２０２３年８月８日

新技術説明会
New Technology Presentation Meetings!

光で分子を不活性化するための新規光増感蛍光タンパク質

～高効率なCALI法実現に向けて～

三重大学大学院医学系研究科生命医科学専攻

竹本　研

CALI法 とは？｜光で分子を特異的に酸化・不活性化する手法

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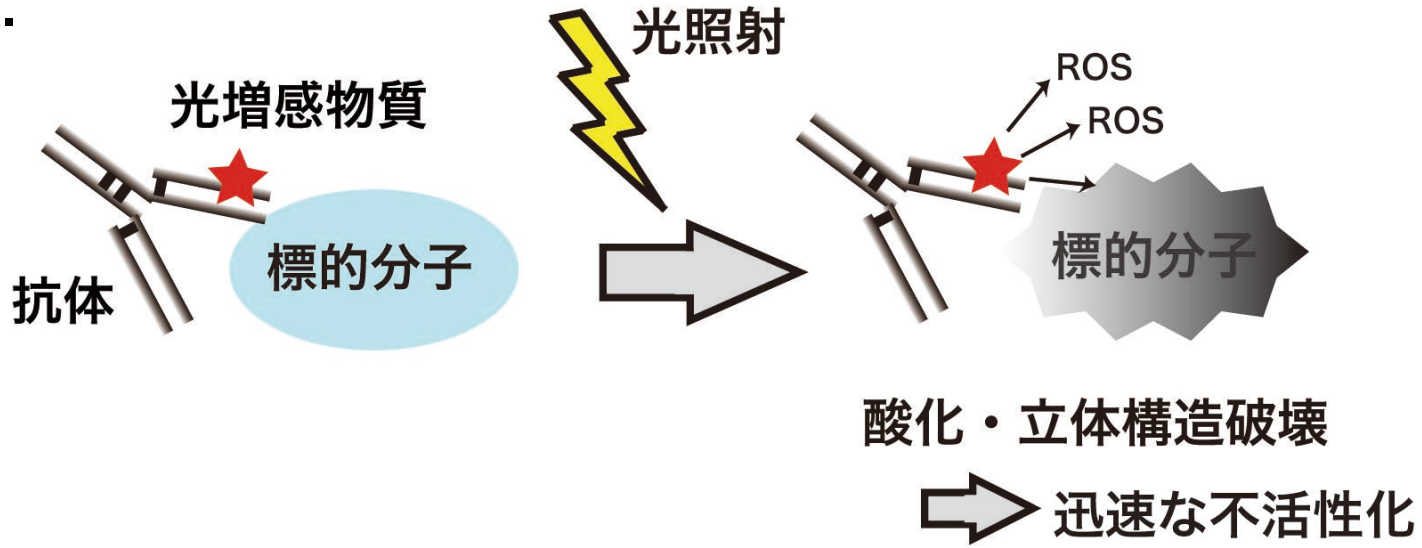
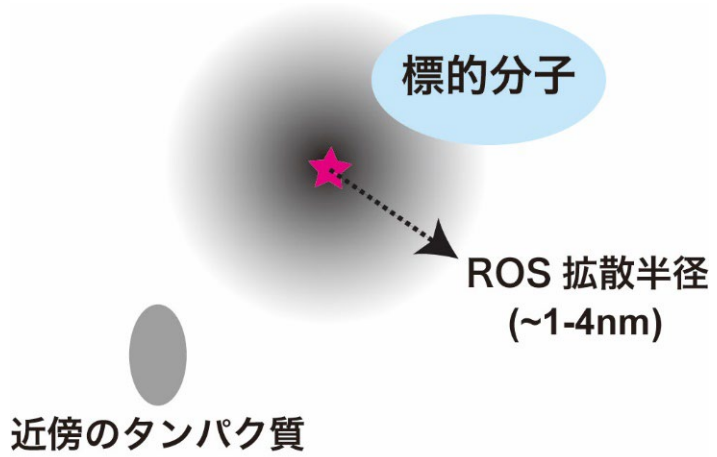
A.



B.



