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History and Basic Technique of Fluorescence Imaging for Hepatobiliary-Pancreatic Surgery

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Abstract

Recently, fluorescence imaging using indocyanine green (ICG) has been used clinically to visualize the vascular/lymphatic anatomy and cancerous tissues in real time during surgery. Potentially, among the best indications for ICG fluorescence imaging are hepatobiliary and pancreatic diseases since not only the fluorescent property of ICG but also its biliary excretion property can be utilized for imaging. In fact, ICG fluorescence imaging is already being used in clinical settings to identify the anatomy of the bile duct during laparoscopic surgery as well as open surgery in cases of liver cancer. 5-aminolevulinic acid is another fluorescent probe that has been administered to humans for identification of malignant glioma, bladder cancer and epidermal tumor, although its application to hepatobiliary and pancreatic diseases has rarely been evaluated. Preclinically, numerous kinds of novel fluorescent probes are being developed to improve the sensitivity and specificity of ICG fluorescence imaging, making in vivo fluorescence imaging one of the most active research fields in the world.

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In vivo fluorescence imaging, aimed at delineating cancerous tissues and anatomic structures for accurate diagnosis and surgical treatment, has become one of the most active areas of medical research. Among the enormous amount of basic research being published, however, very few techniques have reached the level of clinical use, except for real-time fluorescence imaging techniques using indocyanine green (ICG) and 5-aminolevulinic acid (5-ALA). Herein, we review the history of research on fluorescence imaging techniques using ICG and 5-ALA, and introduce recent advances in the development of novel fluorescent probes that could be applied in the near future to the management of hepatobiliary and pancreatic diseases.

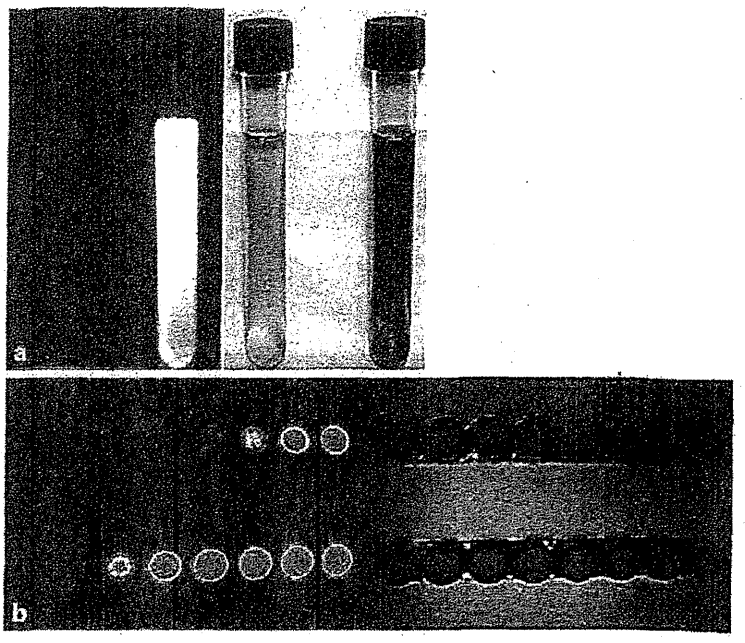

Fig. 1. In vitro fluorescence images of ICG solutions (left) and their gross appearance (right). **a** Although pure ICG solution (0.025 mg/ml) does not show fluorescence (left test tube), it begins to emit strong fluorescence following addition of a small amount of human bile (right test tube). **b** Increasing fluorescence intensity according to the ICG concentrations is visualized on pseudocolor images (left). In the present series, the highest fluorescence intensity was observed at the ICG concentration of 0.025 mg/ml.

