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76.

Figure legends

Figure 1: STRO-1 immuno-reactivity in the radiation induced wound and subsequent scarring from a 58-year-old male patient's back

A:

1: Radiation injury with exposed costal rib and central tissue necrosis at first visit, 2 years after fluoroscopic procedure

2: In the reverse side of injury, scarring was observed at periphery of the base was very hard.

3: 2 years after reconstruction by latissimus dorsi musculo-cutaneous flap (bilobed flap)

4: small fusiform cells were abundant in the scar tissue.

B: Scarred tissue was immunohistological analysis of STRO-1

In the right, scarred tissue explanting cell culture demonstrated Steo-1 immuno-reacivity in cell cytoplasm in the right.

Figure 2: Scheme of Nagasaki University Global Strategic Center for Radiation Health Risk Control

A: Global focal points of collaboration with Nagasaki University

B: Strategy applying to promoting innovative therapy such as ADSC for radiation injury is a part of this project

Figure 3: Schematic principles of progression of chronic radiation injury

A: Once external radiation is administered.

B: For therapeutic radiation, dose is usually greater in deeper in the tissue as the target organ is often located deep

C: Radiated tissue is getting sclerotic in process of chronic radiation injury

D: Surgical debridement is aimed at the wounded and most severe areas

E: ADSC are injected or soaked around wound margins and wound bed

F: Stacked artificial dermis (Terudermis®) and liposuctioned adipose tissue mixed wit ADSC are bridged with the adjacent intact tissue

Figure 4: An 87-year-old female was treated for uterine cervical cancer. A 50 Gy of two gate radiation was performed 40 years previously and wound was reached to the sacral bone, muscle and ligament

1: pre-op

2: intra-op

3: intra-op with artificial dermis

4: 36 days

5: 72 days, linear hypertrophic scar formation

6: 970 days, wound healed and pliable

Figure 5: In vitro cell proliferation and differentiation

1: adipose-derived stem cells in regular cell culture medium at day 2

2: adipose-derived stem cells in ES cell culture medium at day 9

3: adipose-derived stem cells in ES cell culture medium at day 16 at confluent

4: adipose-derived stem cells in ES cell culture medium at day 16 by differentiation induction

5: adipose-derived stem cells in ES cell culture medium at day 16, lipid is marled in red by Oil-Red O staining

