scoring method (Gardner et al., 2000)) were frozen on day 5 (n = 19) or day 6 (n = 22). We vitrified them individually using Yokota's vitrification method (Yokota et al., 2000). Briefly, a blastocyst was exposed to 10% ethylene glycol for five minutes, and then placed in a 50% vitrification solution (Ishimori et al., 1993) for one minute. The vitrification solution contained modified-HFF with 20% Serum Substitute Supplement (Irvine Scientific, Santa Ana, CA, USA), ethylene glycol, and dimethyl sulphoxide at a 2:1:1 ratio. Finally (within 30 seconds), the blastocyst was loaded into a 0.25-ml plastic straw containing the vitrification solution. Both sides of the straw were filled with a thawing solution containing 0.5 mol/l sucrose. An air space was placed between the vitrification solution and the thawing solution (Figure 2). Following heat sealing, the straws were placed in liquid nitrogen vapor for 30 s, and then plunged immediately into the liquid nitrogen. This method is a hermetically closed system with the liquid nitrogen outside of the straw; thus, the embryos are not exposed to infections.

For blastocyst thawing, the straw was warmed by plunging it into a 27°C water bath; this was done after a one-step dilution of the cryoprotectant and was performed using the entire volume of sucrose solution within the straw. Five minutes after thawing, the embryos were placed in the culture medium.

Evaluation of a Blastocyst both Morphologically and via Respiratory Activity

A week or more after freezing, the blastocysts were thawed and cultured for two hours; then evaluated for the degree of blastomere loss and blastocoel re-expansion. We classified thawed blastocysts as "minimally damaged blastocysts" if they had less than

20% blastomere loss; “severely damaged blastocysts” were those with more than 20% blastomere loss. From the viewpoint of a blastocoel recovery, we evaluated rapid re-expanded blastocysts if > 50% of the blastocoels were re-expanded after two hours of culture following thawing. At that time, the respiratory activity was measured again using the SECM. The reason why we measured respiratory activity after two hours of culture was because, in the clinical setting, we transfer thawed embryos after two to four hours of culture. Each blastocyst was documented by a camera connected to the inverted microscope before freezing (at first measurement of respiratory activity), and during the two hours of culture after thawing (at the second measurement of the respiratory activity). We evaluated the oxygen consumption rate and the morphological quality of each blastocyst by estimating their degree of blastomere loss and blastocoel changes both before freezing and after thawing. The oxygen consumption rate is higher at the inner cell mass (ICM) side than it is at the trophoblast (TRP) side (Shiku et al., 2001); therefore, we located the blastocysts ICM and TRP concentrically at the bottom of a microwell.

Statistical Analysis

The correlation of oxygen consumption rates before freezing and after thawing was analyzed. The mean oxygen consumption rates of two groups were compared using the Student’s t-test. $P < 0.05$ was considered statistically significant.

Results

Respiratory activity and morphological recovery of the good-quality group and damaged group after thawing

We found no relationship between respiratory activity before freezing and after thawing; we also found no significant relationship in regard to respiratory activity between the minimally damaged group and the severely damaged group before freezing. However, significantly different rates of oxygen consumption were found between two groups after thawing (minimally damaged group: 0.59×10^{14}/mol·s^{-1}; severely damaged group: 0.39×10^{14}/mol·s^{-1}; $P = 0.00508$; Table 1).

Respiratory activity and morphological recovery of the rapid re-expansion group, the slow or non-re-expansion' groups after thawing

In the preliminary study, we extended the culture period and evaluated the blastocysts on the day after thawing (n = 30). The rapid re-expanded blastocysts with > 50% blastocoel re-expansion (Figure 3 a-c) during the two-hour culture after thawing exhibited good development on the following day (11/13; 85%). In contrast, the slow- or non-re-expansion group (Figure 3 d-f) exhibited poorer development than the former (8/17; 47%) (Figure 4).

