Chapter 2. Evaluation of first and second polar body formation by Biodainamyc Imaging System

2.1 Abstract

Assisted reproductive technologies including *in vitro* maturation (IVM), *in vitro* fertilization (IVF), embryo culture remains challenging. Two major reasons for their low efficiencies are environmental differences between *in vivo* and *in vitro* conditions and the inability to successfully evaluate the viability of the embryo selected for embryo transfer. Many IVF clinics use the presence of the first polar body as a metric of oocyte maturation, which is the first indicator of oocyte maturation and subsequent embryo viability. However, the practice is controversial because it involves the removal of cumulus cells from the surface of the oocytes, and cumulus cells are essential for continued maturation in case the oocyte has not reached the metaphase II stage yet. Fertilization is another crucial process; whose success determines viability of the embryo and its ability to develop to term. An indicator of successful fertilization is the formation of the second polar body. In human IVF, methods that could ensure the selection of cumulus-oocyte complexes having a first polar body without the need of cumulus cell removal (and thus compromising oocyte viability) is highly desirable. The aim of these experiments was to evaluate intracellular dynamic patterns related to the first and second polar body formation by means of Biodynamic Imaging. The Biodynamic Imaging system is a label-free, noninvasive and objective technology that can provide information about the intracellular dynamics of oocytes and embryos as possible parameters of viability. A total of 42 oocyte-cumulus complexes were assess by the BDI to evaluate the first polar body extrusion, and a total of X potential zygotes were used to evaluate second polar body extrusion. We found that immature oocytes (without a first polar body) showed a tendency of having lower S1 and normalized standard deviation of fluctuating intensities than mature oocytes (with an extruded first polar body), however the difference was statistically not significant. In addition,

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2.2 Introduction

Polar bodies are a byproduct of oocyte meiotic division, by which a small portion of the cytoplasm forms a temporary structure in the polar position of an egg and enclose

one-half of the genome (Senger, 1999). Polar bodies contain chromosomes, cortical granules, ribosomes, Golgi complexes, mitochondria, and other cytoplasmic materials (Shuo et al., 2017). In mammals, PB formation will occur on two occasions: the first polar body extrude during oocyte maturation and indicates meiotic competence. It can be detected by stereomicroscopy and for that the presence of it is used as a metric of viability for oocyte selection in IVF clinics (Klatsky et al., 2010). Meanwhile, the second polar body appears after fertilization. The formation of an embryo is associated with factors such as appearance of the second polar body, formation of the pronuclei, and the first mitotic cell division (Zhang et al., 2012).

Maturation of an oocyte is the first viability indicator for further embryonic development; however, this is a complex process and is frequently evaluated (Lasiene, 2009). Currently, most IVF clinics consider oocyte maturation and obtaining meiotic competence to be successful if germinal vesicle breakdown or first polar body extrusion takes place. Unfortunately, this evaluation is currently done after denuding the oocyte from the surrounding cumulus cells (Dr. Aniruddha, personal communication). As mentioned before, embryo culture in IVF clinics is a challenging technique for two reasons: environmental differences between *in vivo* and *in vitro* conditions and the lack of methodologies to asses embryo viability. The removal of the cumulus cells (denuding) from an oocyte can represent a challenge for *in vitro* embryos. COCs have increased levels of intracellular cAMP and meiosis-inducing factors (Mahmoudi, 2005). Even though it is known that a relationship between cumulus investment and oocyte developmental capacity exists, the underlying mechanisms are still poorly understood. Studies have showed that oocytes with partial or complete removal of cumulus cells have lower fertility rates (Robichaud, 2004). In pigs and humans, embryo developmental rates are lower in the case of denuded oocytes; however, in mice and rats the absence of cumulus cells does not seem to not affect embryonic development (Bing et al., 2002; Maedomari et al., 2007). Meanwhile, the extrusion of the second polar body is also an indicator of meiotic competence and also, successful fertilization. After the sperm head decondenses in the ooplasm, it will form a round body of 5 µm and transform into a male pronucleus, which will remain unchanged for 17-24 hours (Ju & Rui, 2012). Normal fertilized embryos contain two pronuclei; however, *in vitro* culture leads to higher

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fertilization abnormalities in the form of monopronuclear or tripronuclear embryos. For this reason, IVF clinics often choose to monitor pronuclear status during culture, to reduce the possibility of the transfer of abnormal and unviable embryos (Porter, et al., 2003).