Figure 1A

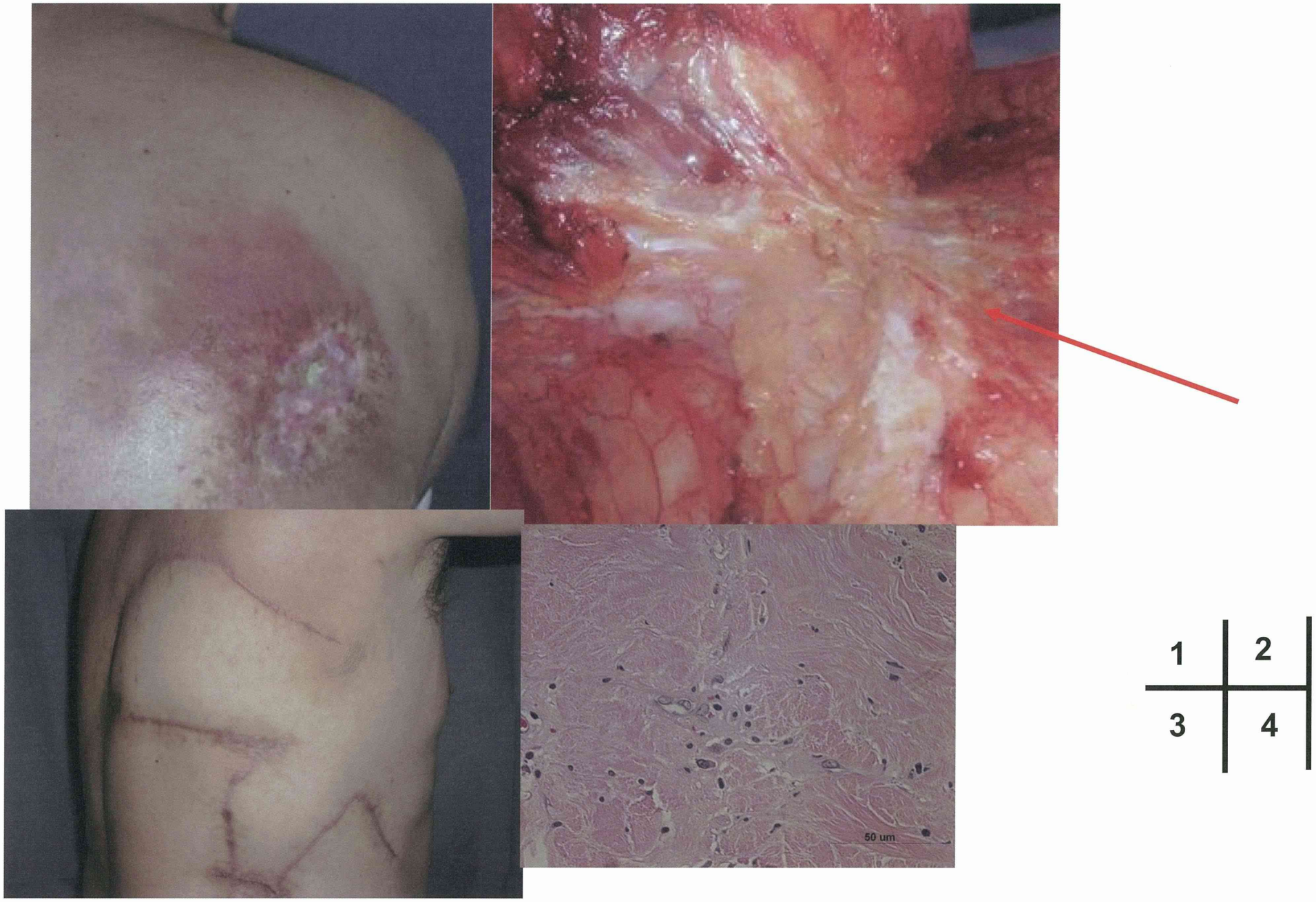


Figure 1B

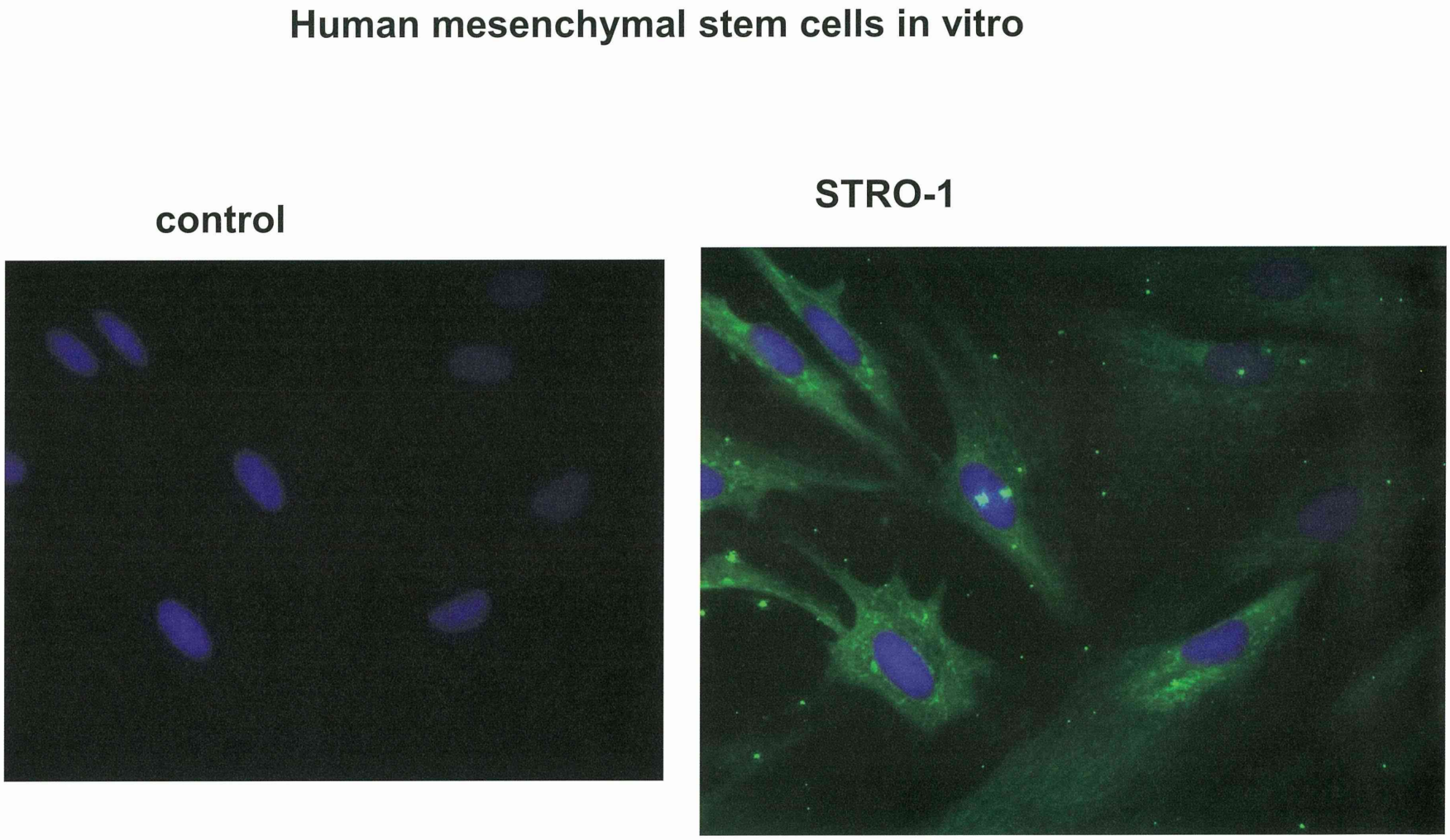


Figure 2A

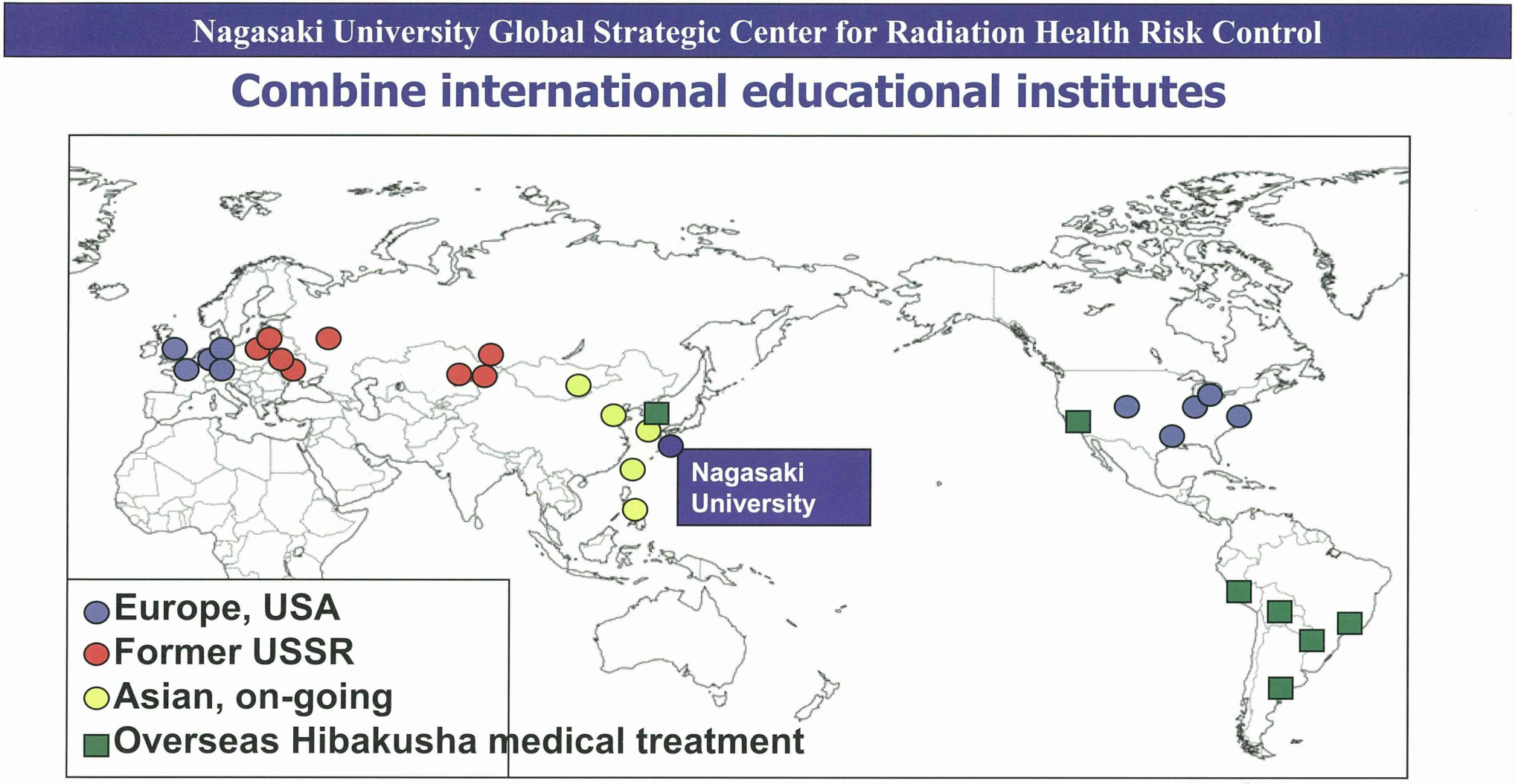

Nagasaki University Global Strategic Center for Radiation Health Risk Control
Combine international educational institutes
Nagasaki University
Europe, USA
Former USSR
Asian, on-going
Overseas Hibakusha medical treatment