CALI法の特徴

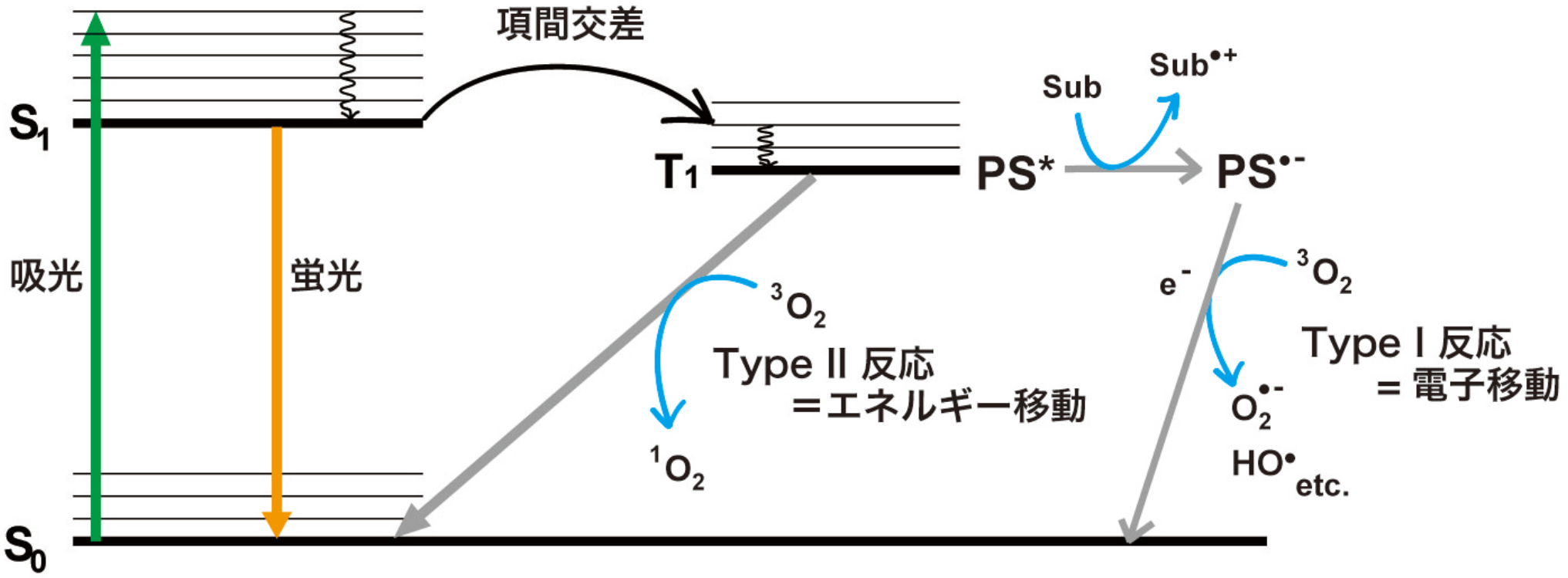
活性酸素の拡散半径が短い ⇒ 高い分子特異性

数秒～数分程度で迅速な不活性化が可能

Takemoto K et al. *ACS. Chem. Biol.* 2011
Takemoto K et al. Sci. Rep. 2013
Takemoto K et al. *Nat. Biotechnol.* 2017

Takemoto K *Proc Jpn Acad Ser B Phys Biol Sci.* 2021を改訂

光増感物質が活性酸素を産生するメカニズム



S_0：基底状態　　PS：光増感物質

S_1：励起一重項　　Sub: 標的分子

T：励起三重項

Takemoto K *Proc Jpn Acad Ser B Phys Biol Sci.* 2021を改訂

光増感物質には合成低分子化合物とタンパク質の2種類がある

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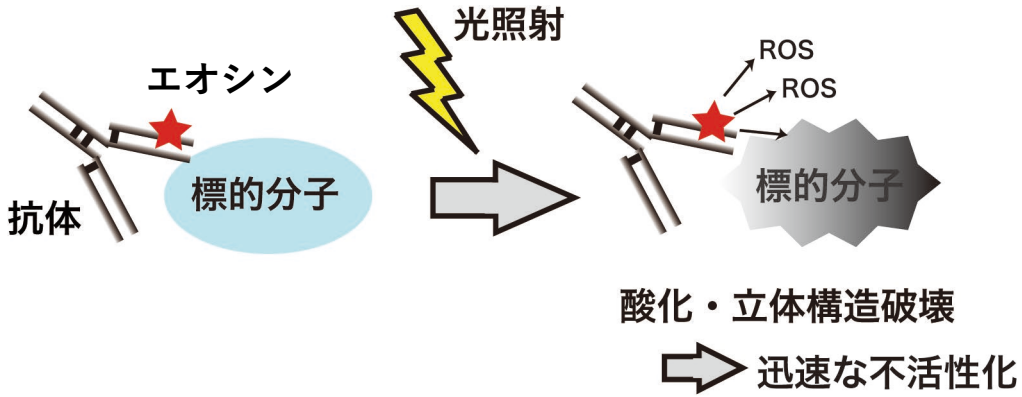
A. 光増感化合物（エオシンなど）によるCALI



Takemoto K et al. *Nat. Biotechnol.* 2017
Takemoto K et al. *ACS. Chem. Biol.* 2011など