A technology with the ability to establish parameters of normal maturation and fertilization of oocytes without compromising their viability status is highly desirable for IVF clinics.

2.3 Materials and methods

2.3.1 Oocyte selection

Ovaries where provided by Indiana Packers Delphi, Indiana USA. They were transported approximately 40 minutes in 0.9 % of saline with 0.75% penicillin and 0.5% streptomycin to maintain temperatures above 30 ºC. Only ovaries that sustain temperatures higher than 30 ºC were used for follicular aspiration at Purdue University, West Lafayette Indiana, USA. Follicles from 3mm-6mm were aspirated with a 10 mL syringe and a 20-gauge hypodermic needle. A total of 35 mL of follicular fluid was collected in 50 mL tubes and placed during 15 minutes in warm water at 39 ºC to create sedimentation of cellular content. The upper liquid was then extracted above 5mL and mix with Hepes-buffered Tyrode's Lactate (TL-Hepes) to be placed in 6 different Petri dishes of 100mm x 15mm. Morphology selection of oocytes for maturation was done by stereomicroscopy, only oocytes with three or more cumulus cells layers were selected.

2.3.2 In vitro maturation (IVM)

COC's were rinsed 3 times in Tissue Culture Medium-199 (TCM-199) supplemented with 0.0007g of L-cysteine (0.1 mg/ml), FSH (0.5 IU/ml), LH (0.5 IU/ml), EGF (10 ng/ml), FGF (40 ng/ml) ,100 uL of IGF (20 ng/ml), 100 uL of and LIF (20 ng/ml). A total of 200 oocytes were incubated during 48 hours in NUNC plates with 500uL of TCM-199 cover with 300uL of mineral oil; at conditions of 39 ºC and 5% CO2.

2.3.3 *In vitro* fertilization

Matured oocytes were denuded in 600 uL hyaluronidase (1 mg/ml) solution and rinsed in TL-Hepes. Morphology evaluation was done by stereomicroscopy; those with an intact plasma membrane and evenly dark cytoplasm were selected to be rinsed three

times in TL-Hepes. Oocytes were transfer in groups of 30, into 50 µl droplets of modified Tris buffered medium (mTBm) covered with 4 mL of mineral oil.

Semen was collected from Purdue University Farm-Swine union and extended with Endura Guard Plus (MOFA) to be stored at 17°C. One mL of semen was added to 9 mL Dulbecco's Phosphate Buffered Saline (DPBS) and centrifuged at 2700 g for 4 mins at room temperature. The supernatant was removed, and the sperm pellet was resuspended into 10 ml DPBS; this process was repeated repeated three times. The pellet was then mixed in 100 µl mTBm and sperm concentration was calculated using a hemocytometer. After concentration evaluation sperm was diluted with mTBm .50 µL of the semen final dilution was added to the 50 µL of mTBm with oocytes.

2.3.4 COC's 1st PB extrusion dynamic evaluation by BDI

Immature(incubation<48 hours) and mature oocytes(incubation=48 hours) COC's oocytes were 3-D evaluated by the Biodainamyc Imaging system. Each oocyte was partially immobilized by TL-Hepes [without PVA] and placed in a 50 uL droplets of TL-Hepes [without PVA] covered with 4 mL of mineral oil. Two different BDI data points were done. First a measurement of 6 different penetration depths from each sample was done to provide a total of 258 data points. The second measurement was focused on an evaluation of the middle point of the sample. Each evaluation under the BDI has a total duration of 30-40 minutes. After BDI evaluation, each embryo was denuded by pipetting in Hyaluronidase enzyme. The presence of the first polar body was observed by means of a stereomicroscope.