Figure 2B

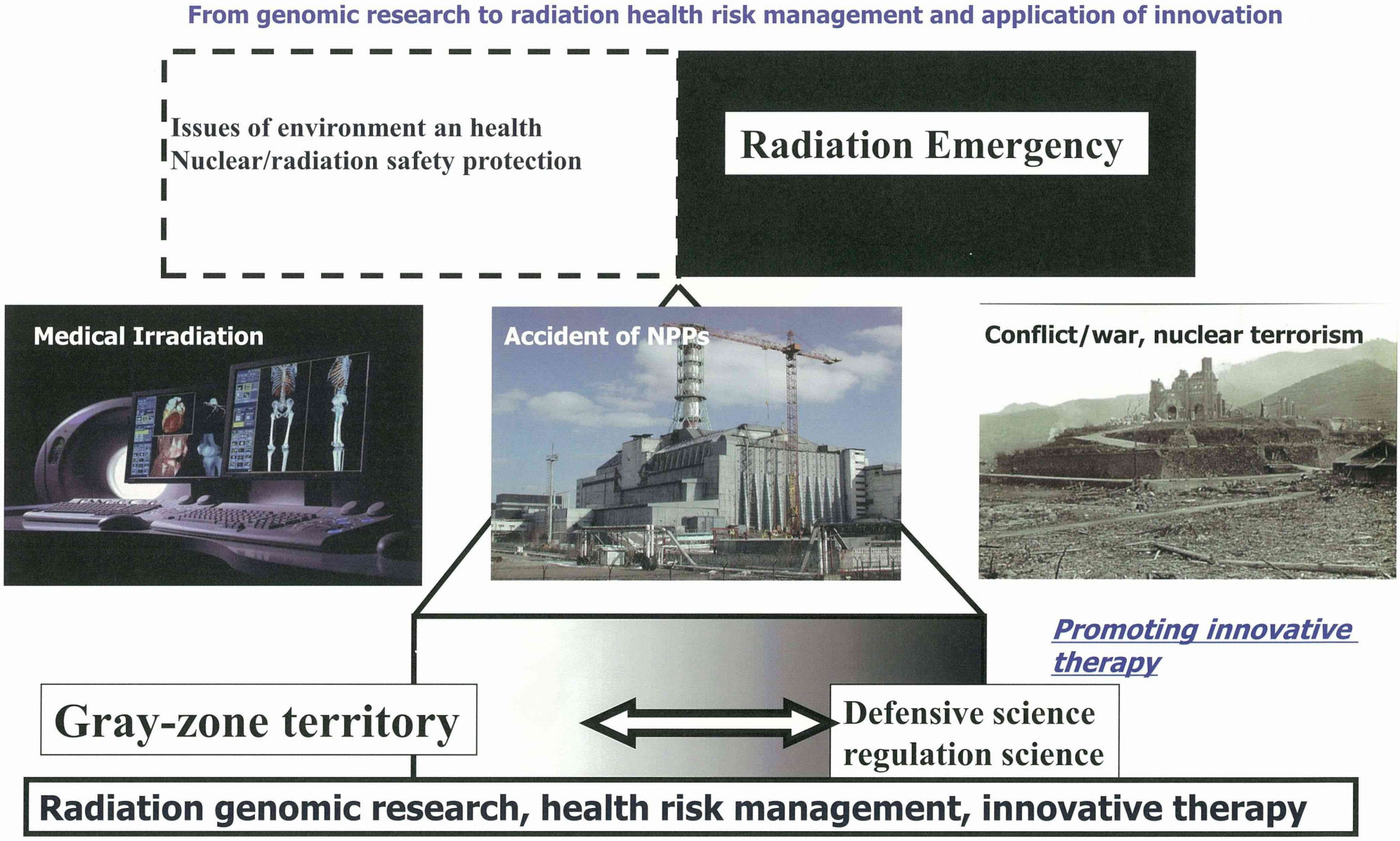

From genomic research to radiation health risk management and application of innovation
Issues of environment an health
Nuclear/radiation safety protection
Radiation Emergency
Medical Irradiation
Accident of NPPs
Conflict/war, nuclear terrorism
Promoting innovative therapy
Gray-zone territory
Defensive science
regulation science
Radiation genomic research, health risk management, innovative therapy