Sixteen blastocysts, which exhibited a rapid re-expansion had significantly higher respiratory activity before freezing than the 25 blastocysts that exhibited slow- or non-re-expansion (rapid: 0.70×10^{14}/mol·s^{-1}; slow- or non-: 0.58×10^{14}/mol·s^{-1}; $P =$

0.01482). Similarly, the former had higher respiratory activity during the two-hour culture after thawing ($0.60 \times 10^{14}/mol \cdot s^{-1}$) than the latter ($0.49 \times 10^{14}/mol \cdot s^{-1}$); the difference was not statistically different (Table 2).

Discussion

We measured the oxygen consumption rate of human blastocysts with the SECM both before freezing and after thawing; furthermore, after thawing, blastocysts that had minimal damage morphologically were shown to have higher respiratory activity (measured after thawing) than severely damaged blastocysts. In addition, blastocysts with rapid blastocoel re-expansion after thawing had a higher respiratory activity (measured before freezing) than those that exhibited slow- or non-re-expansion. In this study, the ages of the embryos were day 5 (n = 19) to day 6 (n = 22); the embryonic stages ranged from early (n = 19) to expanded stages (n = 22) and were grade 3 to 4 by Gardner's blastocyst scoring method. The oxygen consumption rates were not significantly different between embryo ages (day 5: $0.64 \pm 0.18 \times 10^{14}/mol \cdot s^{-1}$; day 6: $0.61 \pm 0.17 \times 10^{14}/mol \cdot s^{-1}$) or blastocyst stages (grade 3: $0.64 \pm 0.19 \times 10^{14}/mol \cdot s^{-1}$; grade 4: $0.61 \pm 0.16 \times 10^{14}/mol \cdot s^{-1}$).

In IVF, patient safety and reassurance are of utmost importance. We must preserve embryos in liquid nitrogen with the avoidance of either infections or contamination. Therefore, we must freeze embryos using a closed container. In regard to this point, we employ a plastic straw in which the ends are closed by heat sealing and a cotton plug. Consequently, the accurate evaluation of the embryo quality without damage is

facilitated. The quantitative prediction of mitochondrial activity was achieved in the embryos evaluated in this study. The SECM developed by Abe (Abe et al., 2004) is a non-invasive and useful system, which can evaluate oxygen consumption rate. The system was based on the spherical diffusion theory; several novel findings have been reported with this system using bovine embryos (Shiku et al., 2001). We previously reported that embryos with the same morphological grade exhibited considerable variation in respiratory rate; therefore, we suggest that this system is of merit for IVF (Utsunomiya et al., 2008). Vitrified blastocysts had significantly lower respiratory activities than non-vitrified blastocysts; furthermore, well-developed blastocysts after thawing were found to have increased respiration compared to arrested or degenerated blastocysts (Yamanaka et al., 2011).

The aim of present study was to demonstrate the correlation between the morphological features and respiratory activity before freezing. There was no significant difference in respiratory activity before freezing between the minimally damaged group and the severely damaged group (Table 1). However, the blastocysts with higher respiratory activity before freezing exhibited a greater potential for recovery (Table 2). Thus, measuring respiratory activity before freezing is an effective method for the prediction of embryo viability after thawing in terms of re-expansion. The clinical efficacy of observing blastocoel re-expansion after thawing was reported by Shu et al. (Shu et al., 2007); they noted that a rapid re-expanded blastocyst (> 50% re-expansion) should be prioritized for transfer. They concluded that rapid re-expanded blastocysts need to be differentiated from slow-and unexpanded blastocysts in post-thaw cultures. To date, the literature contains a few small studies regarding a lower pregnancy rate in the absence

of blastocoel re-expansion (Cohen et al., 1985; Fehilly et al., 1985). Our present study also supports the efficacy of observing the degree of blastocyst re-expansion after thawing to predict the pregnancy outcome.