Indocyanine Green

For more than 50 years since its approval by the Food and Drug Administration (FDA) in 1954, ICG has been used in clinical settings mainly to estimate cardiac output and liver function. The fluorescence property of ICG was characterized in detail in the 1970s, i.e. protein-bound ICG emits fluorescence that peaks at about 840 nm when illuminated with near-infrared light (750–810 nm; fig. 1) [1]. Because this wavelength is hardly affected due to absorption by hemoglobin or water, structures that contain ICG can be visualized through connective tissue thicknesses of up to 5–10 mm by combined use of an appropriate filter and a camera that is sensitive to the infrared region.

By utilizing the potential of ICG as a fluorescent probe to delineate biological structures, fluorescence imaging using ICG began to be applied clinically to fundus angiography in the field of ophthalmology in the early 1990s [2]. In the 21st century, the application of ICG fluorescence imaging has been extended to the field of surgery, as an intraoperative navigation tool to determine the lymphatic flow in the extremities [3], identify sentinel lymph nodes in patients with breast [4] and gastric cancer [5], and track blood flow during coronary artery bypass grafting [6] and clipping of cerebral artery aneurysms [7]. Little attention, however, has been paid to the fluorescence property of ICG in the fields of hepatobiliary and pancreatic surgery (probably because ICG has been widely adopted as a reagent for measuring liver function), except for the use of portal injection of ICG during surgery as a dye to delineate hepatic segments to be resected. In the last years, though, some researchers revisited the first known property of ICG, i.e. its biliary excretion, and developed the technique of intraoperative fluorescence cholangiography (table 1) [8–10].

Table 1. Clinical applications of ICG fluorescence imaging in hepatobiliary and pancreatic surgery

Reference	Indications	Concomitant use
Identification of hepatic segment		
Aoki et al. [35]	liver resection	
Uchiyama et al. [36]	liver resection	
Kasuya et al. [37]	liver resection	
Ishizawa T et al. [38]	laparoscopic liver resection	
Cholangiography		
Mitsuhashi et al. [9]	open cholecystectomy	angiography
Ishizawa et al. [39]	liver resection and open cholecystectomy	
Ishizawa et al. [15]	laparoscopic cholecystectomy	
Aoki et al. [40]	laparoscopic cholecystectomy	identification of hepatic segments
Tagaya et al. [41]	open/laparoscopic cholecystectomy	angiography
Sakaguchi et al. [42]	liver resection	bile leak test
Mizuno and Isaji [43]	liver transplantation (donor)	
Ishizawa et al. [16]	single incision laparoscopic cholecystectomy	
Hutteman et al. [44]	pancreatoduodenectomy	
Kaibori et al. [45]	liver resection	bile leak test
Kawaguchi et al. [46]	liver resection and transplantation	angiography
Sherwinter [17]	laparoscopic cholecystectomy	
Identification of liver cancer		
Ishizawa et al. [11]	liver resection (HCC and colorectal metastasis)	
Gotoh et al. [12]	liver resection (HCC)	
Ishizawa et al. [47]	laparoscopic liver resection (HCC)	
Yokoyama et al. [13]	liver resection (metastasis of pancreatic cancer)	
Ishizuka et al. [48]	liver resection (colorectal metastasis)	
Satou et al. [49]	resection of extrahepatic metastasis of HCC	
Morita et al. [50]	liver resection (HCC)	
Identification of cholecystic venous flow		
Kai et al. [51]	open cholecystectomy	
Identification of cholestatic hepatic segments		
Harada, et al. [52]	liver resection (liver cancer and bile duct cancer)	
Identification of lymphatic drainage		
Hutteman et al. [44]	pancreaticoduodenectomy	
Hirono et al. [53]	pancreaticoduodenectomy	
Angiography		
Kubota et al. [8]	liver transplantation	
Kaneko et al. [54]	laparoscopic cholescystectomy	
Evaluation of liver function in veno-occlusive regions		
Kawaguchi et al. [55]	liver resection and transplantation	

HCC = Hepatocellular carcinoma.

