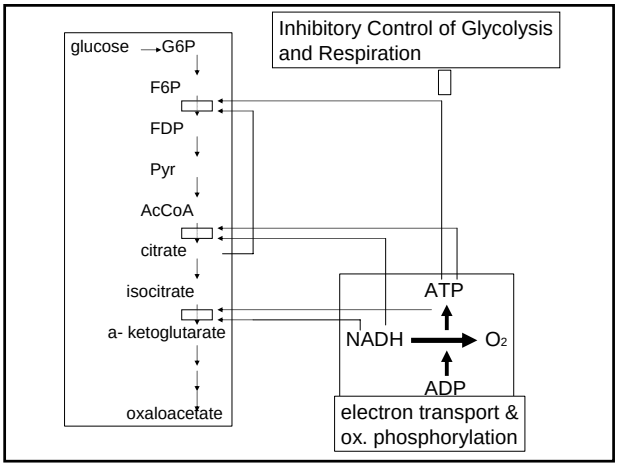
Further Metabolic Considerations

Pyruvate and lactate:

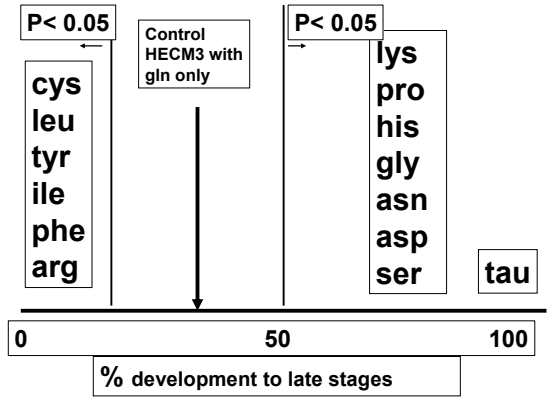
$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xleftrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

1. Conversion regenerates NAD for glycolysis under anaerobic conditions.
2. Cytoplasmic and mitochondrial pools of NADH are not shared.
3. Transfer of reducing power between compartments requires a specific shuttle.





Data from Bavister and McKiernan, 1993



From Gardner and Lane, 1993; Lane and Gardner, 1997

zygotes → 8 cell	> 8 cell
mMTF	mMTF
NEGLN ←	NEGLN
	ESS
	20AA ←
ESS	NEGLN
	ESS
	20AA
20AA	NEGLN
	ESS
	20AA

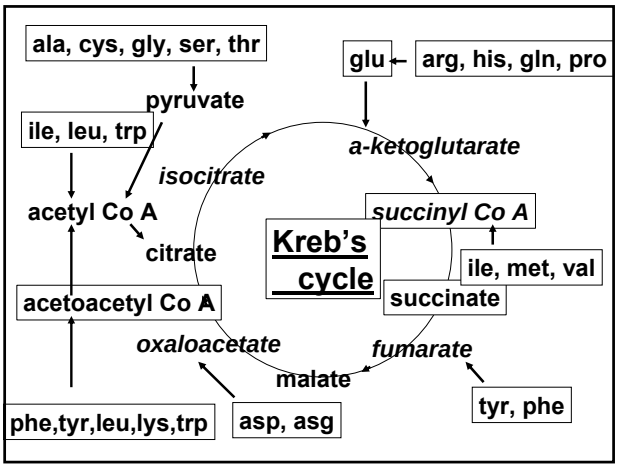
Methionine:

"Essential" amino acid, thus omitted in first interval.

Fuels methylation through synthesis of s-adenosyl methionine

SAM synthesis occurs before genomic activation in humans.

Menezo, YJR. RBM Online 12:616-621, 2006



Adverse effects of ammonium

Ammonium production in embryo culture
Gardner and Lane, 1993

Ammonium in culture induces exencephaly in F1 hybrid mouse
Lane and Gardner, 1994

Ammonium induces multiple problems for mouse blastocyst; ammonium build-up preventable with dipeptide alanyl-gln
Lane and Gardner, 2003

Adverse effects of ammonium

Exposure of mouse embryos to ammonium chloride induces abnormalities, but at a lower rate than previously reported
Sinawat et al., 2003

Reports of effects of ammonium discrepant; studies have produced a biased model of glutamine effects
Biggers et al., 2004

Replacement of glutamine with glycyl-gln enhances mouse preimplantation development
Biggers et al., 2004

EFFECTS OF WHOLE SERUM

Vesicular trophoblast in blastocysts cultured from zygotes in serum:
Gardner, 1994; Gardner et al., 1994
Dorland et al., 1994; Thompson et al., 1995

Abnormal mitochondria,reduced oxidative capacity
Dorland et al., 1994; Thompson et al., 1995

EFFECTS OF WHOLE SERUM

Birth of abnormally large offspring after transfer of embryos grown in serum (LOS in ruminants)

Macromolecular Supplementation

Serum albumin
- **enriched fraction produced by cold alcohol precipitation**
- **recombinant**

Serum albumin plus alpha & beta globulins

Serum albumin plus non-serum polyhydroxylated species (dextran)

Problem: proteins in solution versus oligosaccharides emanating from fixed sites via O-linkage to ser/thre

Common Threads: 1st interval

Sequential culture

1. Reduced glucose with a subset of amino acids (no glucose & taurine or 0.5 mM glucose plus NEAA with glycolytic suppressor)
2. 21 mM lactate
3. 0.33 mM pyruvate

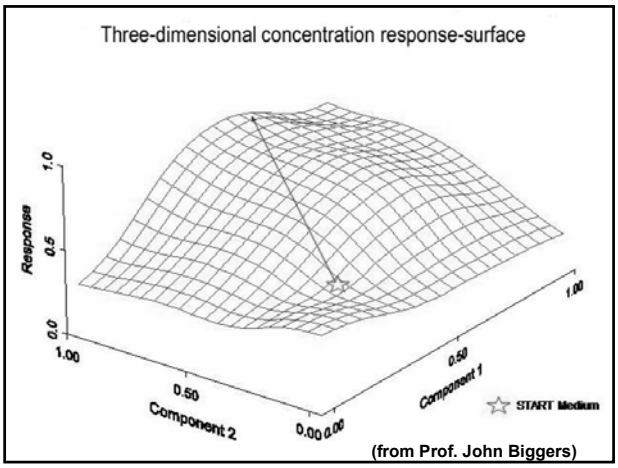
Common Threads - 2nd interval

Sequential culture

1. Elevated glucose (~3 mM) with a complex array of amino acids.
2. Reduced pyruvate (0.1 mM?).
3. Reduced lactate (12 mM?).