In conclusion, the SECM can non-invasively measure the oxygen consumption of a single human blastocyst. This technique contributes to the estimation of embryo viability to better evaluate embryos suitable for freezing. In the near future, information regarding the cutoff value for the oxygen consumption rate is needed.

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Figure Legends

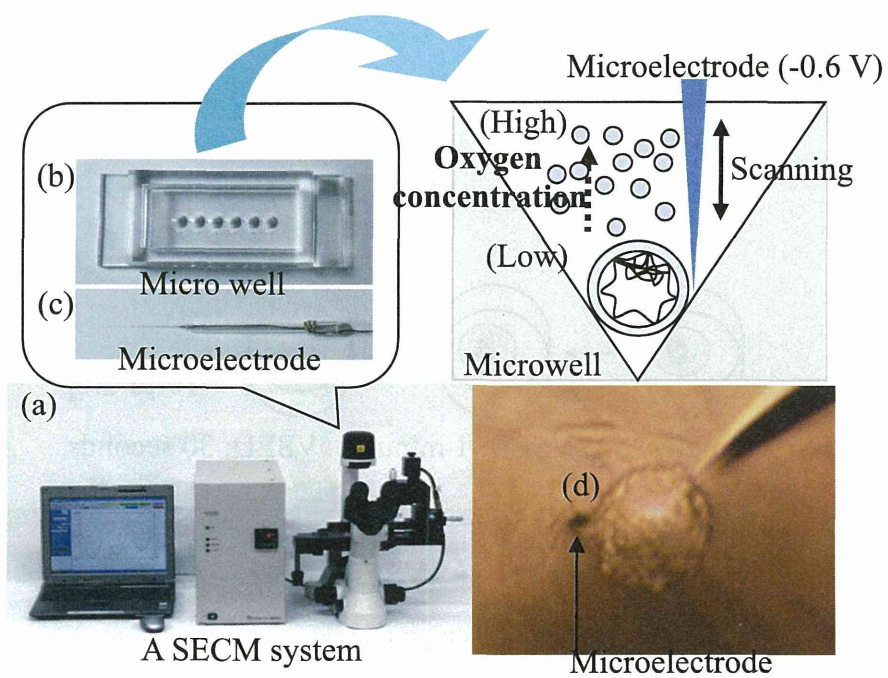
Figure 1. (a) SECM system, (b) a plate, (c) a microelectrode for measuring the respiration activity of the embryos. The embryo is transferred into a microwell filled with medium, the microelectrode sinks down to the bottom of the microwell and remains at (d) the lowest point. Oxygen concentration profiles are calculated with customized software based on the spherical diffusion theory.

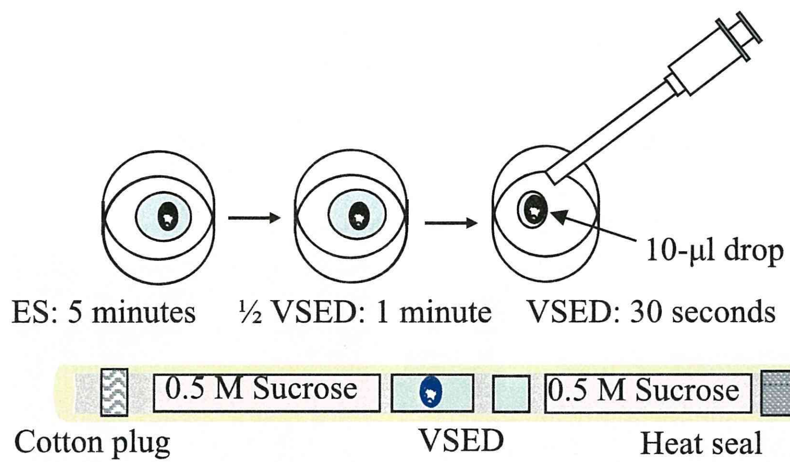
Figure 2 . Vitrification method using a 0.25-ml plastic straw. ES: 10% ethylene glycol.