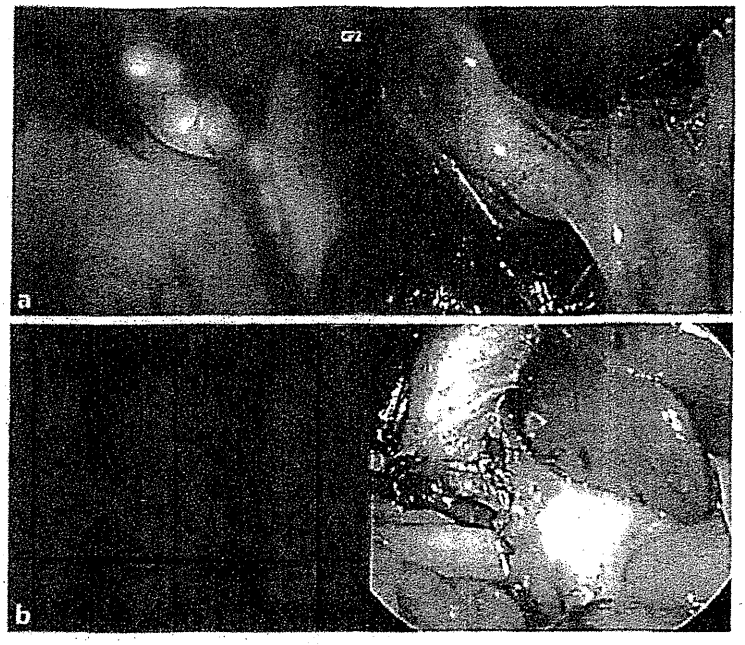

Fig. 2. Fluorescence cholangiography during laparoscopic cholecystectomy. **a** Fluorescence cholangiography before dissection of Calot's triangle using the 1-CCD fluorescence imaging system (Olympus Medical Systems, Tokyo, Japan). **b** Fluorescence cholangiography after dissection of Calot's triangle using the 3-CCD fluorescence imaging system (standard-definition model; Karl Storz, Tuttlingen, Germany).

As a navigational tool during surgery in patients with malignancy, the first clinical applications of ICG fluorescence imaging were identification of sentinel nodes in cases of breast and gastric cancer [4, 5]. Although intraoperative fluorescence imaging of lymph node distribution may have the potential to minimize the dissection area in surgeries for malignancies, fluorescence imaging does not reveal cancer-specific accumulation in metastatic lymph nodes – it only delineates the lymphatic drainage routes from the cancer tissue to the lymph nodes. By contrast, the nature of ICG fluorescence imaging of hepatocellular carcinoma, which was described for the first time in Japan in 2009 [11, 12], is very different from other fluorescence imaging techniques: it allows visualization of the hepatocellular carcinoma itself. Following intravenous administration, the ICG is taken up by the cancer cells, remaining in the cancer tissues at the time of surgery as a result of biliary excretion disorder.

Fluorescence imaging using preoperative intravenous administration of ICG also enables identification of small metastases in the liver, which are difficult to detect by visual inspection, palpation or ultrasonography during surgery [11, 13]. In the case of metastases, ICG accumulates not in the cancerous tissue itself, but in the noncancerous liver parenchyma surrounding the tumor, resulting in the appearance of rim fluorescence around the metastatic cancer on the cut surface of resected specimens [11].

Fluorescence imaging has the potential to be highly suitable for laparoscopic surgery, in which surgeons complete surgical procedures by video imaging. Indeed, since laparoscopic fluorescence imaging systems became commercially available in 2011, the ICG fluorescence imaging technique has begun to be applied to laparoscopic surgery in clinical settings not only in Japan [14–16], but also in the USA [17, 18], Switzerland [19] and Argentina [20], mainly as a tool for navigation of the bile duct anatomy during laparoscopic cholecystectomy (fig. 2). Another application of ICG fluo-

rescence imaging is real-time microscopic visualization of the cellular structure during endoscopic or laparoscopic examinations, which may partly replace pathological diagnosis based on biopsy samples [21].