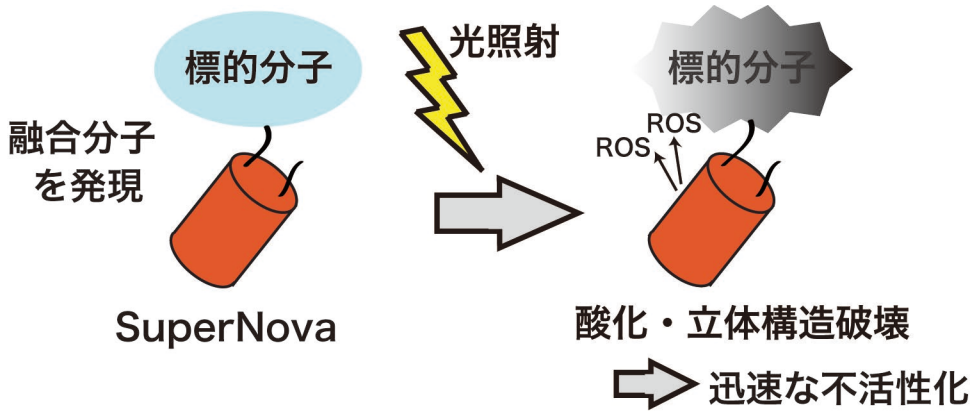
内在性の細胞表面分子を標的にできる

光増感活性が強い

抗体を分子ごとに作る必要（～約1年）

細胞内分子への適用が困難

B. 光増感蛍光蛋白質（SuperNovaなど）によるCALI



Takemoto K et al. *Sci. Rep.* 2013
Riani YD et al. *BMC. Biol.* 2018　など

遺伝子でコードされた実験系

抗体を取得せずに簡便な実験が可能

CALI効率は比較的低い

色変異体は現状2種類しかない

これまでの光増感蛍光タンパク質開発の経緯について

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nature biotechnology

KillerRed

A genetically encoded photosensitizer

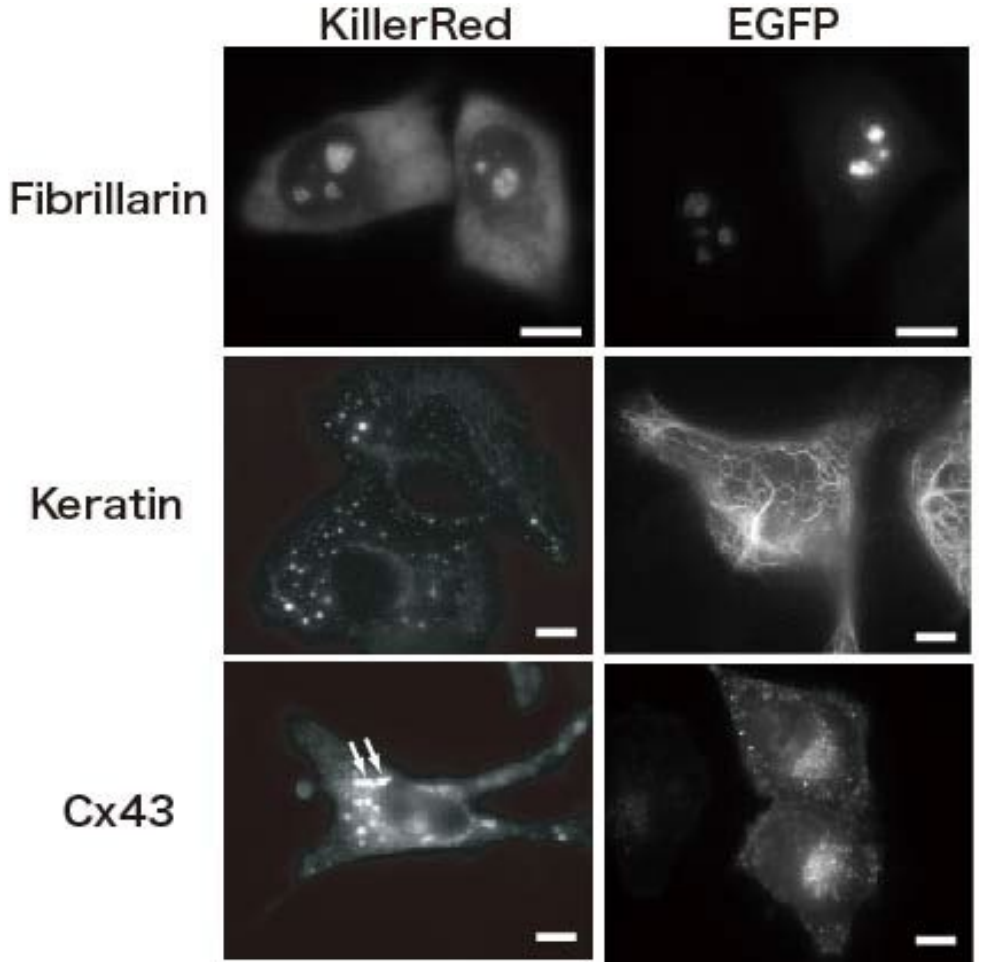
Maria E Bulina[1,3], Dmitriy M Chudakov[1,3], Olga V Britanova[1], Yurii G Yanushevich[1], Dmitry B Staroverov[2], Tatyana V Chepurnykh[2], Ekaterina M Merzlyak[2], Maria A Shkrob[1], Sergey Lukyanov[1] & Konstantin A Lukyanov[1]