Return to Monoculture

Use a complete medium containing electrolytes and energy substrates derived via simplex optimization .



(from Prof. John Biggers)

in either case,

Ensure that glutamine is present in the form of a dipeptide.

Components of a culture system:

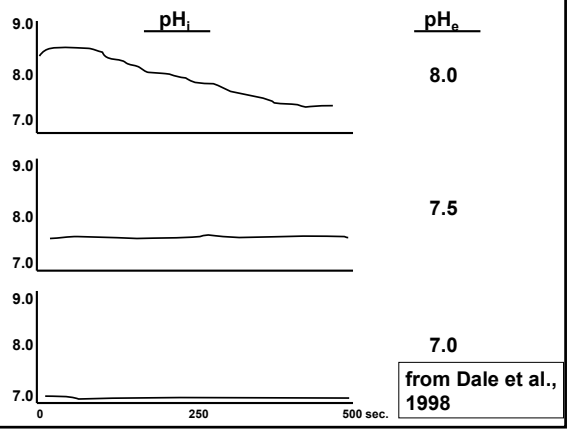
- Culture media
- Macromolecules
- Culture vessel
- Oil overlay
- Incubator
  - temperature
  - humidity
  - gas phase

Take Home Lesson:

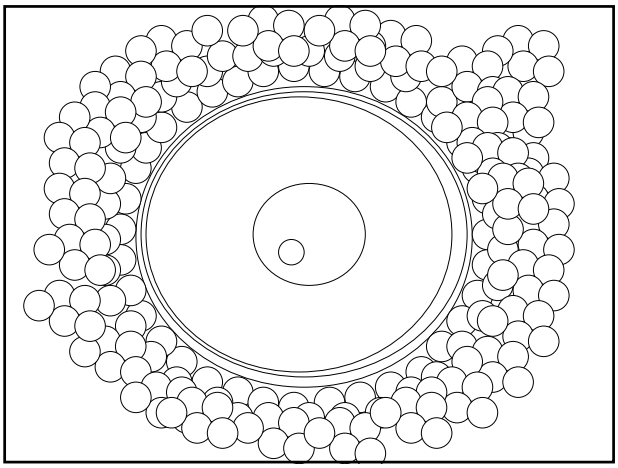
One cannot consider the effects upon outcome of a culture medium formula without simultaneously considering the influence of all other elements of the culture system.

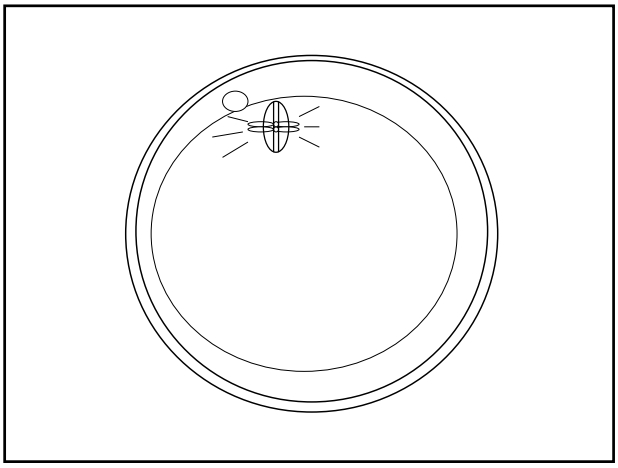
Enhanced and Reproducible Performance from Embryo Culture

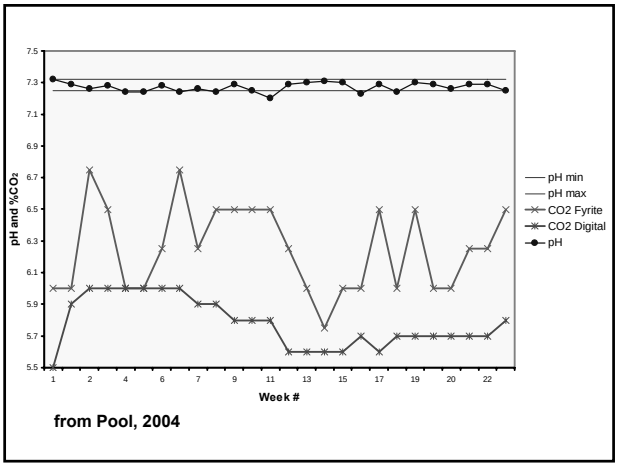
1. **Monitor temperature**
2. **Understand, measure, monitor and manage pH.**



from Dale et al., 1998

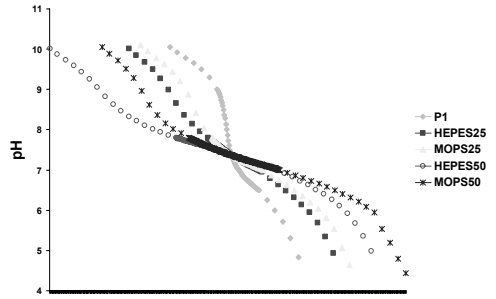




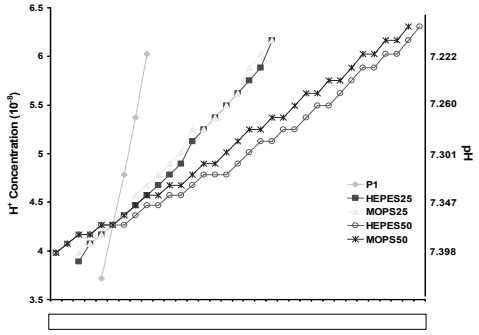


from Pool, 2004

Dose Titration



Dose Titration



The problematic in-vitro embryo in the age of epigenetics

M.H. Johnson, RBMOnline 10 (Suppl. 1):88-96, 2005

Adapting means getting past the cumulative stresses of the culture environment.