5-Aminolevulinic Acid

5-ALA is the natural precursor of the heme pathway. In noncancerous cells, exogenous application of 5-ALA results in production of protoporphyrin IX (PPIX) with a fluorescent property; however, PPIX is rapidly metabolized to nonfluorescent heme. In contrast, in malignant cells, administration of 5-ALA can cause overproduction of PPIX, probably as a result of increased activity of porphobilinogen deaminase and/or decreased activity of ferrochelatase [22, 23], enabling identification of cancerous tissues using 5-ALA-induced PPIX fluorescence.

Oral 5-ALA has been approved as an agent for photodynamic therapy of keratosis by the FDA and as an optical imaging agent for intraoperative identification of malignant glioma [24] in Europe and Korea. Intravesical administration of a 5-ALA derivative has also been used for the detection of bladder cancer [25]. However, there are few reports of fluorescence imaging using 5-ALA in the field of digestive surgery, except for its application to the detection of metastasis from gastric [26] and colorectal cancer [27]. A previous report revealed that the tissue concentration of protoporphyrin in the liver and pancreas gradually increases to reach its peak 7–10 h after oral administration of 5-ALA [28]. The author's group also confirmed fluorescence of PPIX in swine liver, pancreas and bile by naked-eye observation following oral 5-ALA administration (fig. 3), suggesting the potential use of 5-ALA as a fluorescent agent for intraoperative navigation during hepatobiliary and pancreatic surgery. The major advantage of 5-ALA-induced PPIX fluorescence is that it lies within optical regions (excitation: 440 nm; emission: 575–675 nm), while this fluorescent property may also pose a limitation with respect to tissue permeability to delineate deeply located cancerous tissues and bile ducts covered with connective tissue.

Novel Fluorescent Probes

Recently, there have been reports on numerous kinds of novel fluorescent probes for in vivo imaging of biological structures every month. Among these, close-to-clinical application techniques are introduced herein (please see the section 'Near-future technology').

One of the most promising indications of intraoperative fluorescence imaging is fluorescence cholangiography to avoid bile duct injury, or at least reduce the need for conventional radiological cholangiography during laparoscopic cholecystectomy, which has become one of the most popular surgical procedures worldwide. Although