Photosensitizers are chromophores that generate reactive oxygen species (ROS) upon light irradiation[1]. They are used for inactivation of specific proteins by chromophore-assisted light inactivation (CALI) and for light-induced cell killing in photodynamic therapy. Here we report a genetically encoded photosensitizer, which we call KillerRed, developed from the hydrozoan chromoprotein anm2CP, a homolog of green fluorescent protein (GFP). KillerRed generates ROS upon irradiation with green light. Whereas known photosensitizers must be added to living systems exogenously, KillerRed is fully genetically encoded. We demonstrate the utility of KillerRed for light-induced killing of *Escherichia coli* and eukaryotic cells and for inactivating fusions to β-galactosidase and phospholipase Cδ1 pleckstrin homology domain.

variants of different colors, nonfluorescent chromoproteins and circularly permuted variants. The majority of proteins tested had little or no effect on the viability of bacterial cells (**Fig. 1**). Mutant W94F of the chromoprotein asulCP from *Anemonia sulcata* showed a weak phototoxic effect, resulting in decreased bacterial survival. Only one of the proteins, KillerRed, showed a strong phototoxic effect (**Fig. 1a,b**).

KillerRed (GenBank accession number AY969116) is a dimeric red fluorescent protein with fluorescence excitation/emission maxima at 585/610 nm, an extinction coefficient of 45,000 $M^{-1}cm^{-1}$ at 585 nm and a fluorescence quantum yield of 0.25 (**Fig. 1c**). We derived it from the hydrozoan chromoprotein anm2CP[20] by including the substitutions T145N and C161A (corresponding to positions 148 and 165 in *Aequorea victoria* GFP), which are spatially close to the chromophore and drastically affect protein fluorescent properties, as well as the folding- and brightness-improving mutations

Bulina et al. *Nat. Biotechnol.* 2006



凝集体を形成
生理的局在が喪失

- 世界初の光増感蛍光タンパク質
- 二量体構造のため融合分子の局在・機能が失われる場合が多い

(Takemoto K et al. *Sci. Rep.* 2013)

これまでの光増感蛍光タンパク質開発の経緯について

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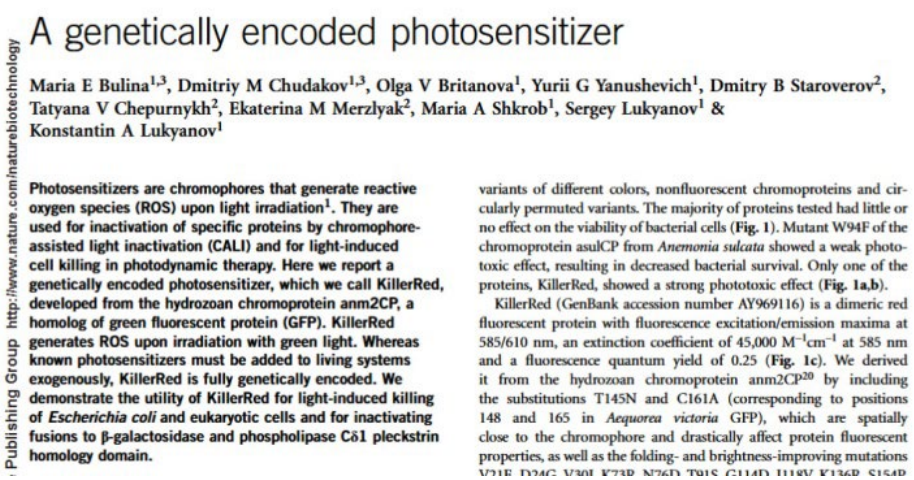
KillerRed


nature biotechnology

A genetically encoded photosensitizer

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Bulina et al. *Nat. Biotechnol.* 2006

遺伝子変異
スクリーニング

SuperNova


SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
CALCIUM SIGNALLING
FLUORESCENCE IMAGING

Received
7 March 2013

Accepted

SuperNova, a monomeric photosensitizing fluorescent protein for chromophore-assisted light inactivation

Kiwamu Takemoto[1]*§, Tomoki Matsuda[1]†§, Naoki Sakai[2]‡§, Donald Fu[3], Masanori Noda[4], Susumu Uchiyama[4], Ippei Kotera[3], Yoshiyuki Arai[1]†, Masataka Horiuchi[5], Kiichi Fukui[4], Tokiyoshi Ayabe[2], Fuyuhiko Inagaki[5], Hiroshi Suzuki[3] & Takeharu Nagai[1]†