Figure 1. Non-polar body in mature oocyte (NPB group)

Figure 2. First polar body in mature oocyte (PB group)

2.4.5 Evaluation of 2nd PB extrusion by BDI

After one hour of co-incubation of the sperms and oocytes in mTBm one potential zygotes was rinsed in TL-Hepes [without PVA] and placed in a 50µL droplet cover with

mineral oil. BDI evaluation was done during 18-24hours at 39 C. The sample was then removed from BDI to evaluate the second polar body presence under stereomicroscopy.

2.4.6 BDI biomarkers

Each embryo is characterized by its Doppler power spectrum. This spectrum is characterized by 13 parameters; these parameters are the potential biomarkers (Table 1) of embryo viability.

Table 1. Biodynamic biomarkers wave properties

BIOMARKERS	PROPERTIES
Backscatter brightness (BB)	Intensity of the reflected light
Dynamic range (Dy or Dr)	Ratio from the largest to the smallest wave intensity
Knee frequency	Maximum amplitude or roll-over frequency in the curve
Normalize standard deviation (NSD)	$= \frac{Normalize\ standar\ deviation\ of\ intensity}{average\ intensity}$
Nyquist Floor	Lowest amplitude
r^1	R^2 range(0.01-0.08Hz)
r^2	R^2 range (0.08-0.4Hz)
r^3	R^2 range (0.4-2.0Hz)
s^1	Slope range (0.01-0.08Hz)
s^2	Slope range (0.08-0.4Hz)
s^3	Slope range (0.4-2.0Hz)
Slope (S)	Change at the curve at midfrequency
R2	proportion of the variance in the dependent variable that is predictable from the independent variable

2.5 Results

Oocytes were evaluated at different depths and layers in order to recognize a specific intra-dynamic pattern related to the presence of the 1st PB and maturation. A total of 42 samples were evaluated in three different groups Immature oocytes (N_{IM}:8), Po;ar Body oocyte (N_{PB}: 24) and Non-polar body(N_{NPB}: 11).

2.5.1 Six layers COC's evaluation

Each COC oocyte was evaluated by 13 biomarkers in six different layers to identify the exact position of the 1st PB. Several biomarkers shown a significant difference within each other; having the potential to become an assessment for polar body presence. A "ONE-WAY ANOVA" was performed to analyze differences among the IM,

NP and PB on each of the dependent variables used (biomarkers) a. In those cases, in which an intergroup effect was found, a Bonferroni Post Hoc test was ran to determine whether the difference was between two groups or among all three.

The "ONE-WAY ANOVA" revealed a group effect on variable(Figure 1A-1H) on [1] **Knee**, F(2,243)=12.819;p=.000; [2] **Slope**, F(2,243)=8.489; p=.000; [3**] Dy**, F(2, 243) = 12.55; p =.000]; [4] **s[1]**, F(2,243)=16.846; p=.000;[5] **Floor,** F(2,243)=15.136; p=.000;[6]**NSD;**F(2,243) =32.7;p=0.000 **[7]s [2]**, F(2,243) =5.44; p=0.005;[6**,** F(2,243) [8] **s[3]**, F(2,243) 15.959 p=0.00. The Bonferroni test detected that for the variable "Slope" the lower value (p<.05) was found for the group PB (M= 1.8262, SD= 0.2413). Such value was lower than that found for group NP (M= 1.932, SD= 0.3241) and NM (M= 1.9920, SD =0.2413).

The relationship between the measured variables was assessed through a Pearson Product Moment Correlation. A positive relationship was found between the variable "slope" and "NSD" (r= 0.127, p=0.047) and "s1" (r= 0.215, p=0.001). On the other hand, the variable "Slope" correlated negatively with the variables "r1" (r= -0.388 p =.000), "s2" (r= -0.273, p= 0.000), "and "s3" (r= -0.345, p=0.000).

Figure 1. Average of biomarker measurements between PB, IM and NPB.