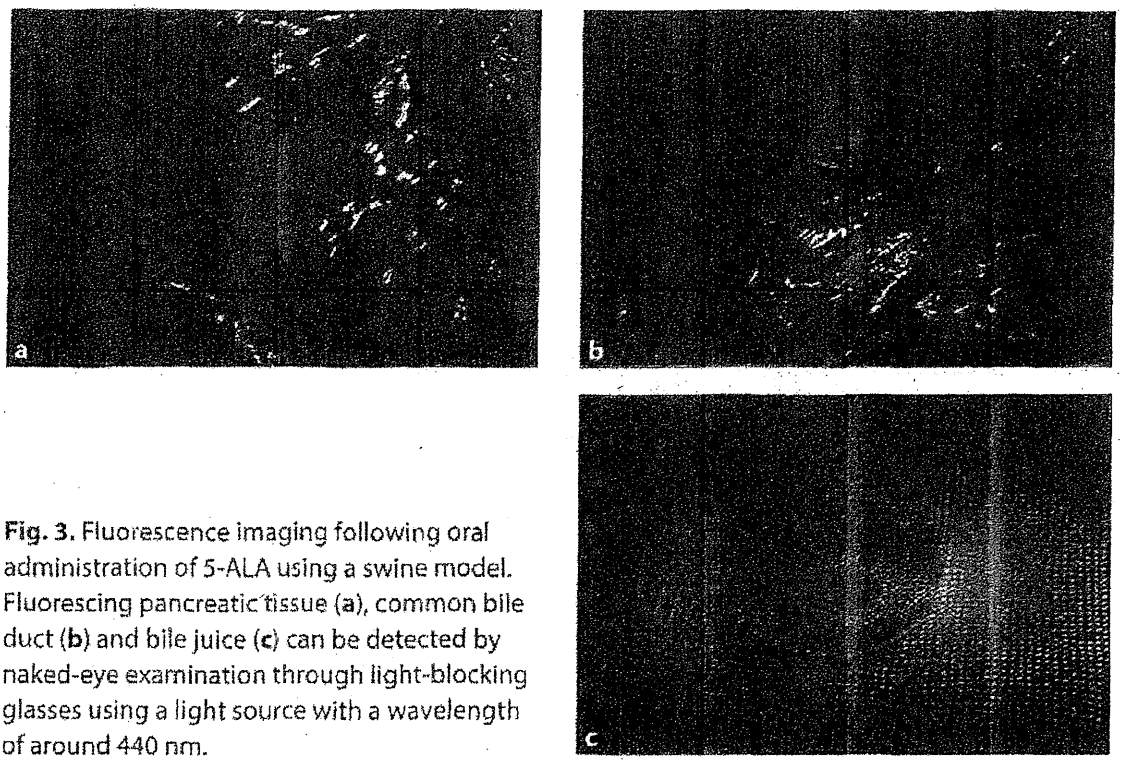

Fig. 3. Fluorescence imaging following oral administration of 5-ALA using a swine model. Fluorescing pancreatic tissue (**a**), common bile duct (**b**) and bile juice (**c**) can be detected by naked-eye examination through light-blocking glasses using a light source with a wavelength of around 440 nm.

ICG seems to have ideal properties for fluorescence cholangiography, novel preclinical agents with optimized pharmacokinetics and tissue permeability have also been developed for use in fluorescence cholangiography [29]. Frangioni and colleagues [30] have developed novel fluorescent probes to be used with ICG and/or methylene blue for simultaneous identification of the bile duct and vascular anatomy using a dual-channel near-infrared imaging system.

Considering the fact that ICG is almost the only fluorescent agent that can be administered intravenously to human subjects and will therefore remain the mainstay for the next few decades, Kobayashi and colleagues [31] at the National Institutes of Health continue to develop antibody-ICG conjugates to visualize specific receptor expressions in cancerous tissues in real time. In contrast, another group has produced novel fluorescent agents by conjugating known antigenic carbohydrates, such as carcinoembryonic antigen, with commercially available fluorophores other than ICG, enabling detection of metastatic lesions from pancreatic cancer in animal models [32]. Instead of utilizing the antigen-antibody reactions between fluorescent agents and cancerous tissues, Urano et al. [33] focused on cancer-specific overexpression of membrane enzymes and developed γ-glutamyl hydroxymethyl rhodamine green, which is completely quenched by spirocyclic caging, but is activated rapidly by a one-step enzymatic reaction in the presence of γ-glutamyltranspeptidase. This kind of probe may be applicable not only to detection of cancerous tissues overexpressing γ-glutamyltranspeptidase [34] during digestive surgery, but also for real-time visualization of pancreatic leaks based on the peptidase activities in pancreatic juice.

Conclusion

Fluorescence imaging using ICG has been used clinically to visualize the lymphatic drainage, liver cancer and bile duct anatomy in real time during surgery, and is beginning to be applied to laparoscopic surgery. In order to further enhance the sensitivity and specificity of intraoperative fluorescence imaging, we need to improve the imaging systems used for visualizing the fluorescence of ICG and also develop novel fluorescent agents that would enable cancer-specific identification of other hepatobiliary and pancreatic malignancies besides hepatocellular carcinoma.