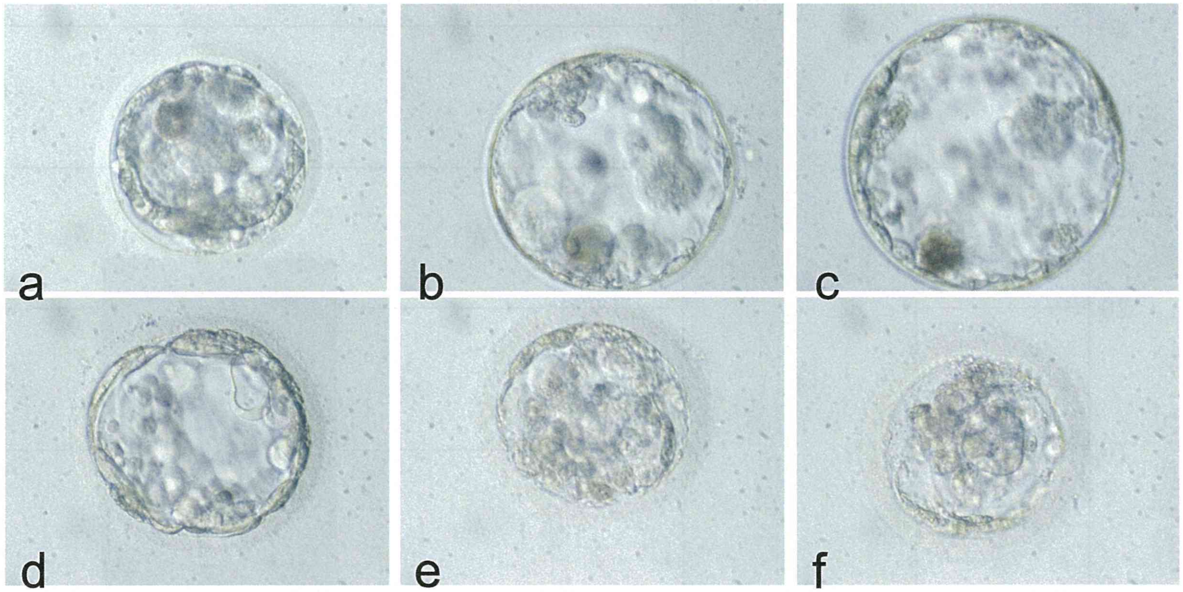
1/2VSED: 12.5% ethylene glycol + 12.5% dimethyl sulfoxide. VSED: 25% ethylene glycol + 25% dimethyl sulfoxide.