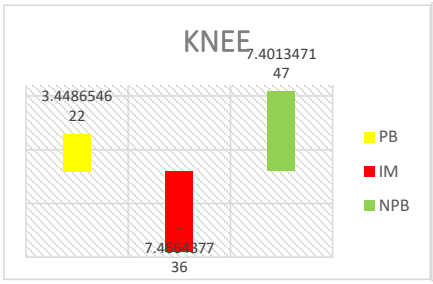


Figure 1A. Average of KNEE PB, IM and NPB measurements

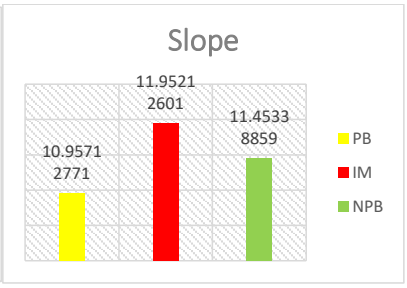


Figure 2A. Average of Slope PB, IM and NPB measurements

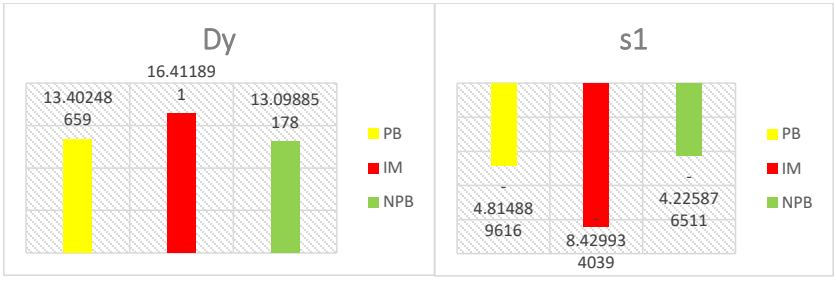


Figure 1C. Average of Dy PB, IM and NPB measurements

Figure 1D. Average of s1 PB, IM and NPB measurements

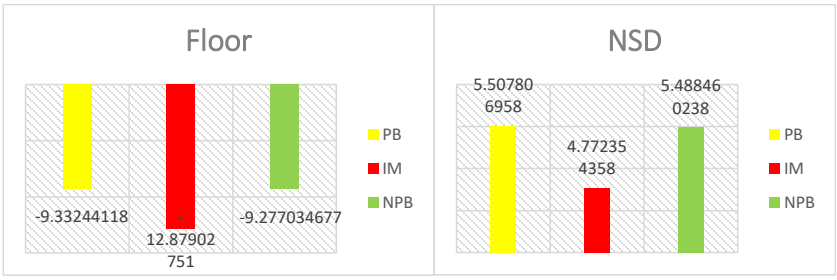


Figure 1E. Average of Floor PB, IM and NPB measurements

Figure 1F. Average of NSD PB, IM and NPB measurements

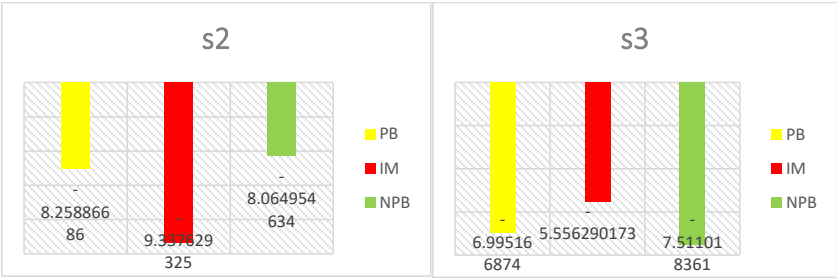
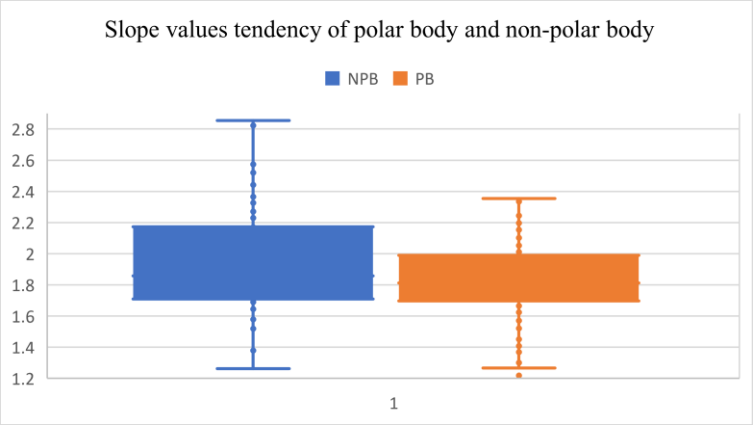