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第8章　霊長類ES/iPS細胞の凍結保存・輸送・解凍

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1　はじめに

培養細胞を取り扱う研究において，細胞の凍結保存は必須の技術である。1949年にグリセロールが凍結保存された精子を保護する効果を持つ[1]ことが発見された後，様々な凍結保護剤が見出され，細胞の凍結保存が可能になったことで細胞生物学の実験結果の再現性が確認できるようになっただけでなく，生殖・細胞移入医療や家畜産業なども大きく発展した。細胞の凍結保存の手法は，一般に緩慢凍結法と急速凍結法（ガラス化法）に大別される。

緩慢凍結法は，グリセロールやジメチルスルホキシド（DMSO）を凍結保護剤とし，1分に1℃程度に温度を下げて徐々に凍結する方法であり，多様な細胞を凍結保存できることが報告されている[2~5]。緩慢に冷却することで細胞内の水分子が凍結保護剤と置換し脱水され，細胞内および細胞周辺部の氷結晶の成長が抑えられ，細胞膜・細胞内構造の損傷や，タンパク質の変性・切断を防ぐ[6]（図1）。プログラムフリーザーを使用し厳密に温度調節することが必要とされていたが，胚の凍結保存を除き一般的な細胞では，プログラムフリーザーを使用せずに発砲スチロールの箱や市販の細胞凍結ボックスを用いて緩慢に凍結する方法が研究室や細胞バンクなどで広く使われている。また，解凍時は37℃のウォーターバスで急速に融解する。このような手法を簡易緩慢凍結法と呼ぶ場合もある。操作が容易であり，多くの細胞種や細胞株でこの緩慢凍結法が用いられている。

一方，ガラス化法は1937年に報告されてから実用化までの技術開発に長い期間を要したが，1985年に高濃度の凍結保護剤を用いる方法が開発[7]され，マウス初期胚の高効率な凍結保存が可能になったことから緩慢凍結法に代わる方法として認知されるようになった。この凍結法は，DMSO，アセトアミド，プロピレングリコール，ポリエチレングリコールを組み合わせ凍結保存液とし，細胞を液体窒素に直接浸漬し急速に凍結することで細胞内外の氷結晶の生成を抑える方法である。緩慢凍結法では困難であったウシ胚[8,9]やブタ胚[10]の凍結保存がこの凍結法で可能になったことから，胚バンクをはじめ多くの機関で用いられている。

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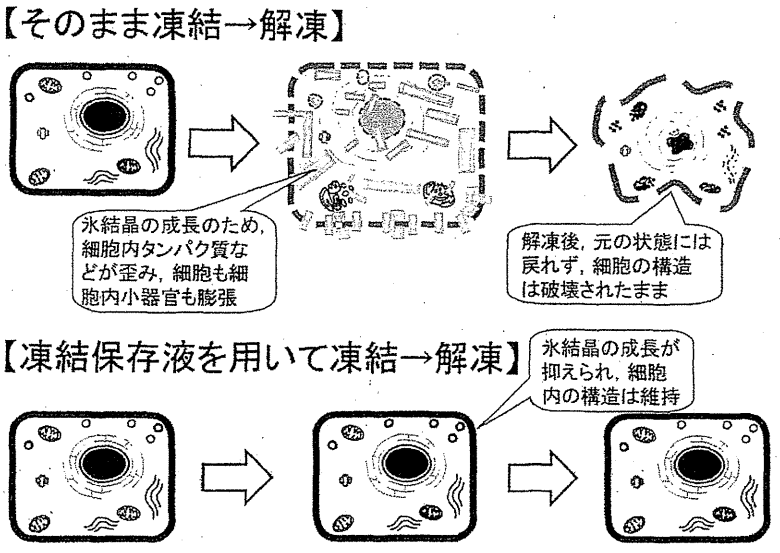

図1　細胞の凍結保存
細胞をそのまま凍結すると細胞内・細胞周辺部で氷結晶が成長し，細胞膜・細胞内構造が破壊されてしまう。凍結保存液を用いると，凍結保護物質により氷の結晶が抑えられ，細胞膜・細胞内構造が維持される。