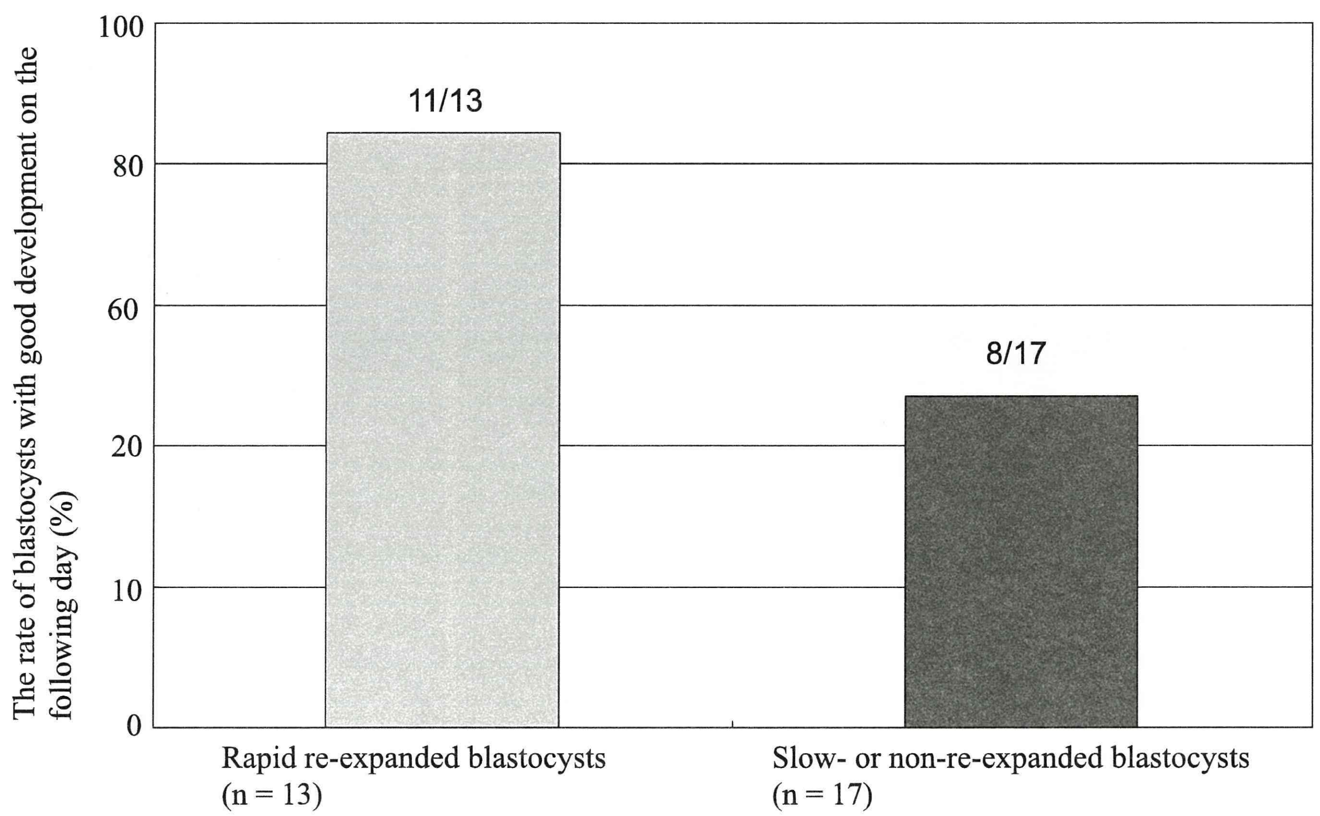
Figure 3. Morphological changes of frozen blastocysts before freezing (a, d), after thawing for 2 hours (b, e), and after thawing for 24 hours (c, f). (a, d) Same initial morphological level. (a–c): (a) Before freezing. (b) Two-hour culture after thawing shows >50% of blastocoel re-expansion. (c) Following day (24 hours of thawing), blastocyst develops to fully expanded blastocyst. (d-f): (d) Before freezing. (e) Two-hour culture after thawing shows no blastocoel re-expansion. (f) Following day, it arrests development. (Original magnification ×400)

Figure 4. Eleven out of 13 rapid re-expanded blastocysts with > 50% blastocoel re-expansion exhibited good development on the following day. Eight out of 17 slow- or non-re-expanded blastocysts exhibited good development.


Microelectrode (-0.6 V)
(High)
Oxygen
concentration
(Low)
Scanning
Microwell
(b)
Micro well
(c)
Microelectrode
(a)
A SECM system
(d)
Microelectrode


10-μl drop
ES: 5 minutes
½ VSED: 1 minute
VSED: 30 seconds
0.5 M Sucrose
0.5 M Sucrose
Cotton plug
VSED
Heat seal


a
b
c
d
e
f


The rate of blastocysts with good development on the following day (%)
100
80
60
20
10
0
11/13
8/17
Rapid re-expanded blastocysts
(n = 13)
Slow- or non-re-expanded blastocysts
(n = 17)

II. 分担研究報告書

3. 新規受精卵呼吸測定装置の開発に関する研究

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厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