Takemoto et al. *Sci. Rep.* 2013

- 世界初の光増感蛍光タンパク質
- 二量体構造のため融合分子の局在・機能が失われる場合が多い

世界初の完全に遺伝子でコードされた<u>単量体</u>光増感タンパク質

これまでの光増感蛍光タンパク質開発の経緯について

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SuperNova

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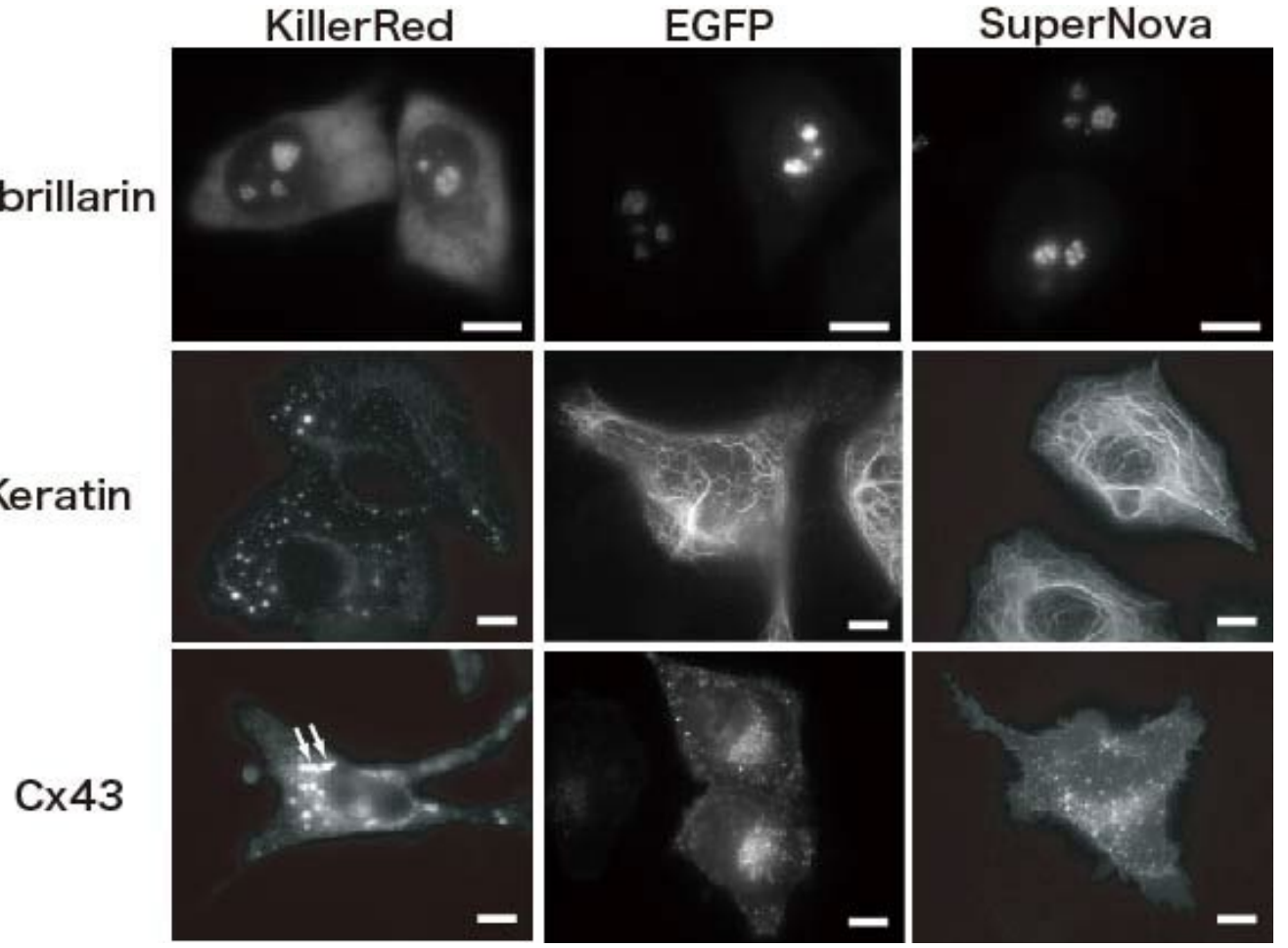

正しい局在

- 世界初の単量体光増感蛍光タンパク質
- 融合分子の局在・機能を乱さない

(Takemoto K et al. *Sci. Rep.* 2013)

従来の技術｜SuperNovaによるCALI法

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CALCIUM SIGNALLING
FLUORESCENCE IMAGING

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Cofilinに対するCALI法を記憶研究に応用

（Goto A et al *Science* 2021）

NEUROSCIENCE

Stepwise synaptic plasticity events drive the early phase of memory consolidation

Akihiro Goto[1,2], Ayaka Bota[1,2,3], Ken Miya[2,4,5], Jingbo Wang[1], Suzune Tsukamoto[1], Xinzhi Jiang[1], Daichi Hirai[2], Masanori Murayama[2,6], Tomoki Matsuda[7], Thomas J. McHugh[2,6], Takeharu Nagai[7], Yasunori Hayashi[1,2,8]*

Arl13bへの適用

（Gabriela TT et al *Sci. Adv.* 2020）

SCIENCE ADVANCES | RESEARCH ARTICLE

DEVELOPMENTAL BIOLOGY

Primary cilium remodeling mediates a cell signaling switch in differentiating neurons

Gabriela Toro-Tapia and Raman M. Das*

CamkII β への適用

Neuron
Article

（Kim K et al *Neuron* 2015）

CellPress

A Temporary Gating of Actin Remodeling during Synaptic Plasticity Consists of the Interplay between the Kinase and Structural Functions of CaMKII

Karam Kim,[1] Gurpreet Lakhanpal,[2] Hsiangmin E. Lu,[3,4] Mustafa Khan,[2] Akio Suzuki,[1] Mariko Kato Hayashi,[6] Radhakrishnan Narayanan,[6] Thomas T. Luyben,[2,7] Tomoki Matsuda,[8] Takeharu Nagai,[8] Thomas A. Blanpied,[3,4,5] Yasunori Hayashi,[1,6,9,10,11,*] and Kenichi Okamoto[2,6,7,11,*]

従来のSuperNovaを用いてCALI法が成功した分子は少ない

Table 2. Examples of molecular inactivation by CALI using photosensitizing proteins.