2　霊長類ES細胞の性質と凍結保存の現状

マウス胚性幹（Embryonic Stem：ES）細胞は1981年に樹立[11]されて以来の歴史の中で，樹立・培養・凍結の最適条件が見出されてきた。マウスES細胞は遺伝子導入後のスクリーニング待ちの間，プレート上でコロニーのまま保存[12]することも可能なくらい，他の一般的な細胞株よりも凍結抵抗性に優れているようにも思える。しかし，霊長類ES細胞はサルで1995年[13]，ヒトでは1998年[14]に樹立されたこともあり，これらの細胞取扱い技術は開発途上である。マウスES細胞の場合はキメラマウスを作製し，そのマウスのキメラ率や生殖系列伝達を確認できることでES細胞としての性質の標準化が明確である。しかし，霊長類ES細胞の場合はそのような実験が不可能なために標準化が曖昧になっていることが，霊長類ES細胞の培養や凍結の最適条件が決まらない理由でもある。またこのような状況の中で，2007年にヒトES細胞とほとんど等価であるとされるヒト人工万能性幹（induced Pluripotent Stem：iPS）細胞が体細胞から人工的に作出され[15]，ヒトES細胞と同様な条件で取り扱われている。

マウスとヒト（霊長類）で同じようにES/iPS細胞と呼ばれているが，それらが有する細胞生物学的な性質において異なる点が指摘されている[16]。その一つとして，マウスES/iPS細胞は未分化維持のためにはコロニーを酵素処理により単一細胞にして継代を行うが，霊長類ES/iPS細胞を単

一細胞に解離させてしまうと細胞死（アポトーシス）が誘導[17,18]されるために，コロニーを10～50程度の細胞塊にして細胞間接着を保ったまま行わなければならない。継代時だけでなく，凍結・解凍する際も同様にコロニーを分割した状態で行う。もう一つは，緩慢凍結法における解凍後の回復率の違いがあげられる。マウスES/iPS細胞は，操作の簡便性や解凍後の回復率の良さなどを考慮して市販の凍結保存液を使用する研究者も多いが，一般の細胞株と同様に10% DMSOを含有した培地／ウシ胎仔血清を用い緩慢凍結法により凍結保存することができる。一方，霊長類ES/iPS細胞の細胞塊を10% DMSO含有培地で緩慢凍結法により凍結保存すると，解凍後の回復率が著しく低い[19]。細胞内に形成される氷結晶が細胞構造に損傷を与えている可能性がある[6]が，胚のような細胞集団を凍結するときもこの方法では凍結できないことを考えると細胞塊の状態は緩慢凍結法には向いていないのかも知れない。そのため，新規の凍結保存液の開発が望まれている。

細胞質の容積が他の細胞に比べて大きい，または単一細胞ではなく複数の細胞（割球）で構成されている初期胚はガラス化法で凍結保存する。細胞塊の状態で凍結保存をするヒトES細胞の凍結保存もガラス化法が用いられるようになった[20]。これは，ガラス化保存液中に細胞塊を懸濁し，哺乳類の初期胚などを凍結保存する際に用いるストロー内に移した後に液体窒素に直接浸漬して急速にガラス化状態にする方法である。また，将来的な医療への応用を考慮し，ガラス化保存液に含まれているウシ胎仔血清をヒトアルブミンに置き換えることで異種成分を排除することも可能となった[21]。しかし，これらのストローを用いるガラス化法は，凍結・解凍の操作が煩雑であり，またごく少量の細胞しか凍結保存できないという欠点があった。その後，ストローの代わりに凍結チューブを用いても凍結保存が可能であることが示され，ガラス化保存液の組成を最適化することにより，緩慢凍結法よりも劇的に回復率が向上したという報告[19]がある。この凍結法を簡易ガラス化法と呼ぶ場合もある。この方法により，従来のガラス化法よりも操作性が向上し，さらにより多くの細胞を凍結保存できるようになった。日本国内では霊長類ES/iPS細胞の凍結保存には主にこの方法が用いられている。なお，米国・カナダでは，回復効率が悪くても，一般的な10% DMSO含有培地で操作が簡便な緩慢凍結法により凍結保存している研究者が多い。