Figure 3A

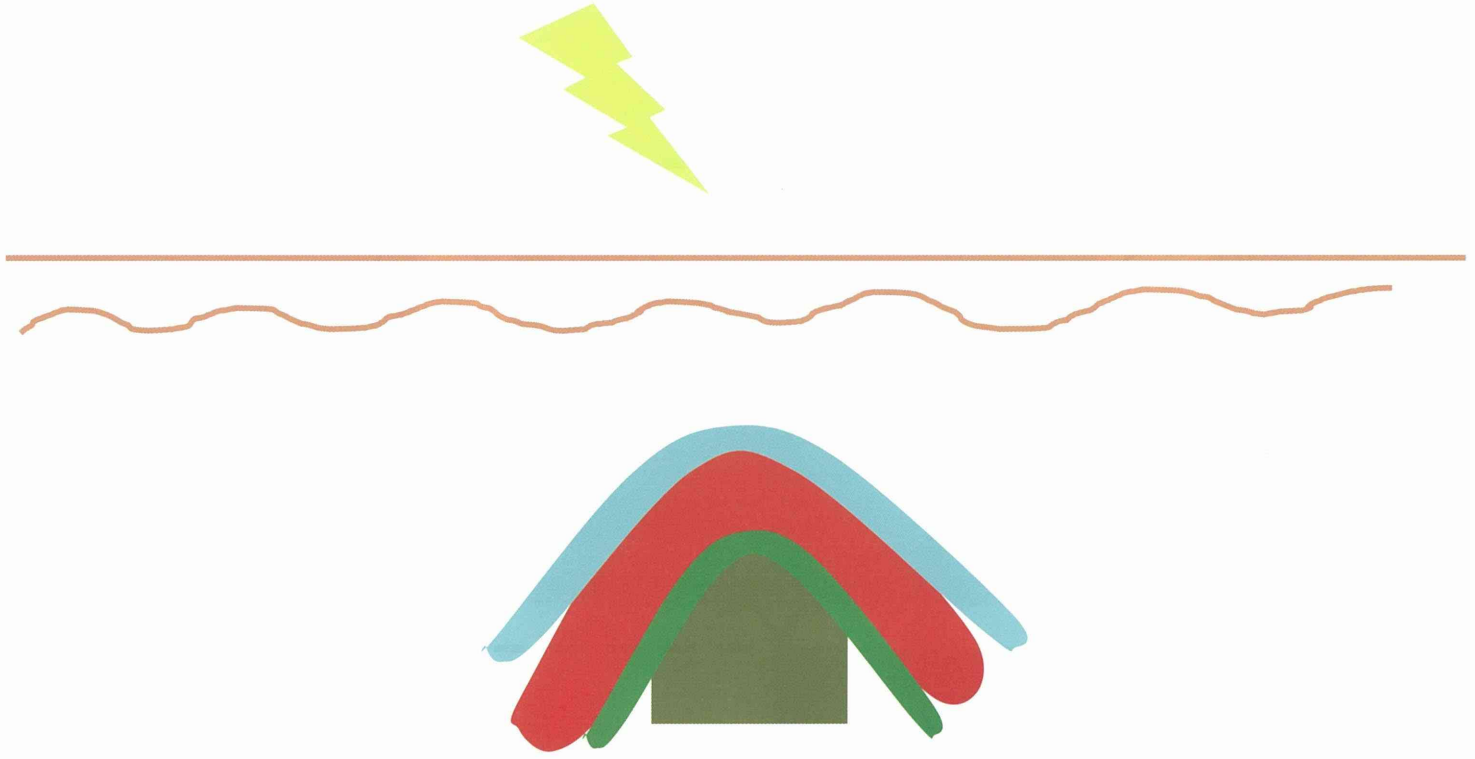


Figure 3B

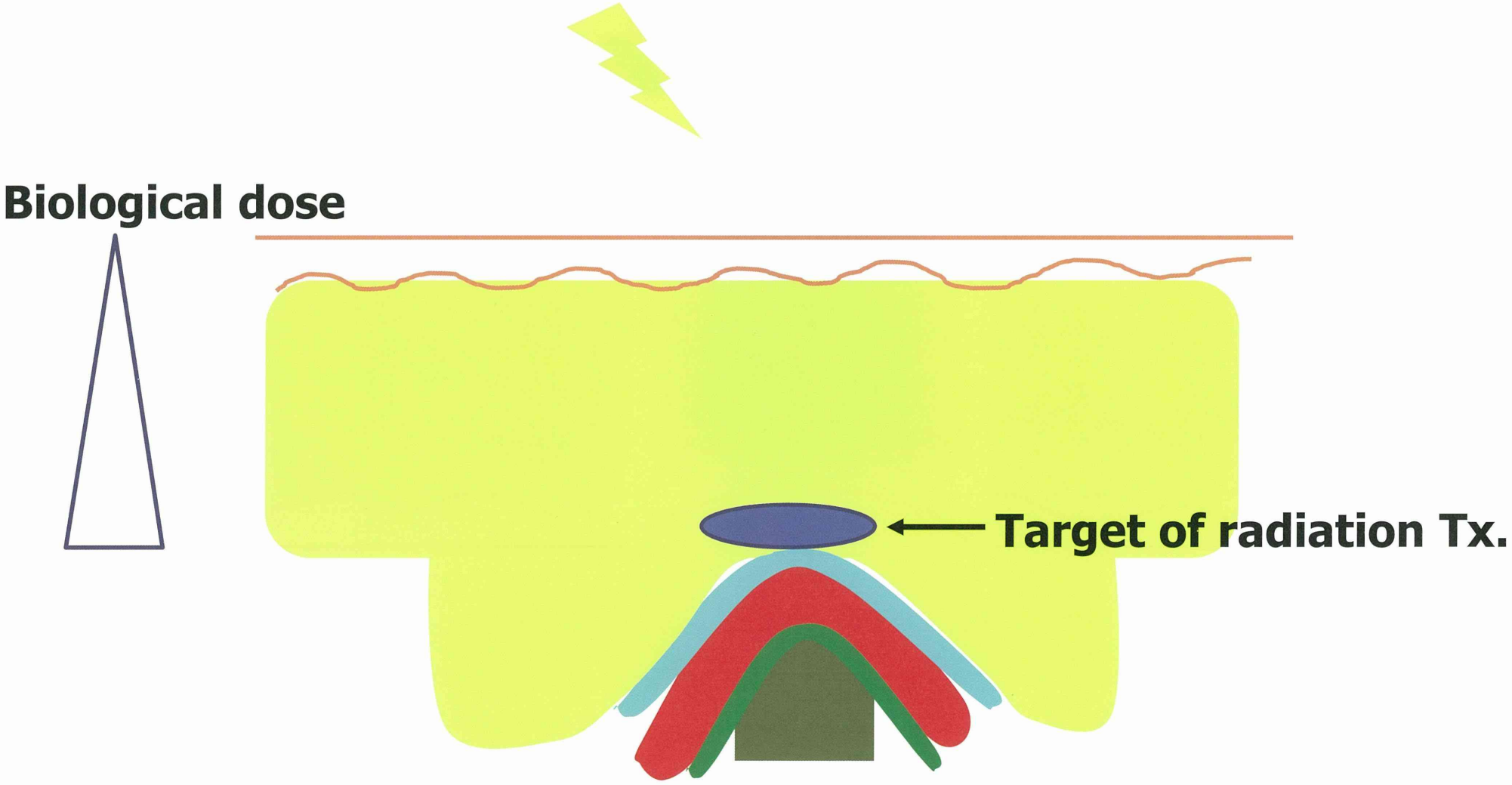