Photosensitizer	Protein of interest	Function	Targeting method	CALI Sample	Reference
KillerRed	PLCδ1	lipid metabolism etc.	fusion	*in vitro*	38
	β1-integrin	invadasome structure	fusion	*in vitro*	47
	Centrin2	replication of centromere	fusion	brain slice	48
	Histon H2B	component of nucleosome	fusion	*in vitro*	49
	RBMX	chromosome morphogenesis	fusion	*in vitro*	50
	Sec13	biogenesis of COPII-coated vesicle	fusion	*in vitro*	51
	Aquaporin1/4	water transport	fusion	*in vitro*	52
	Cofirin	actin cfilament disassembly	fusion	*in vitro*	53
	Rab7	endocytosis	fusion	*in vitro*	54
	GON domain	protein secretion from ER	fusion	*in vivo (c.elegance)*	76
	GRASP55/65	fomation of Golgi ribbon	fusion	*in vitro*	55
tandem KillerRed	Ran	membrane targetting of RhoA	fusion	*in vitro*	56
SuperNova	cofirin	actin filament disassembly	fusion	*in vitro*	15
	mDia1	Rho effector etc.	fusion	*in vitro*	57
	CamKIIβ	LTP induction	fusion	brain slice	58
	Synapsin	component of synaptic vesicle	fusion	*in vitro*	59
	Synaptophysin	component of synaptic vesicle	fusion	*in vitro*	59
	SDHB/SDHC	Mitochondrial electron transport Chain complex II	fusion (CRISPER)	*in vivo (c.elegance)*	63
	Myosin II	growth of cell junction	fusion (CRISPER)	*in vivo (Drosophila)*	64
	Arl13b	primary cillium formation	fusion	chick embryonic slice	60
SuperNova-Green	PLCδ1	lipid metabolism etc.	fusion	*in vitro*	44