Might the embryo accumulate non-lethal defects that later have inappropriate fetal, neonatal or adult consequences?

"primum non nocere"

Epidemics: **"As to diseases, make a habit of two things – to help, or at least to do no harm"**

Epigenetic deregulation of genomic imprinting

Allele-specific expression of imprinted genes:

- **depends on inheritance from mother or father**
- **controlled by DNA methylation in ICR**
- **epigenetic "life cycle" (germline erasure, germline establishment, somatic maintenance) disrupted in some diseases**

Arnaud P. and Feil R., Birth Defects Res.,75:81-97, 2005

Epigenetic deregulation of genomic imprinting

Examples of such diseases:
Beckwith-Wiedemann syndrome (BWS)
Prader-Willi syndrome (PWS)
Angelman syndrome (AS)
Hydatiform mole
Rett syndrome (ICR mediation of imprinted expression perturbed)

Arnaud P. and Feil R., Birth Defects Res.,75:81-97, 2005

What has precipitated the concern?

- Ruminant LOS.
- H19 imprinting errors induced in mouse models with certain media.
- Altered gene expression in bovine embryos.
- Elevated incidence of certain imprinting disorders after ART.
- Imprinting disorders inherited in ESC.

Cautions for interpreting model data:

- Faulty media formulations induce errors.
- Inappropriate medium supplements can induce errors.
- Inappropriate gas phase produces errors.
- Genetic strain influences imprinting errors.
- Ammonium induces imprinting errors.
- Was superovulation used in the study?

"primum non nocere"

Epidemics: **"As to diseases, make a habit of two things – to help, or at least to do no harm"**

"pluralitas non est ponenda sine neccesitate"

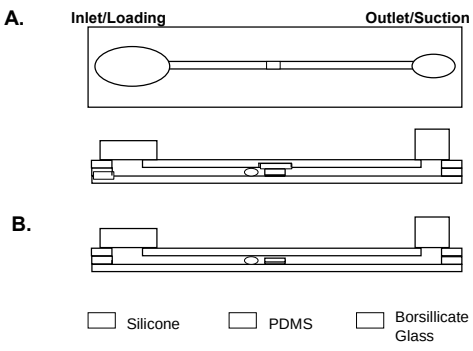
"plurality should not be posited without necessity"

What's wrong with the microdrop?

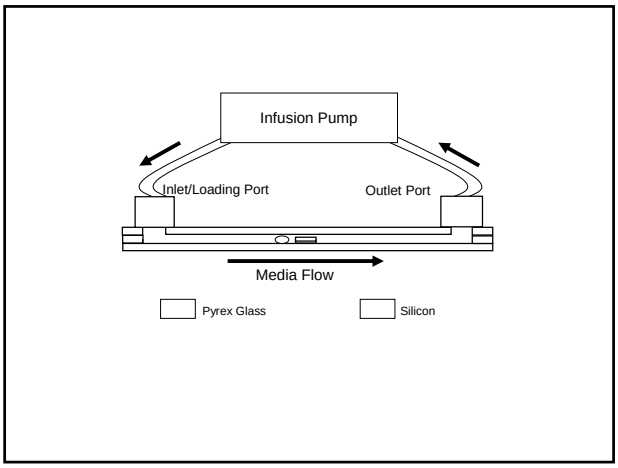
Maximum concentration of solutes obtainable is determined by solubility.

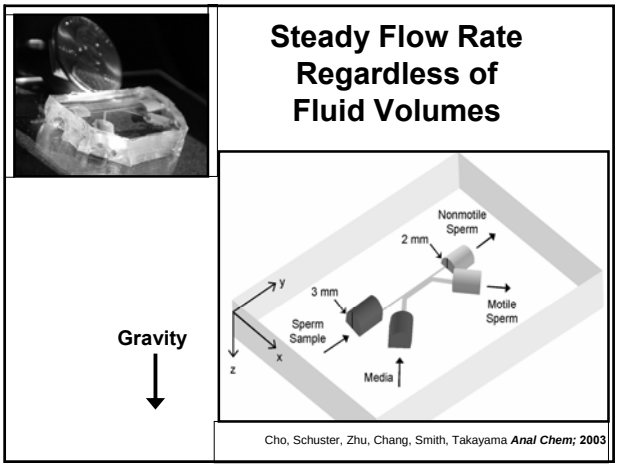
Functionally static

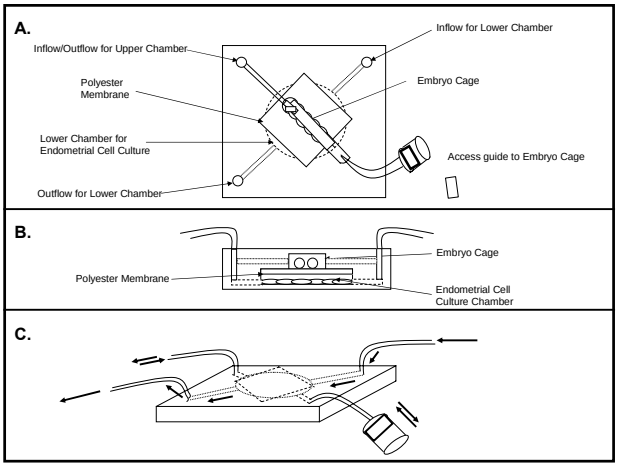
Third dimension not available for surface chemistry.



- from the work of David J. Beebe, Mathew Wheeler et al.


Infusion Pump
Inlet/Loading Port
Outlet Port
Media Flow
Pyrex Glass
Silicon


Steady Flow Rate Regardless of Fluid Volumes
Gravity
Nonmotile Sperm
2 mm
3 mm
Motile Sperm
Sperm Sample
Media
y
x
z
Cho, Schuster, Zhu, Chang, Smith, Takayama Anal Chem; 2003


A.
Inflow/Outflow for Upper Chamber
Inflow for Lower Chamber
Polyester Membrane
Embryo Cage
Lower Chamber for Endometrial Cell Culture
Access guide to Embryo Cage
Outflow for Lower Chamber
B.
Polyester Membrane
Embryo Cage
Endometrial Cell Culture Chamber
C.