Figure 3C

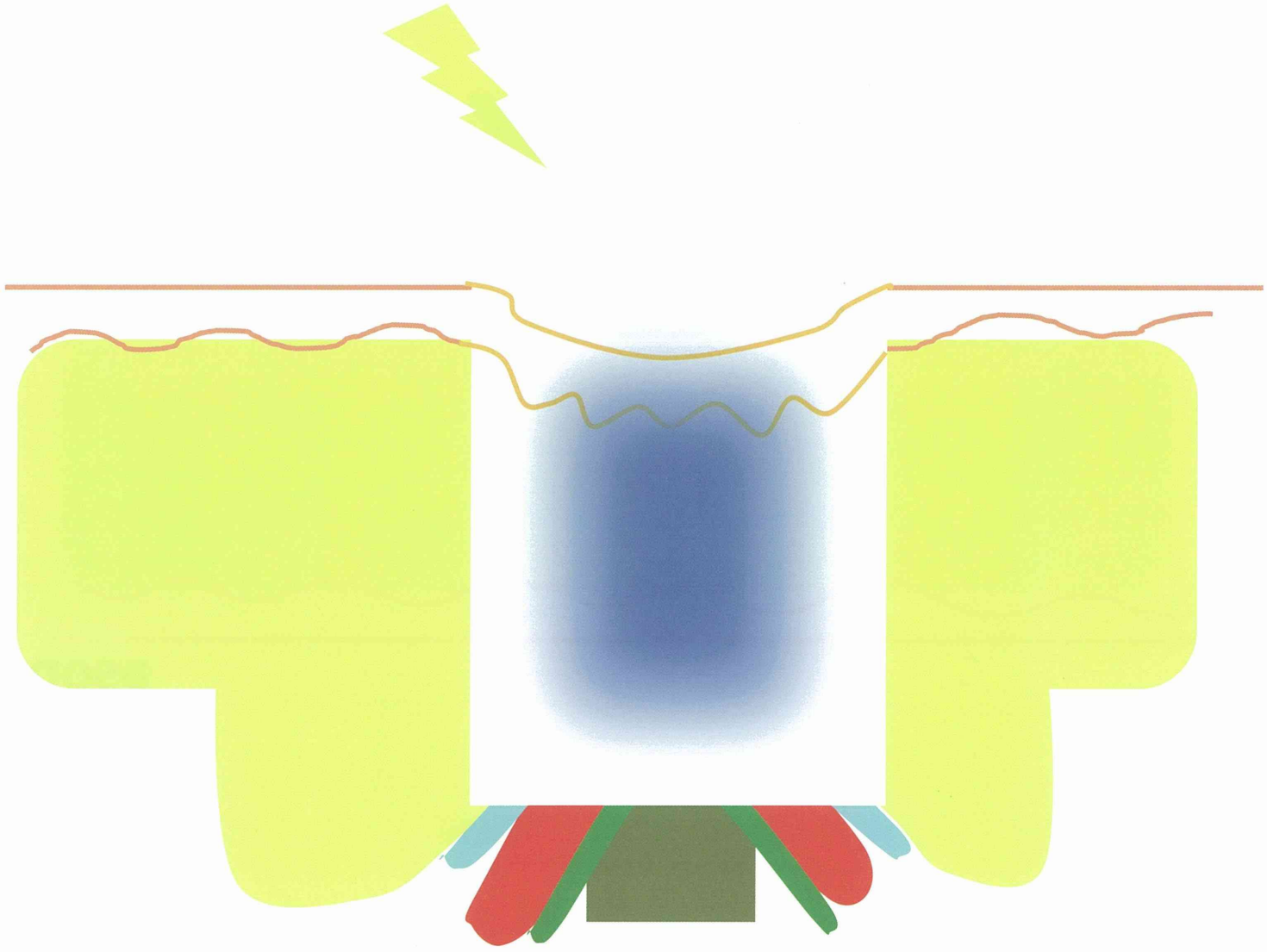
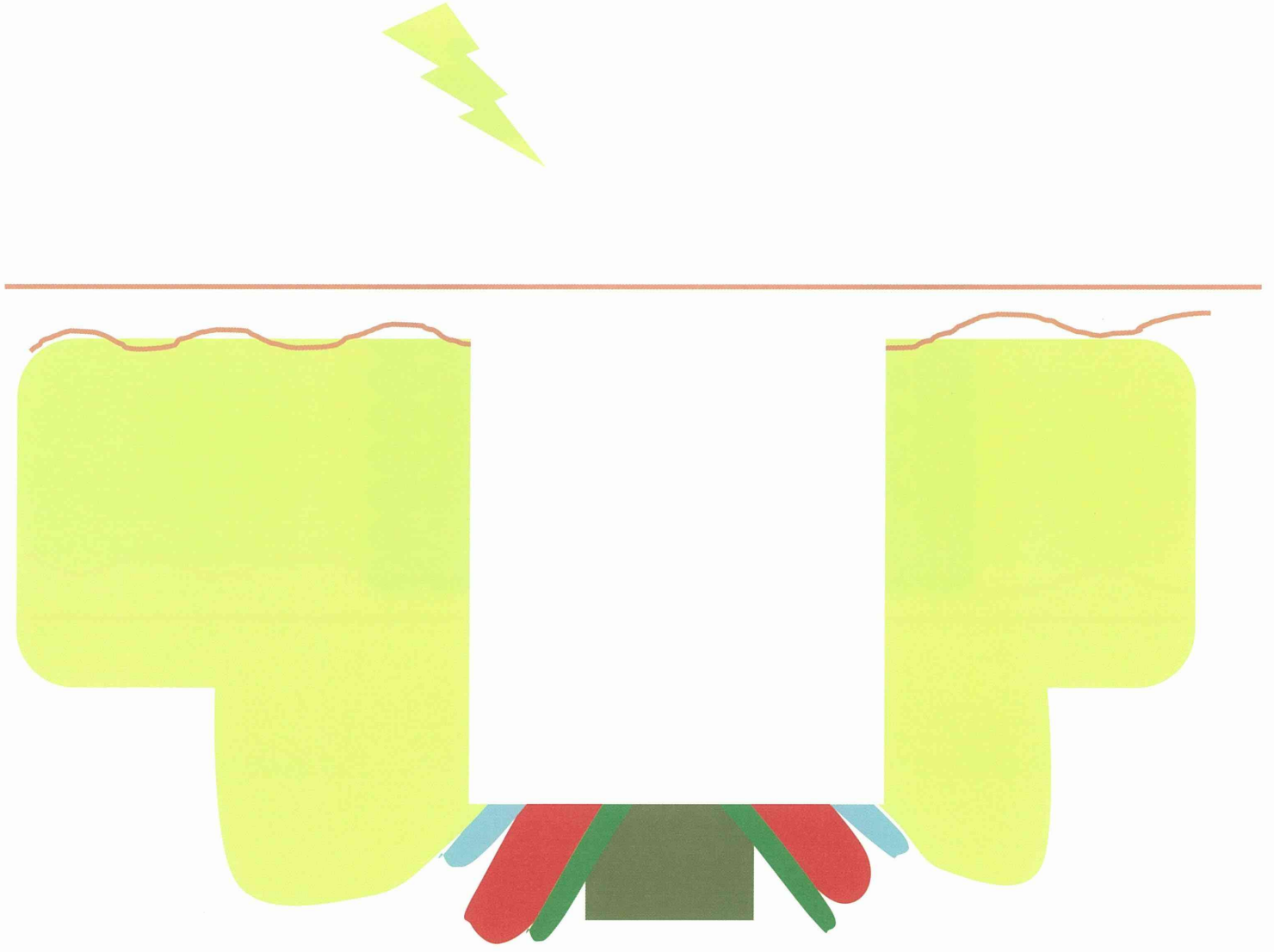