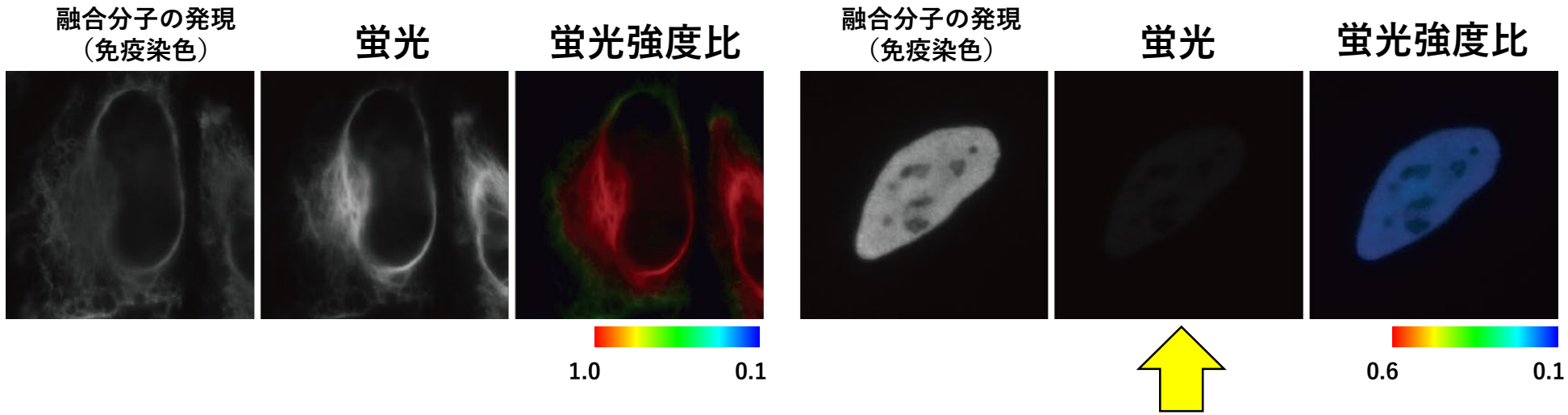
なにがボトルネックになっているか？

Takemoto K *Proc Jpn Acad Ser B Phys Biol Sci.* 2021を改訂

従来のSuperNovaの問題点| 37℃で立体構造形成効率が低い

細胞骨格分子X（細胞内半減期>300hr）　　転写因子Y（細胞内半減期=0.5hr）

SuperNova
融合分子



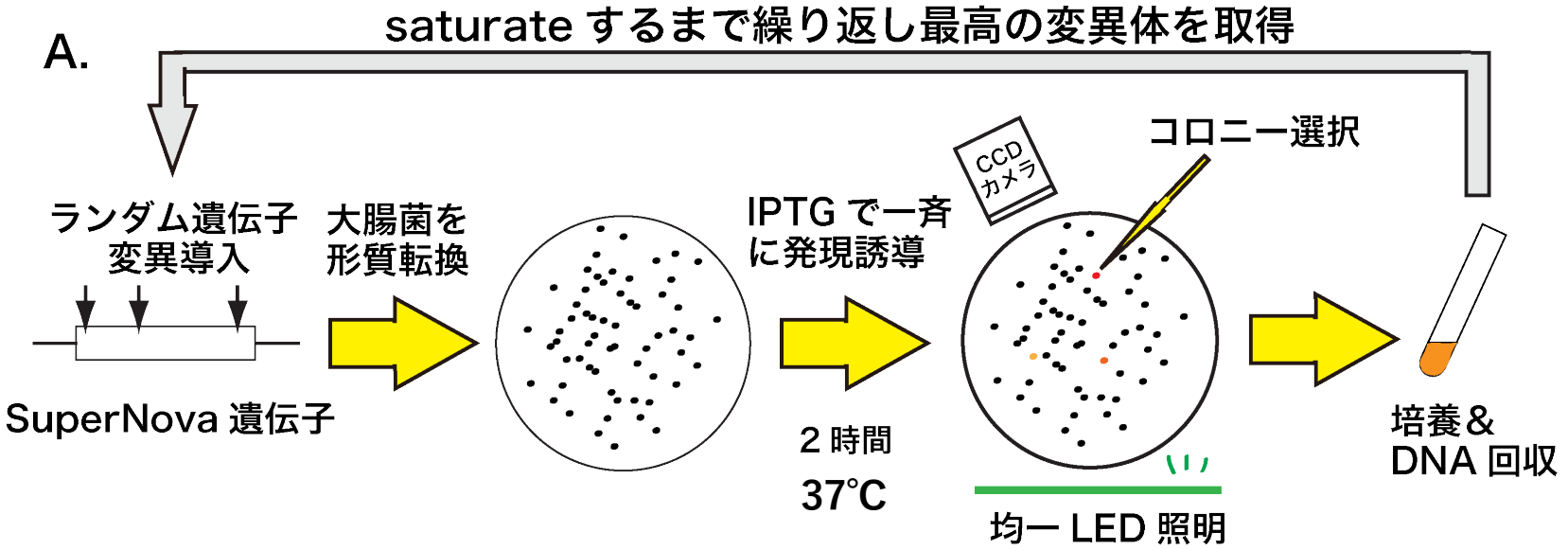
半減期が短い標的分子と融合し37℃で発現すると蛍光が極めて弱い

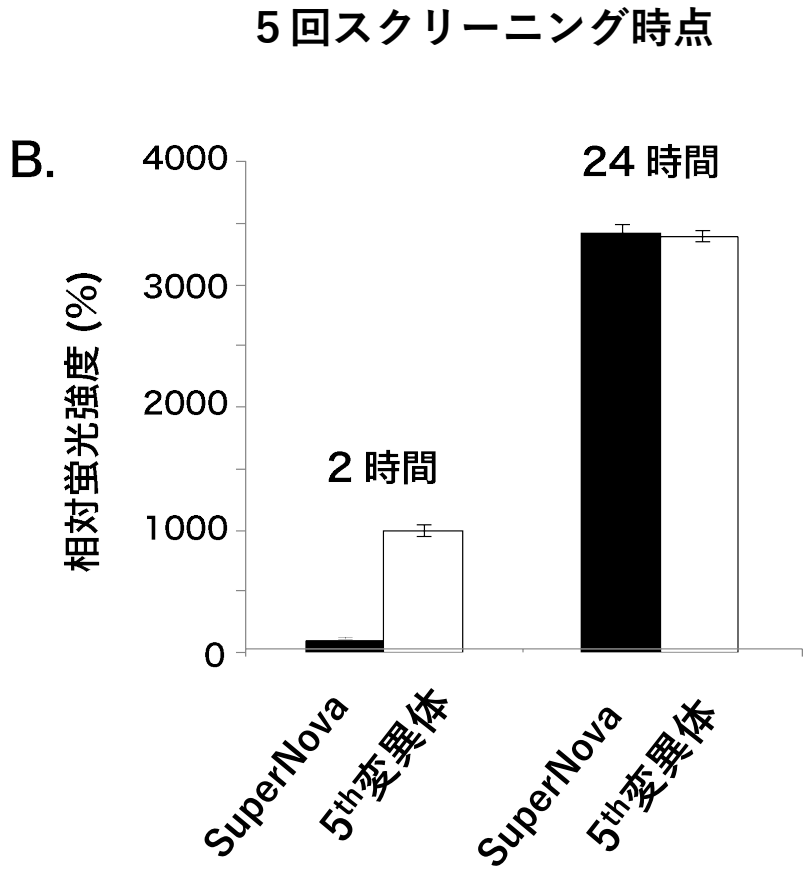
光らないので生細胞・顕微鏡下では発現が確認できない。

多くの分子でCALIができない

SuperNova変異体開発によりこれを改善できないか？

37℃での立体構造形成効率が高いSuperNova変異体スクリーニング





13個のアミノ酸置換変異を持つ新規変異体”HyperNova”を取得

HyperNovaは37℃での立体構造形成効率が高い

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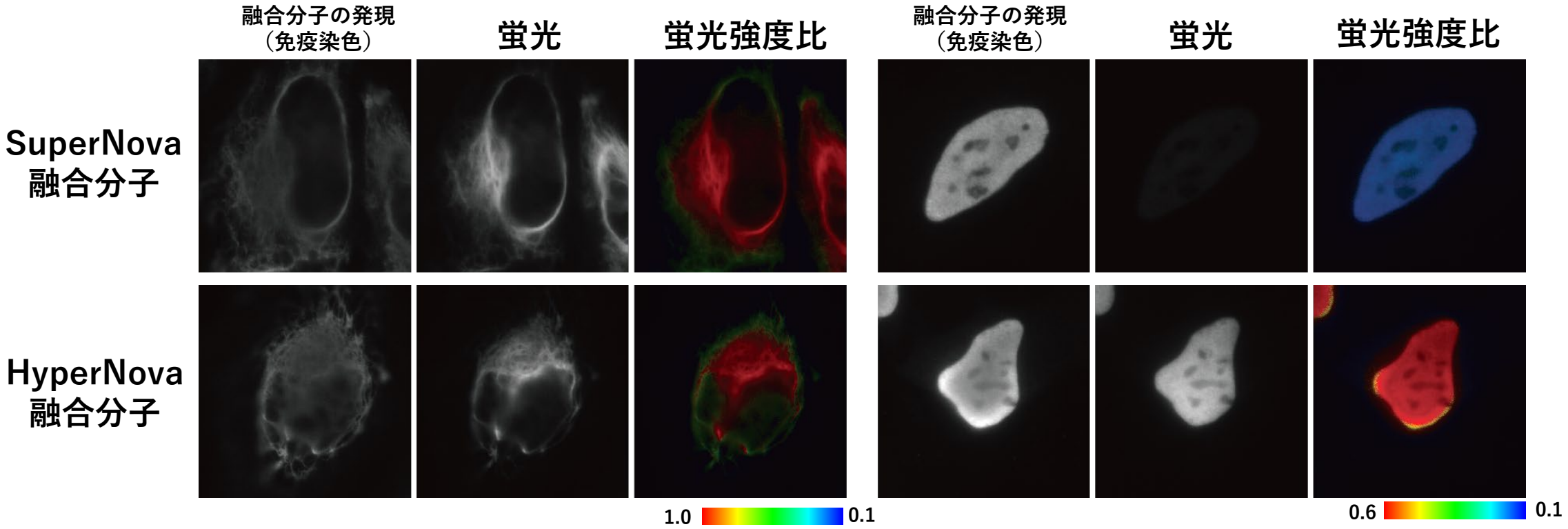
細胞骨格分子X（細胞内半減期>300hr）　　転写因子Y（細胞内半減期=0.5hr）



半減期が長い分子を融合すると差はない

半減期が短い分子ほど顕著に改善

➡ 蛍光特性ではなく立体構造形成効率が顕著に向上した

本発明・HyperNova光増感蛍光タンパク質の概要

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1）立体構造形成効率が高い新規光増感蛍光タンパク質を発明した。