Embryo Culture in Microchannels

Raty *et al.*, Theriogenology, 2000

Stage	µF	µdrop
16c/mor. –24h	23.5*	4.7
Blast. – 48h	17.6**	2.4
Blast. – 72h	72.9**	42.9
Hatched – 72h	4.1*	0
Hatched – 96h	26.5**	8.8

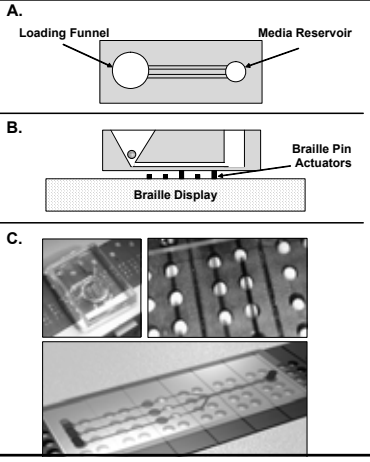
*p<0.05; **p<0.01
In each arm, n = 170

Microfluidics For Sperm, Eggs, and Embryos

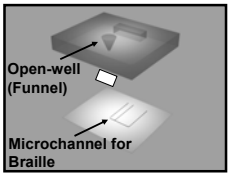
University of Michigan Interdisciplinary Team

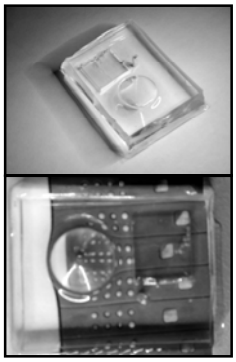
Shuichi Takayama, Ph.D.
Departments of Biomedical Engineering & Macromolecular Science and Engineering
Specializes in Microfluidics for Cell Culture and Analysis

Gary D. Smith, Ph.D.
Department of OB/GYN, Urology, and Physiology
Laboratory Director:
University of Michigan Fertility Clinic
Huntington Reproductive Center of Brazil



Current Embryo Culture Chip

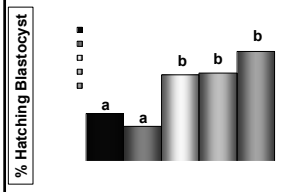




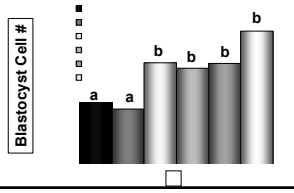
- Easy in, easy out
- 100% recovery
- Quick (reduced pH shifts)
- User friendly

Courtesy of G. Smith / S. Takayama

Microfluidic Embryo Culture



- Improved mouse blastocyst hatching

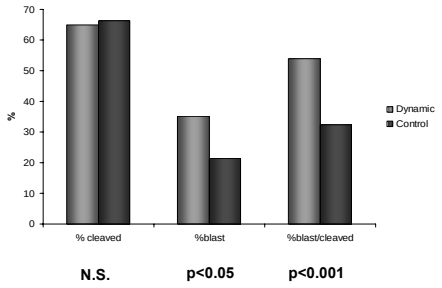


- Increased blastocyst cell #

Cabrera et al. 2006

Bormann *et al.*, 2007

Bovine zygotes, produced in vitro, randomized into 50 µl of KSOM w/ 3 mg/mL BSA in either static drops (n=145) or dynamic culture (n=97) in a microfluidic device, cultured 10 per group.

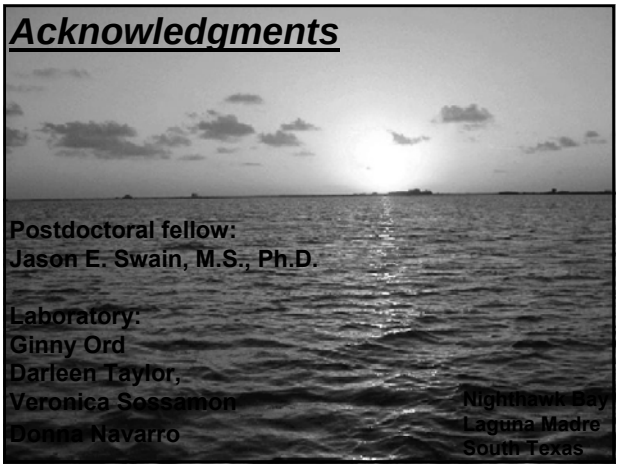


N.S. p<0.05 p<0.001

Potential Applications of Microfluidics

- Micro-culture volumes with flow.
- Medium change by gradient.
- High macromolecular:solvent ratios.
- Fixed-site, 3-dimensional presentation.
- Micro-environmental self-regulation.
- Integration of real-time analytical capabilities (metabolome, secretome) with flow controlled solid-phase, micro-volumetric culture in a single platform.

Acknowledgments



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CLINICA VALLE GIULIA, Rome

24th Annual Meeting of ESHRE
Barcelona - 2008

PRE-CONGRESS COURSE:
The Human IVF Lab in 2008 and beyond

Safe and Efficient vitrification methods for human oocytes: how to do it?