新規受精卵呼吸測定装置の開発の関する研究

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研究主旨

クリノ株式会社が開発した受精卵細胞呼吸活性測定装置（CRAS-1.0）は、哺乳類における受精卵の呼吸活性を非侵襲的かつ定量的に測定するための機器である。ヒトに関しても生殖医療の需要は著しく増加しているが、着床能の高い優良な受精卵を選別することには限界があるのが実情である。従来、受精卵の形態学的評価のみで品質評価を行ってきたが、主観性が強く観察者間での結果に差が生じる可能性が高い。そのため、客観的で再現性のある高精度の評価方法が切望されている。今回、従来の主観的な形態学的評価に受精卵呼吸測定装置を用いた客観的な機能評価を加えることにより、優良卵の選別が可能になると考え、全自動受精卵呼吸測定装置の開発を試みた。

従来機器は、呼吸活性を測定する方法として針式のマイクロプローブを受精卵の近傍に移動して上下動の走査で測定する手動方法を採用している。非常に高感度で侵襲もないと考えられたが、正確な呼吸量測定には手技の習得に時間を要する。そのため、従来機器の有用性が証明できたとしても標準診療に取り入れるためにはハードルが高く、普及の妨げになることが予想される。よって、本研究では初心者でも再現性の高い正確な結果が得られ、測定者による差異解消や測定のスピードアップを図る。

測定時に受精卵を測定ウェルにセットした後、全自動で測定出来るようにすることを目標にチップの設計・試作を行った。この容器は受精卵をセッティンングし培養器の中に置くだけで呼吸量が測定可能なため操作性は著しく改善する。そして、ターゲットとなる構造（各部の寸法等）を絞り込むため、構造に関して数項目の最適化を行った。それぞれの寸法を持つチップパターンを評価用プロセスマスクに組み込んでおり、一括でそれぞれのチップを作成することができるため、迅速な構造絞込みが可能となっている。最終的には、このチップが測定数に合わせて数個（6個程度）並んだ形で樹脂プレートに埋め込む計画である。

今後、本機器の有用性・安全性・操作性・経済性を検討しながら改良を加えていき、日常臨床への実用化につなげていきたいと考えている。

研究協力者

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A・研究目的

クリノ株式会社が開発した受精卵細胞呼吸活性測定装置（CRAS-1.0）は、哺乳類における受精卵の呼吸活性を非侵襲的かつ定量的に測定するための機器である。近年、生殖医療の需要は著しく増加しているが、多胎妊娠による母体合併症や低出生体重児の増加が大きな社会問題となり、日本産科婦人科学会は生殖補助医療における多胎妊娠防止に関する見解をまとめ、原則として単一受精卵（胚）のみを移植することが提唱された。そして今後、着床能の高い優良な受精卵を選別することが非常に重要になると考えられている。従来、受精卵の形態学的評価のみで品質評価を行ってきたが、主観性が強く観察者間での結果に差が生じる可能性が高い。そのため、客観的で再現性のある高精度の評価方法が切望されている。今回、従来の主観的な形態学的評価に受精卵呼吸測定装置を用いた客観的な機能評価を加えることにより、優良卵の選別が可能になると考え、全自動受精卵呼吸測定装置の開発を試みた。

B・研究方法

従来機器は、呼吸活性を測定する方法として針式のマイクロプローブを受精卵の近傍に近付けて上下動の走査で測定する手動方法を採用している。非常に高感度で侵襲もないと考えられたが、正確な呼吸量測定には手技の習得に時間を要する。そのため、従来機器の有用性が証明できたとしても標準診療に取り入れるためにはハードルが高く、普及の妨げになることが予想される。また、従来機器は培養器から受精卵と取り出して測定を行うため、本来の培養環境における呼吸量を測定できているか疑問が生じる。よって、本研究では初心者でも再現性の高い正確な結果が得られ、測定者による差異解消や測定のスピードアップを図ることを目的に研究を行った。