Figure 3D



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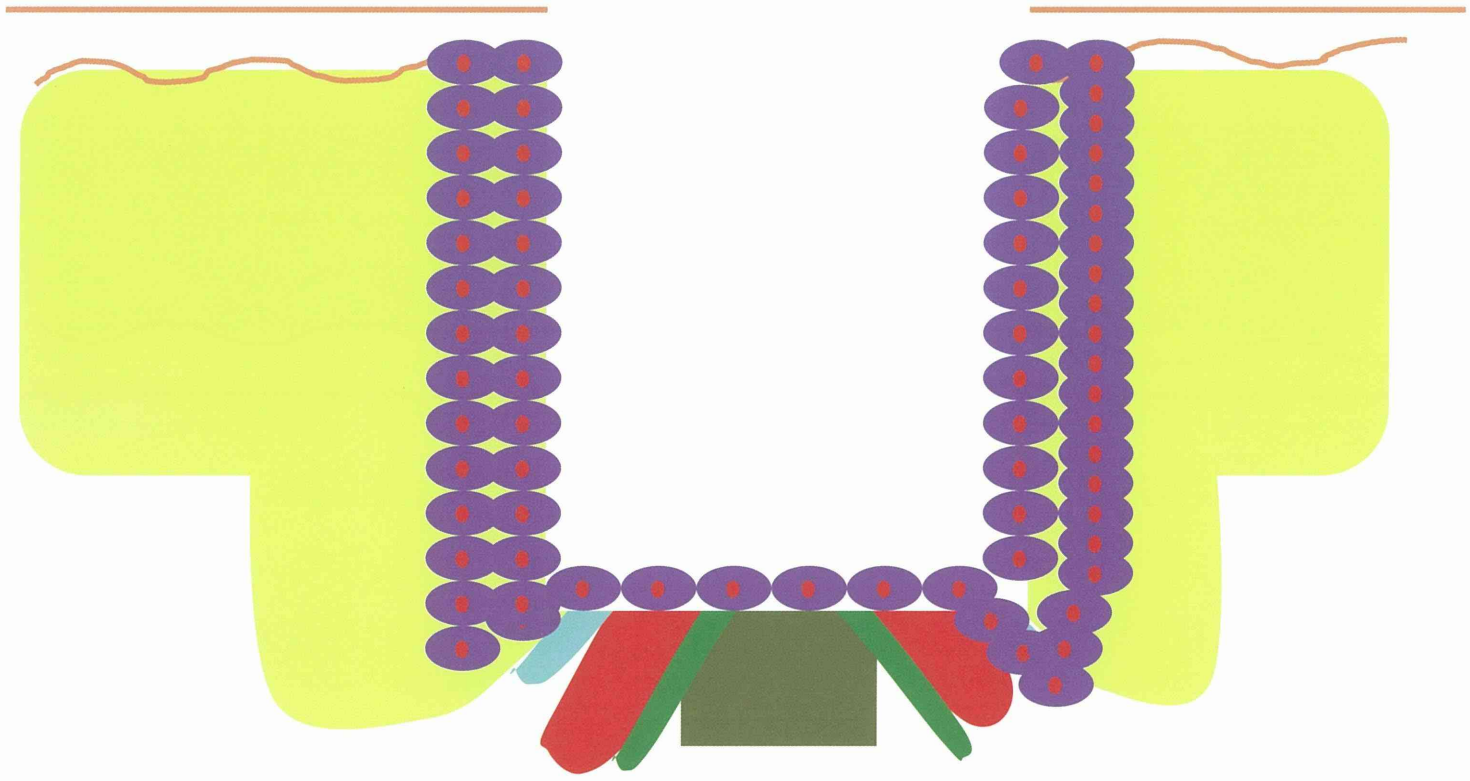