2）従来型のSuperNovaでは不可能であった分子を標識することができ、CALI法の適用が可能になった。

3）HyperNovaにより世界で初めて、細胞増殖にかかわるリン酸化酵素、細胞死等に係るリン酸化酵素、細胞分裂関連酵素のCALI法の開発に成功した。

※　本発明は論文投稿中のため、詳細なデータはCDA下でお見せしディスカッションが可能です。
ライセンスや共同研究をご希望の企業の方からのご連絡をお待ちしています。

期待・想定される用途

1）これまで操作できなかった分子を光で操作する基礎研究ツール

2）ゲノム編集によりHyperNovaをノックインすることで、内在性分子の操作を可能に

3）上記のノックインマウスを用いた疾患等のin vivo解析

企業への期待

・HyperNova発現ベクターの販売・開発

・ゲノム編集用HyperNovaノックインベクターの販売・開発

・上記ノックインマウスの販売・開発

本技術に関する知的財産権

新技術説明会
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・発明の名称　：立体構造形成能が高い光増感タンパク質

・出願番号　：特願2022-103027

・出願人　：国立大学法人三重大学、公立大学法人横浜市立大学

・発明者　：竹本研

お問い合わせ先

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三重大学
みえの未来図共創機構
知的財産マネジメント部門

ＴＥＬ ０５９－２３１－５４９５
ＦＡＸ ０５９－２３１－９７４３

e-mail chizai-mip@crc.mie-u.ac.jp