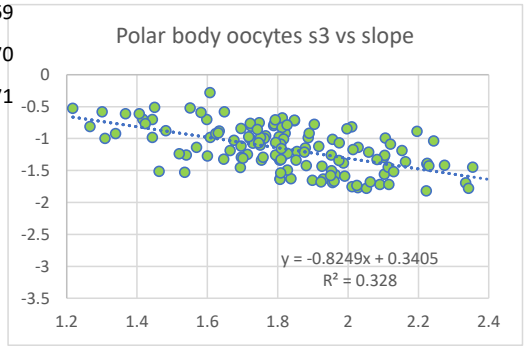
Figure 1G. Average of s2 PB, IM and NPB measurements

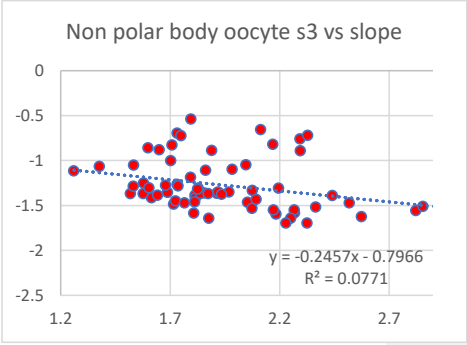
Figure 1H. Average of s3 PB, IM and NPB measurements

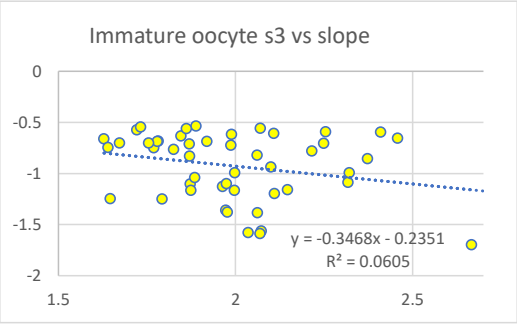
Figure 2. Slope values tendency of polar body and non-polar body

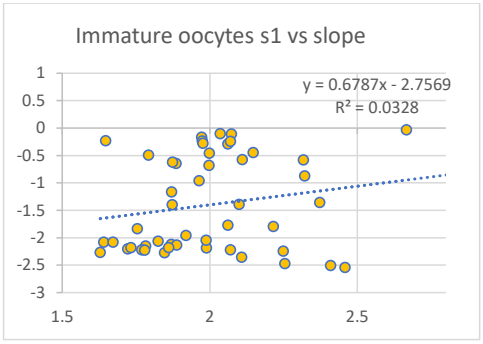


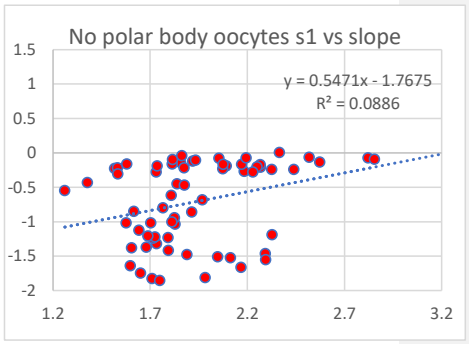
A total of 199 data points are represented in a box plot to show the tendency of the group PB and NPB. Bonferroni analysis detect the biomarker "Slope" has a significant value of PB over NPB and IM group.