具体的には、下記の要件を満たすことが必要となる。

1）受精卵への安全性を考慮し、受精卵検査に伴い用いられる微弱電流が従来機器と同等かそれ以下であること。また、使用する素材が受精卵に悪影響を与えないこと。
2）受精卵の呼吸測定感度が、従来機器と同等かそれ以上であること。
3）従来機器で受精卵を測定する際に用いるディスポーザブル極細プローブは、非常に高価である。しかも機器の操作性が悪いために、プローブを破損する可能性がある。よって、従来のプローブと比較して価格が低廉であり、且つ耐久性に勝ること。
4）従来機器と比較して、受精卵測定時の初期設定が簡便かつセットが容易であり、操作性に優れ、初心者でも高い再現性を得ることが可能で、全自動化を実現することにより検査時間の短縮を図るものであること。

C・研究結果

図１に、検討予定のチップ構造の一例を示す。この容器は受精卵をセッティングし培養器の中に置くだけで呼吸量が測定可能なため操作性は著しく改善する。最終的には、このチップが測定数に合わせて数個（6個程度）並んだ形で樹脂プレートに埋め込まれる形に決定した。

ターゲットとなる構造（各部の寸法等）を絞り込むため、以下の構造に関して最適化を行っている。各部の名称に関して図２に示す。それぞれの寸法を持つチップパターンを評価用プロセスマスクに組み込んでおり、一括でそれぞれのチップを作成することができるため、迅速な構造絞込みが可能である。

① ダイアフラム厚み（5～15um）
② チップ径（φ1mm、φ2mm、φ5mm）
③ 電極サイズ（2um角，5um角）
④ 電極距離（受精卵位置からの距離）（5～300um）
⑤ 貫通孔サイズ（10，20，50，100um）
⑥ キャビティ構造（垂直、テーパー、受精卵からの距離）
⑦ 酸化膜形成（有り、無し）
⑧ 拡散孔形成（有り、無し、拡散孔配置および大きさ）

現段階ではダイアフラム厚みを可能な限り薄くし、貫通孔サイズを大きくして酸素濃度勾配が大きくなるよう検討している。

そして、安全性に関しても従来機と比較し微弱な電流のみで測定可能な予定である。そして、溶媒中に溶出するような素材の使用も想定していない。

また、大量生産が可能となれば、デバイス単価も現行機器以下に抑えることが可能となる。

D・考察

現段階では試作品第１弾の電気化学的検証が行われている。そこで幾つかの課題は提示されているが、概ね諸条件を満たした試作品が完成した。安全性や経済性に関しても引き続き考慮しながら、より一層効果的な測定機器の開発に取り組んでいく。