Laura Rienzi, Rome, Italy

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Learning objectives

1) SAFETY
- Possible injuries to the oocyte during cryopreservation
- Possible contamination of the oocyte

2) EFFICIENCY, WHERE ARE WE?
- oocyte cryosurvival
- oocyte/embryo development post vitrification

3) HOW TO IMPROVE IT?
- Factors that may influence the efficiency

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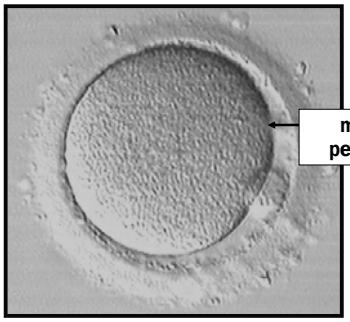
Possible injuries



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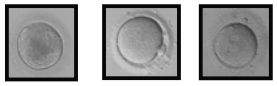
Membrane permeability



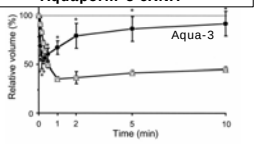
60-65% 35-40% 75-80%

Survival rates of human oocytes frozen with the same slow freezing protocol
(Lassalle et al., 1985)

Aquaporin-9, a protein channel that can transport water and other solutes through the plasmalemma is expressed in rat GV-stage but not mature oocytes (Ford et al., 2000)

+	+	Permeability
+	-	Aquaporin-9 expression

Osmotic response to glycerol of mouse oocytes injected with Aquaporin-3 cRNA



Edashige et al., 2003

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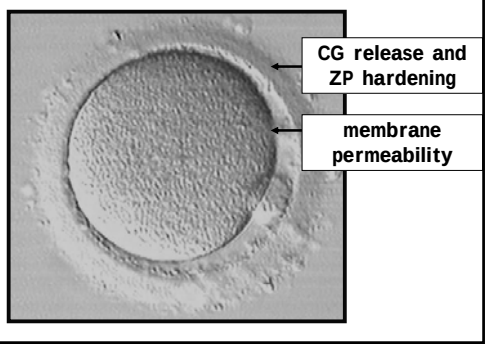
Possible injuries



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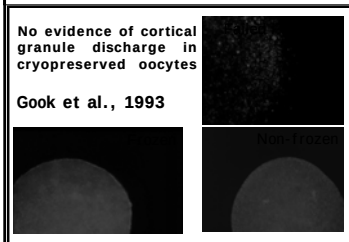
Cortical granules release

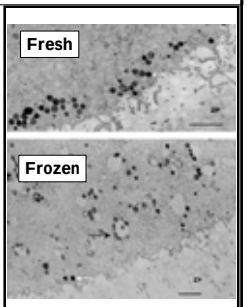
No evidence of cortical granule discharge in cryopreserved oocytes

Gook et al., 1993



"The immunostaining examination for CG of the frozen–thawed oocytes did not reveal evidence of the premature release of CG."

Li et al., 2005



Ghetler et al., 2006

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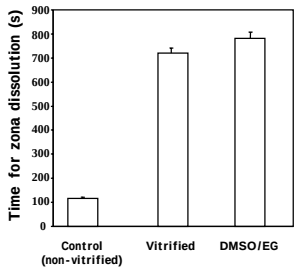
Zona Pellucida Hardening



Larman et al., 2006

n = greater than 60 oocytes per treatment with 3 replicates

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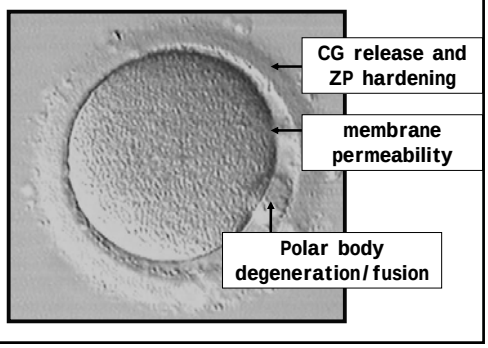
Possible injuries



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Aneuploidy and PB retention

Early reports on failure of PBII extrusion and increase of aneuploidy in thawed mouse oocytes

Glenister et al, 1987; Carroll et al., 1989

	No. of Oocytes (%)		
Frozen	Scored	% Aneuploidy	% Retention PB
+	352	6.4	2.6
-	218	8.0	4.4

No increase in the rates of aneuploidy/digyny in parthenogenetically activated mouse oocytes after cryopreservation with DMSO/slow freezing

Bos-Mikich and Whittingham, 1995

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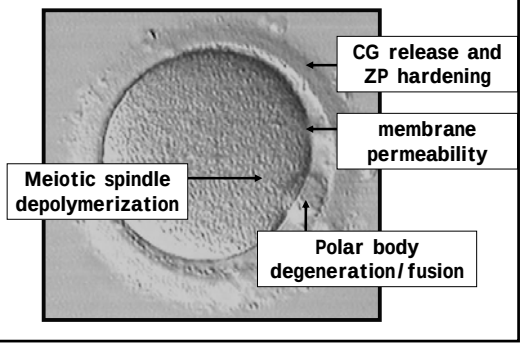
Possible injuries



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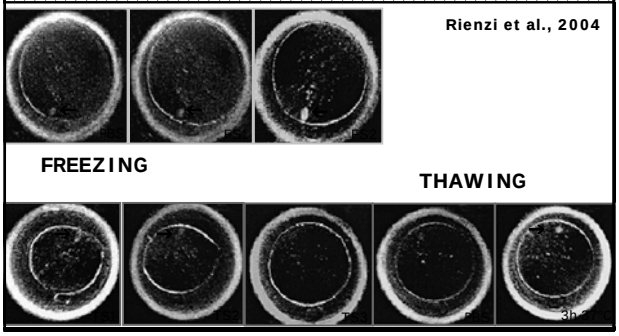
Meiotic spindle analysis during slow freezing



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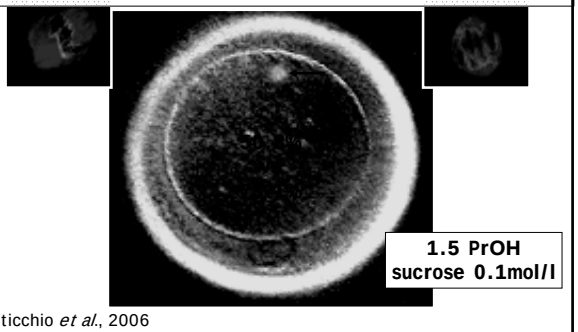
Meiotic spindle analysis during slow freezing