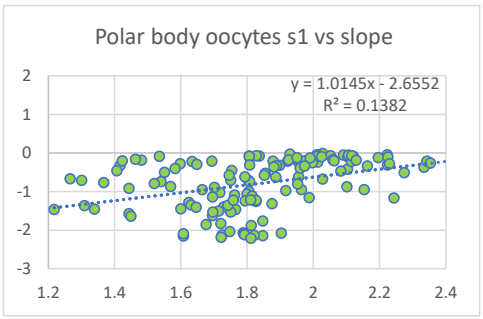


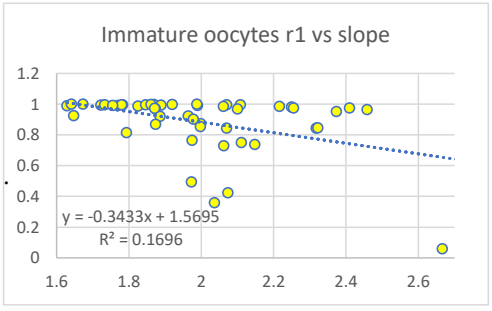


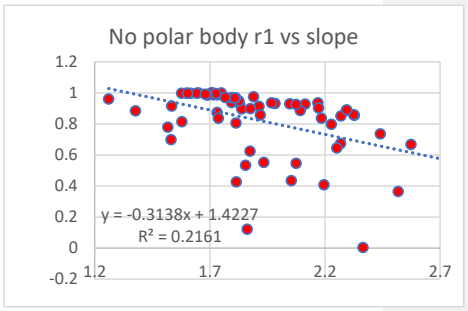


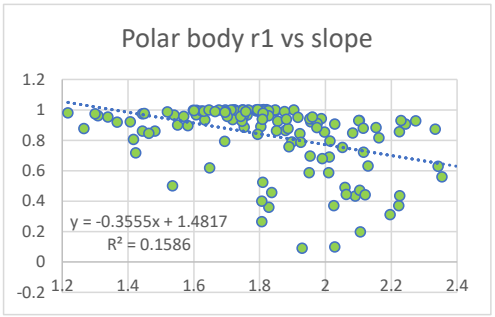

Immature oocytes s1 vs slope
y = 0.6787x - 2.7569
R² = 0.0328

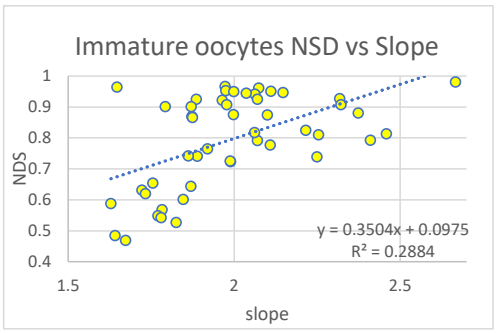

No polar body oocytes s1 vs slope
y = 0.5471x - 1.7675
R² = 0.0886

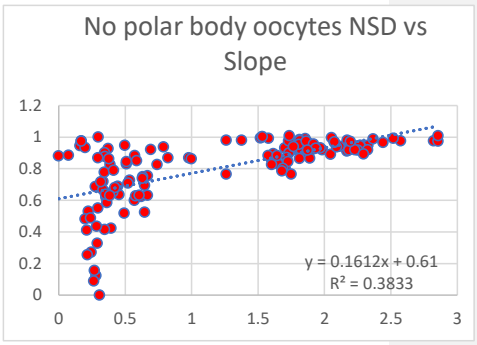

Polar body oocytes s1 vs slope
y = 1.0145x - 2.6552
R² = 0.1382

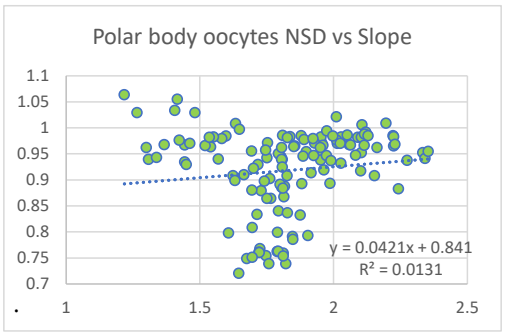

Immature oocytes r1 vs slope
y = -0.3433x + 1.5695
R² = 0.1696

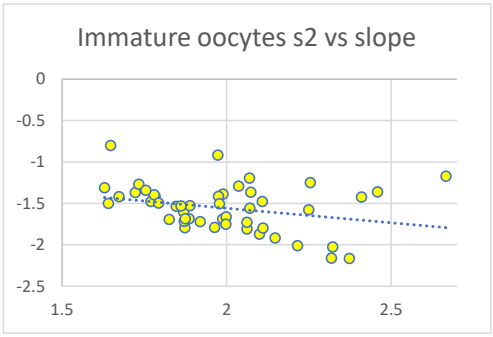

No polar body r1 vs slope
y = -0.3138x + 1.4227
R² = 0.2161

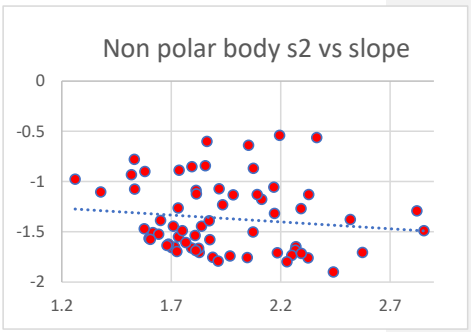