E・結論

今回、操作性を著しく改善し、さらに培養器内で測定可能な試作品を完成することができた。今後、さらに精度を向上させた機器の開発を行っていく。

G・研究発表

特記事項なし

H・知的財産権の出願・登録状況

特記事項無し

図 1. チップの構造の一例

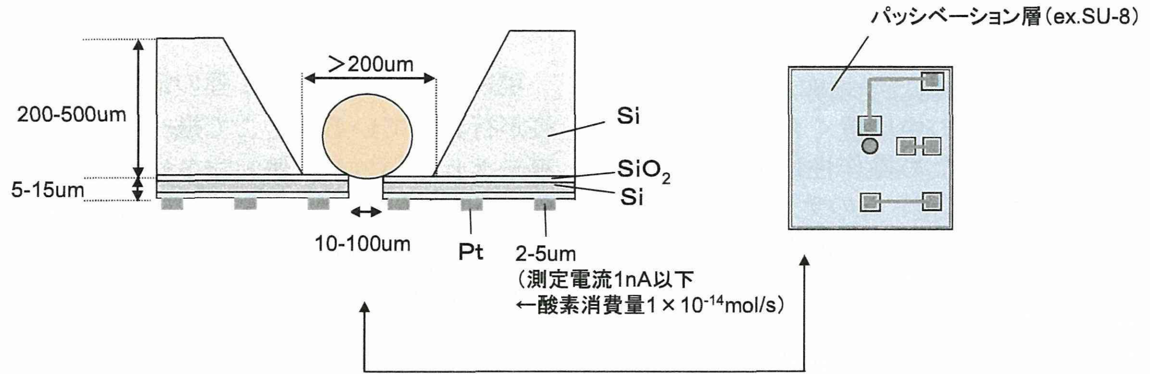
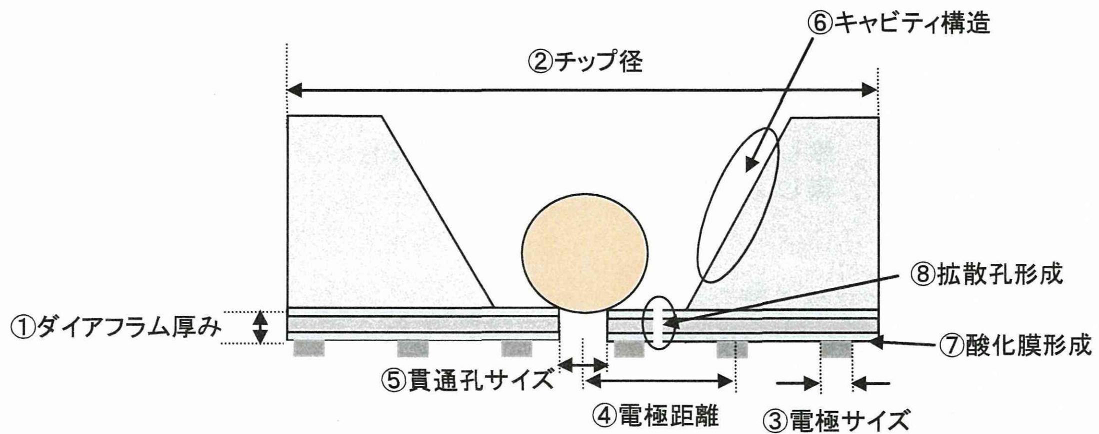


図 2. チップの各部の名称



III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
阿部宏之	胚の呼吸能の測定	柴原浩章・森本義晴・京野廣一編著	図説よくわかる臨床不妊症学入門・生殖補助医療編 第2版	中外医学社	東京	2012	59～68

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tokunaga H, Nagase S, Yoshinaga K, Tanaka S, Nagai T, Kurosawa H, Kaiho-Sakuma M, Toyoshima M, Otsuki T, **Utsunomiya H,** Takano T, Niikura H, Ito K, Yaegashi N.	Small Cell Carcinoma of the Uterine Cervix:Clinical Outcome of Concurrent Chemoradiotherapy with a Multidrug Regimen.	Tohoku J Exp Med.	229	75-81	2013
Tanaka S, Nagase S, Kaiho-Sakuma M, Nagai T, Kurosawa H, Toyoshima M, Tokunaga H, Otsuki T, **Utsunomiya H,** Takano T, Niikura H, Ito K, Yaegashi N.	Clinical outcome of pelvic exenteration in patients with advanced or recurrent uterine cervical cancer.	Int J Clin Oncol.	In press		2013
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Ito K, **Utsunomiya H,** Niikura H, Yaegashi N, Sasano H.	Reply to Comments on "Inhibition of estrogen actions in human gynecological malignancies: New aspects of endocrine therapy for endometrial cancer and ovarian cancer	Mol Cell Endocrinol.	363	133	2012
Nagai T, Niikura H, Kurosawa H, Tanaka S, Otsuki T, **Utsunomiya H,** Nagase S, Takano T, Ito K, Kaiho Y, Nakagawa H, Arai Y, Yaegashi N.	Individualized Radical Hysterectomy Procedure Using Intraoperative Electrical Stimulation for Patients With Cervical Cancer.	Int J Gynecol Cancer.	22	1591-6	2012