Coticchio *et al.*, 2006

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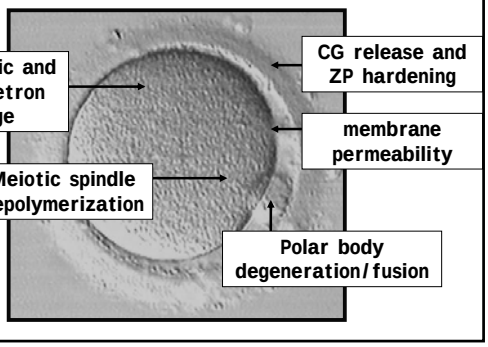
Possible injuries



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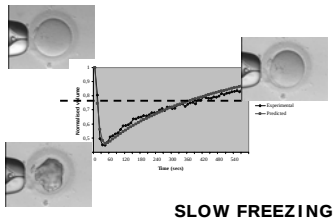
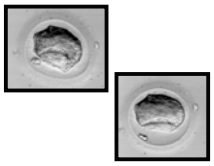
Osmotic toxicity

Coticchio et al., 2004



SLOW FREEZING

VITRIFICATION



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Osmotic toxicity

OOCYTE OSMOTIC TOLERENCE AND OOLEMMA PERMEABILITY

- Temperature of exposure influence shrinking (swelling) patterns
- Oocyte shrinkage tolerance is about 30% of their initial volume
- At 22°C, EG has a lower permeability coefficient relative to DMSO and PG
- The membrane is more selective for EG and DMSO than for PG (mean reflection coefficient Sigma lower for PG)
- Permeability coefficients of individual oocytes varied substantially (inherent biological variability)

Van den Abbeel et al., 2007

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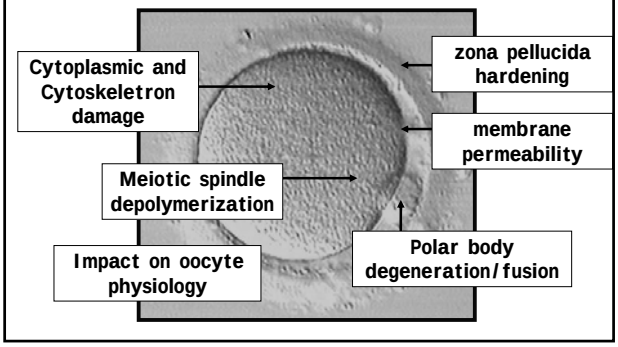
Possible injuries



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Oocyte metabolism post-cryopreservation

METABOLISM MONITORING THROUGH PYRUVATE UPTAKE (mouse oocytes):

Mouse oocytes and developing embryos following slow freezing were metabolically impaired compared with those that were vitrified

…although vitrification was also associated with a decrease in nutrient utilization by the oocyte compared to controls the decrease was significantly smaller than that induced by slow freezing.

Lane and Gardner., 2001; Lane et al., 2002

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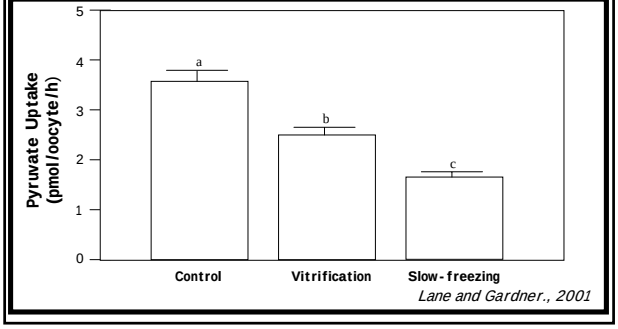
Oocyte metabolism post-cryopreservation



Lane and Gardner., 2001

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Oocyte protein profile post-cryopreservation

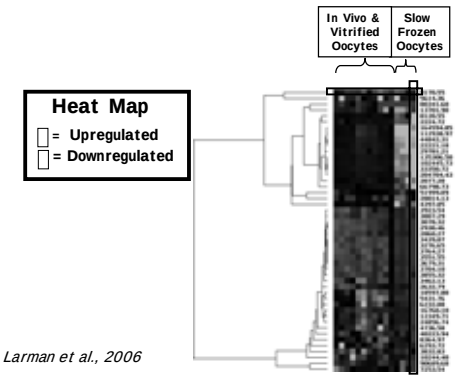
PROTEOMIC ANALYSIS OF OOCYTE PROTEIN PROFILES (mouse oocytes) by SELDI-TOF MS:

Mouse oocytes following slow freezing revealed major alterations compared with those that were vitrified.

Vitrified oocyctes appeared to be similar to the non-cryopreserved control oocytes...

Larman et al., 2006

Hierarchical Clustering of Anionic Protein Profile



Larman et al., 2006

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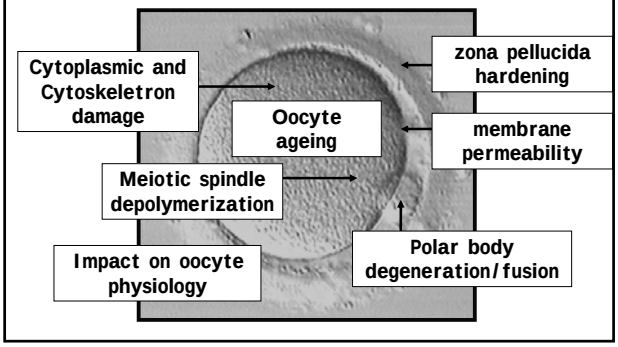
Possible injuries



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Learning objectives

1) SAFETY
- Possible injuries to the oocyte during cryopreservation
- Possible contamination of the oocyte

2) EFFICIENCY, WHERE ARE WE?
- oocyte cryosurvival
- oocyte/embryo development post vitrification

3) HOW TO IMPROVE IT?
- Factors that may influence the efficiency

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Principles of vitrification

1. High levels of cryoprotectants.
2. Extremely fast rates of cooling.
3. No ice crystal formation or damage; straight to a glass.

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TOOLS

Small volume is necessary for extremely rapid cooling rate (about 20,000°C/min).

- Electron microscopic grids
- Open-pulled straw
- Stripper trip
- Cryoloop
- Hemi-straw system
- Cryotop
- Cryoleaf

} OPEN SYSTEM

- Cryotip

...