Polar body r1 vs slope
y = -0.3555x + 1.4817
R² = 0.1586

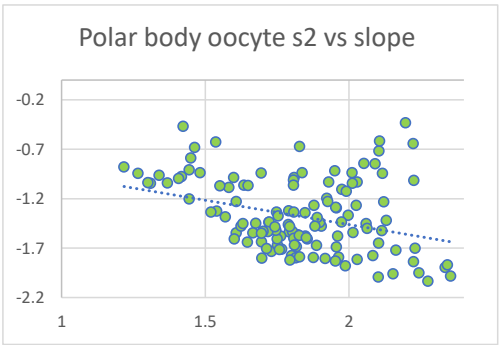

Immature oocytes NSD vs Slope
NDS
slope
y = 0.3504x + 0.0975
R² = 0.2884


No polar body oocytes NSD vs Slope
y = 0.1612x + 0.61
R² = 0.3833


Polar body oocytes NSD vs Slope
y = 0.0421x + 0.841
R² = 0.0131


Immature oocytes s2 vs slope


Non polar body s2 vs slope


Polar body oocyte s2 vs slope

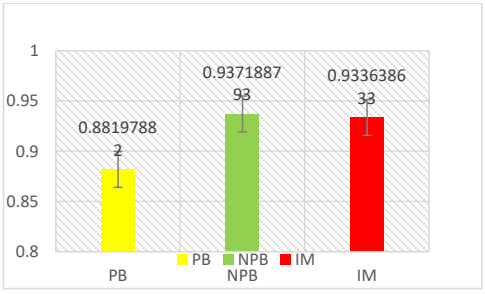


Figure 4A. Average of middepoin NSD of PB, NPB & IM

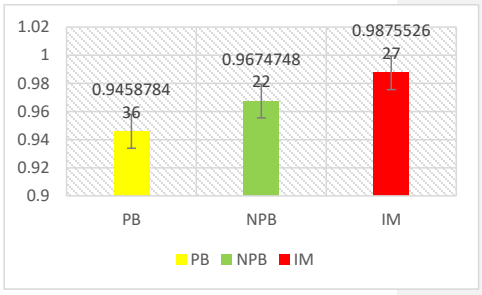


Figure 4A. Average of middepoin r2 of PB, NPB & IM

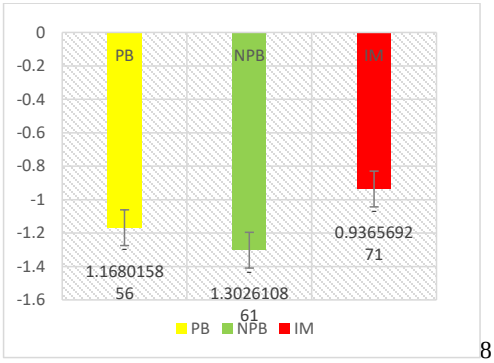


Figure 4C. Average of middepoin s3 of PB, NPB & IM