3　ドライアイス輸送を考慮した霊長類ES/iPS細胞用凍結保存液の開発

我国内では霊長類ES/iPS細胞の凍結保存は，ガラス化法が主流である。しかしながらガラス化法は，ガラス化保存液の高い浸透圧のため細胞に対する毒性が強く，細胞をガラス化保存液に懸濁してから液体窒素に浸漬するまで15～30秒程度という短時間に行わなければ解凍後の回復率が極端に低くなってしまう[19]。そのため，操作には熟練を必要とする。また，ガラス化保存液は－130℃より高い温度で保存するとガラス化した水が脱ガラス化し，細胞内外で再結晶することによって細胞膜・細胞内構造に損傷を与える。したがって，ガラス化細胞はドライアイス輸送ができず，液体窒素中で輸送しなければならない。特別な液体窒素輸送箱が必要となり，輸送費用もかさむ。

そこで筆者らは，上記の問題を克服するため，DMSOとヒト血清アルブミンの2つの成分に着目し，霊長類ES/iPS細胞の緩慢法凍結保存液を開発した（製品名：バンバンカー®hRM 以下開発品とする）。そして，サル（コモンマーモセット）ES細胞を用いて開発品の保存，解凍後の回復率，およびドライアイス輸送における安定性を検討した。

セミコンフルエント状態のサルES細胞コロニーを小さく分割した後培養容器から剥離し，開発品を用いて緩慢凍結法で凍結した。コントロールとして，一つに既存のガラス化保存液でガラス化法，もう一つに10% DMSOを含む霊長類ES/iPS細胞用培地で緩慢凍結法によりそれぞれ凍結した。その後，液体窒素で数日保存した後，各々最適条件により解凍・播種した。そして培養4日目にアルカリホスファターゼ染色を行い，未分化状態の生着コロニー数を比較した。その結果，開発品は，既存のガラス化保存液と比較し，生着コロニー数が約2倍多いことが示された。また，10% DMSOを含む培地と比較し，生着コロニー数が約4倍多いことが示された（図2）。

さらに，開発品で凍結した細胞をドライアイスで輸送が可能か否かを検討した。開発品で凍結した細胞を液体窒素保存後，液体窒素からドライアイス上に移して24時間後，最適条件で解凍・播種した。培養4日目にアルカリホスファターゼ染色を行い，未分化状態の生着コロニー数を測定した。その結果，ドライアイス保存後でも未分化状態を維持した生着コロニーが十分得られることが確認できた。しかしながら，ガラス化法で凍結した細胞を同様にドライアイス上で放置後に解凍した場合，生着コロニーはほとんど確認できなかった。ガラス化法で凍結保存したサルES細胞はドライアイス輸送ができないことを確認した。以上の結果より，開発品を用いることにより，操作が容易な緩慢凍結法でもサルES細胞の高効率な凍結保存が可能であり，凍結細胞をドライアイスで輸送できることが示された。

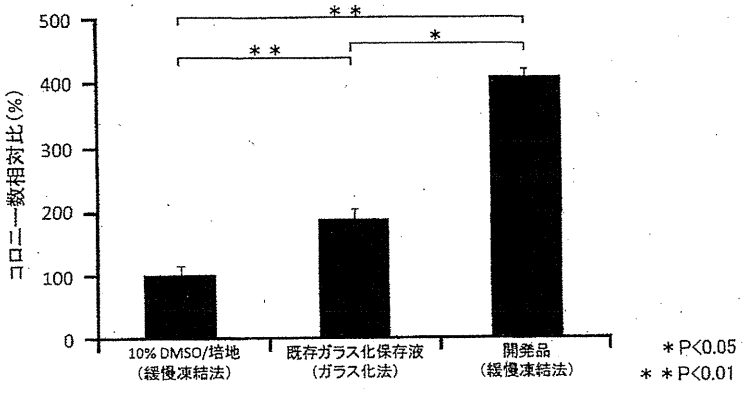

図2　各凍結保存液の解凍後生着コロニー数相対比