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Direct contact with nitrogen

OOCYTE CONTAMINATION:

- Not sterile procedure
- Liquid nitrogen may be contaminated by the surface of straws/cryovials or other tools
- Risk of liquid nitrogen mediated disease transmission

Tedder et al., 1995; Fountain et al., 1997; Berry et al., 1998

SOLUTIONS:

- Use of sealed system to avoid direct contact (ref)
- Cooling in liquid nitrogen vapours

Larman et al. 2006, Cobo et al., 2007

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Learning objectives

1) SAFETY
- Possible injuries to the oocyte during cryopreservation
- Possible contamination of the oocyte

2) EFFICIENCY, WHERE ARE WE?
- oocyte cryosurvival
- oocyte/embryo development post vitrification

3) HOW TO IMPROVE IT?
- Factors that may influence the efficiency

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Results of vitrification

Author	Study	Patients	Clinical pregncncies	Abortions	Ongoing pregnancies	Gestational sacs	Freezing protocol
Kuleshova, 1999	ICSI	4	1	0	1	1	VF
Cha, 1999	ICSI	1	1	0	1	1	VF
Yoon, 2003	ICSI	34	6	0	6	7	VF
Katayama, 2003	ICSI	2	2	0	2	2	VF
Chian, 2005	ICSI	25	11				EAF
Kim, 2005	ICSI	13	7				ds
Ruvalcaba, 2005	ICSI	NA	8				
Okimura, 2005	ICSI	NA	12				
Lucena, 2006	ICSI	73	13				OP
Kuwayama, 2005	ICSI	29	12				OP
Kyono, 2005	ICSI	1	1	0	1	2	VF CRYOTOP
Selman, 2006	ICSI	7	2	0	2	2	VF OPS
Yoon, 2007	ICSI	28	13	2	11	17	VT SN2 grids
Cobo, 2007	ICSI	23	14	3	11	20	VF CRYOTOP
Antinori, 2007	ICSI	120	36	8	28	39	VF CRYOTOP
Chang, 2008	ICSI	2	2	0	2	3	VF CRYOTOP
Chen, 2008	ICSI	1	1	1	1	2	VF

117 ongoing pregnancies/deliveries

149 sacs/live births

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Results of vitrification

Variable	Slow Freezing literature 1996-2005	Vitrification literature 2003-2005
Age, mean	33.7	32.3
Fertilization rate	64.9 (2,478/3,818)	74.2 (637/859)
Clinical pregnancies per thawed oocyte	2.3 $x10^{-2}$ (153/6720)	4.5 $x10^{-2}$ (61/1354)
Clinical Pregnancies per injected oocytes	4.0 $x10^{-2}$ (153/3818)	7.2 $x10^{-2}$ (61/859)
Clinical Pregnancies per transfer	20.6 (153/742)	45.5 (61/134)
Implantation rate	10.1 (185/1828)	17.2 (81/473)

Oktay *et al.*, 2006

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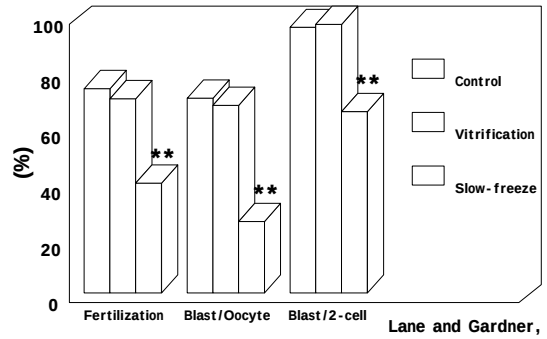
Mouse oocyte vitrification



Lane and Gardner, 2001

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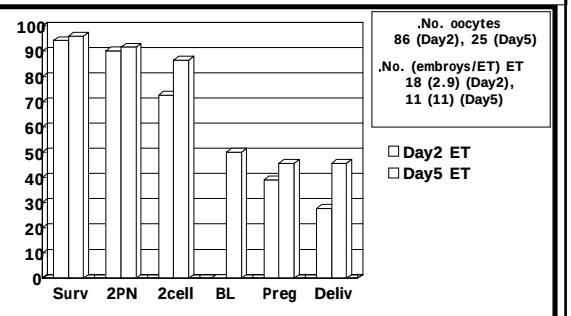
Masa Kuwayama, ESHRE, 2006



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Factors that may influence oocyte vitrification efficiency

1) CRYOPROTECTANTS and intracellular Ca2+

2) TEMPERATURE and meiotic spindle

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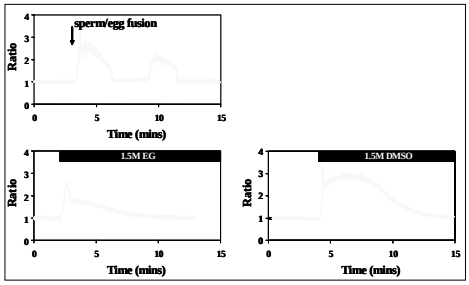
Cryoprotectant and intracellular Ca2+



Larman et al., 2006

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Cryoprotectant and intracellular Ca2+

Increase in intracellular Ca2+ triggers activation:

- Block to polyspermy
- Completion of meiosis and start of mitotic divisions
- Down-regulation of cell cycle proteins
- Apoptosis

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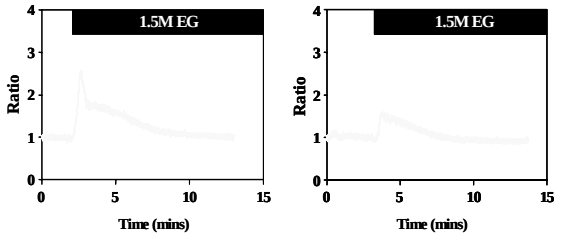
Effect of removing extracellular Ca2+ on EG challenge



Larman et al., 2006

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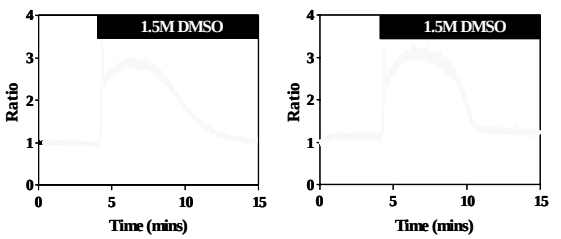
Effect of removing extracellular Ca2+ on DMSO challenge



Larman et al., 2006

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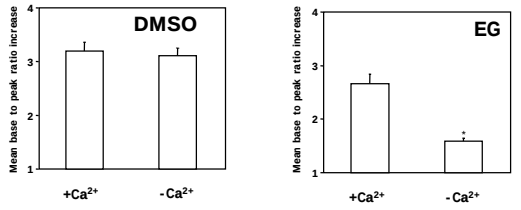
Effect of removing extracellular Ca2+ on cryoprotectant challenge



n = 12 for each treatment with 4 replicates *; p= <0.01

Larman et al., 2006

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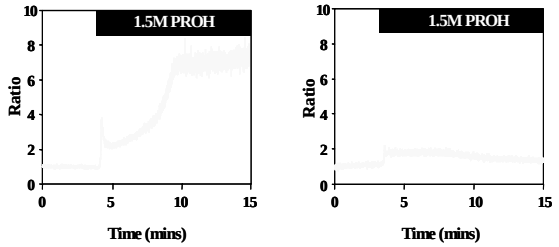
Effect of removing extracellular Ca2+ on PROPANDIOL challenge



Larman et al., 2006

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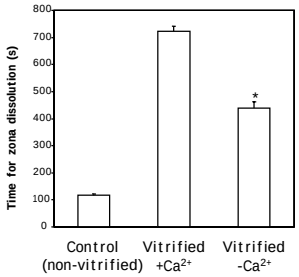
Effect of removing extracellular Ca2+ on ZONA HARDENING



Larman et al., 2006

n = greater than 60 oocytes per treatment with 3 replicates *; p = <0.01

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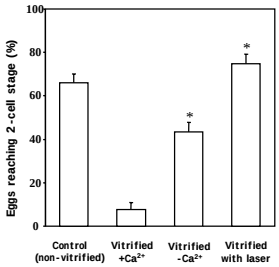
Effect of removing extracellular Ca2+ on FERTILIZATION RATE



Larman et al., 2006

n = greater than 200 oocytes per treatment with 3 replicates *; p = < 0.01

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Factors that may influence oocyte vitrification efficiency

2) TEMPERATURE and meiotic spindle

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Meiotic spindle analysis during vitrification

MEIOTIC SPINDLE AND VITRIFICATION TEMPERATURE:

Non invasive visualization of the meiotic spindle in living mouse oocytes following cryoprotectant exposure at room temperature (RT) and 37°C.

Non invasive visualization of the meiotic spindle in living mouse and human oocytes following vitrification procedure performed at 37°C.

Colorado Centre for Reproductive Medicine & European Hospital Rome

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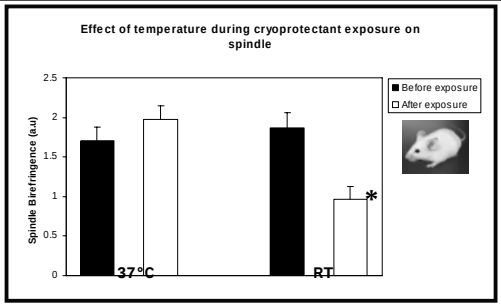
Meiotic spindle analysis during vitrification



Larman, RBM on line 2007

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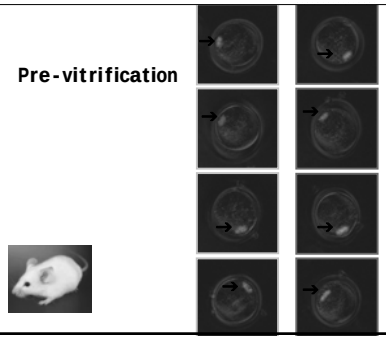
Meiotic spindle analysis during vitrification



Larman, RBM on line 2007

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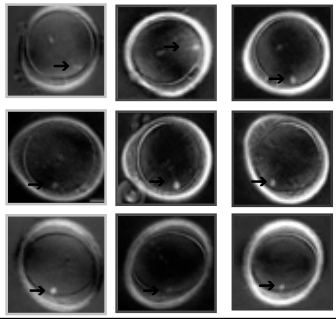
Meiotic spindle analysis during vitrification



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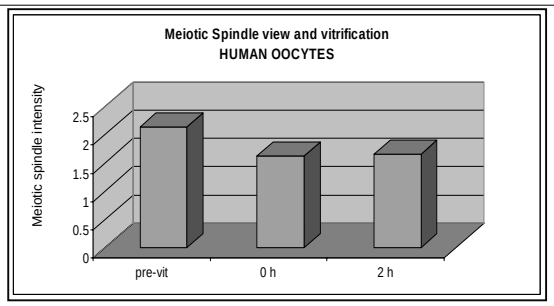
Meiotic spindle analysis during vitrification



Larman, RBM on line 2007

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CONCLUSIONS

Oocyte cryopreservation poses certainly specific problems:

- **The oolemma and not the size of MII oocyte is the key to explain the low survival rates obtained with slow freezing.**
- **Release of cortical granules (controversial)**
- **Chemical toxicity from cryoprotectants (type specific)**
- **Osmotic toxicity**
- **Meiotic spindle depolymerization**
- **Oocyte physiology alteration (metabolism and protein profile)**

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CONCLUSIONS

Safe and Efficient vitrification methods for human oocytes: how to do it?

- **The actual vitrification methods seems already to offer high survival rate. Deriving embryo viability has however to be determined on larger scale.**
- **The choice of the cryoprotectant is very important to minimize oocyte damage and activation.**
- **To reduce MS damage, vitrification procedure has been proposed to be performed at 37°C.**

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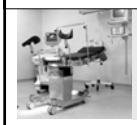
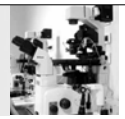
CLINICA VALLE GIULIA, Rome



ginecologia
endocrinologia
embriologia
riproduzione
assistita



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