Figure 3F

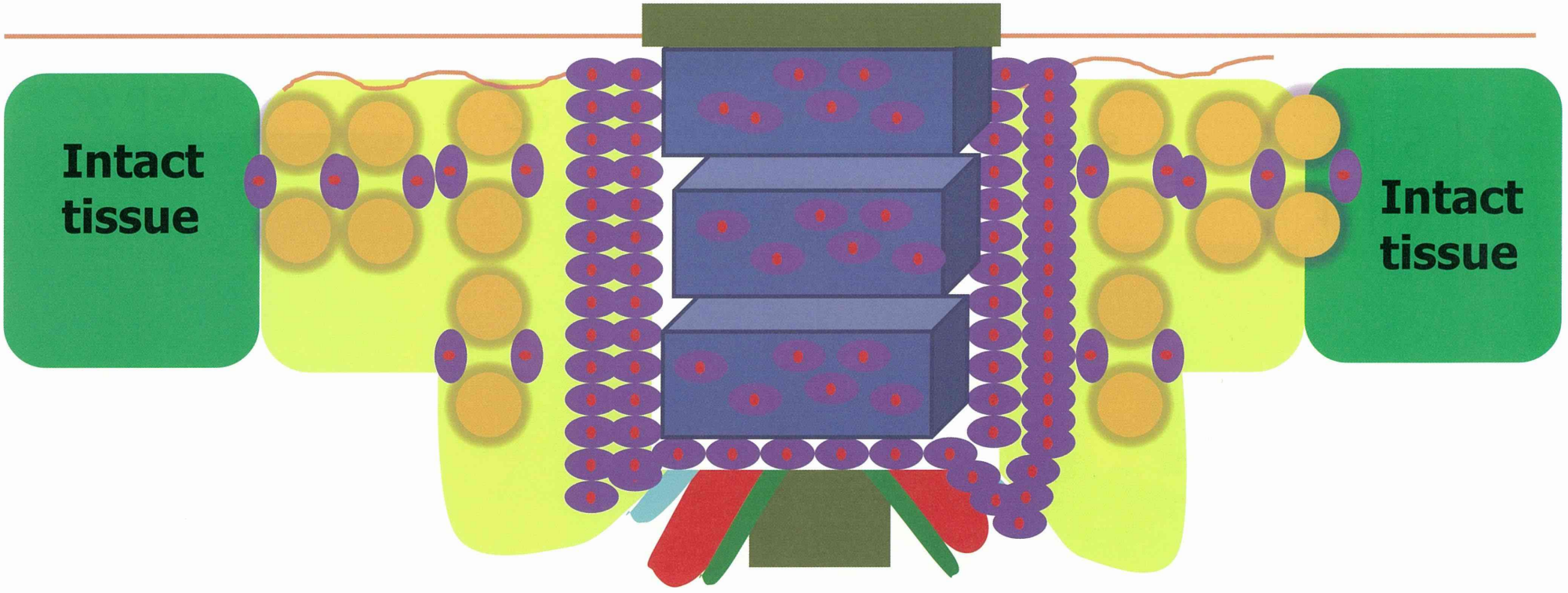


Figure 4

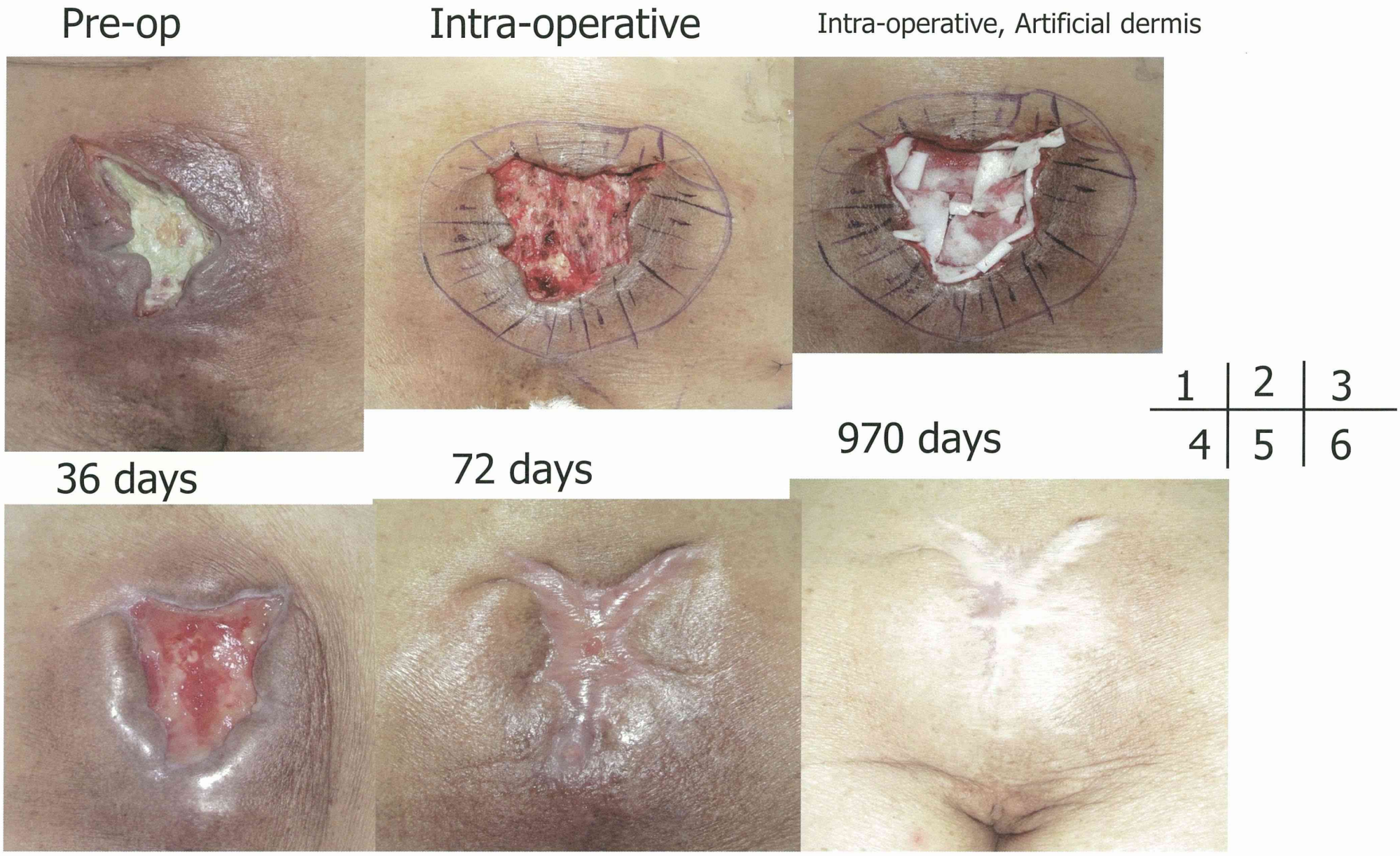
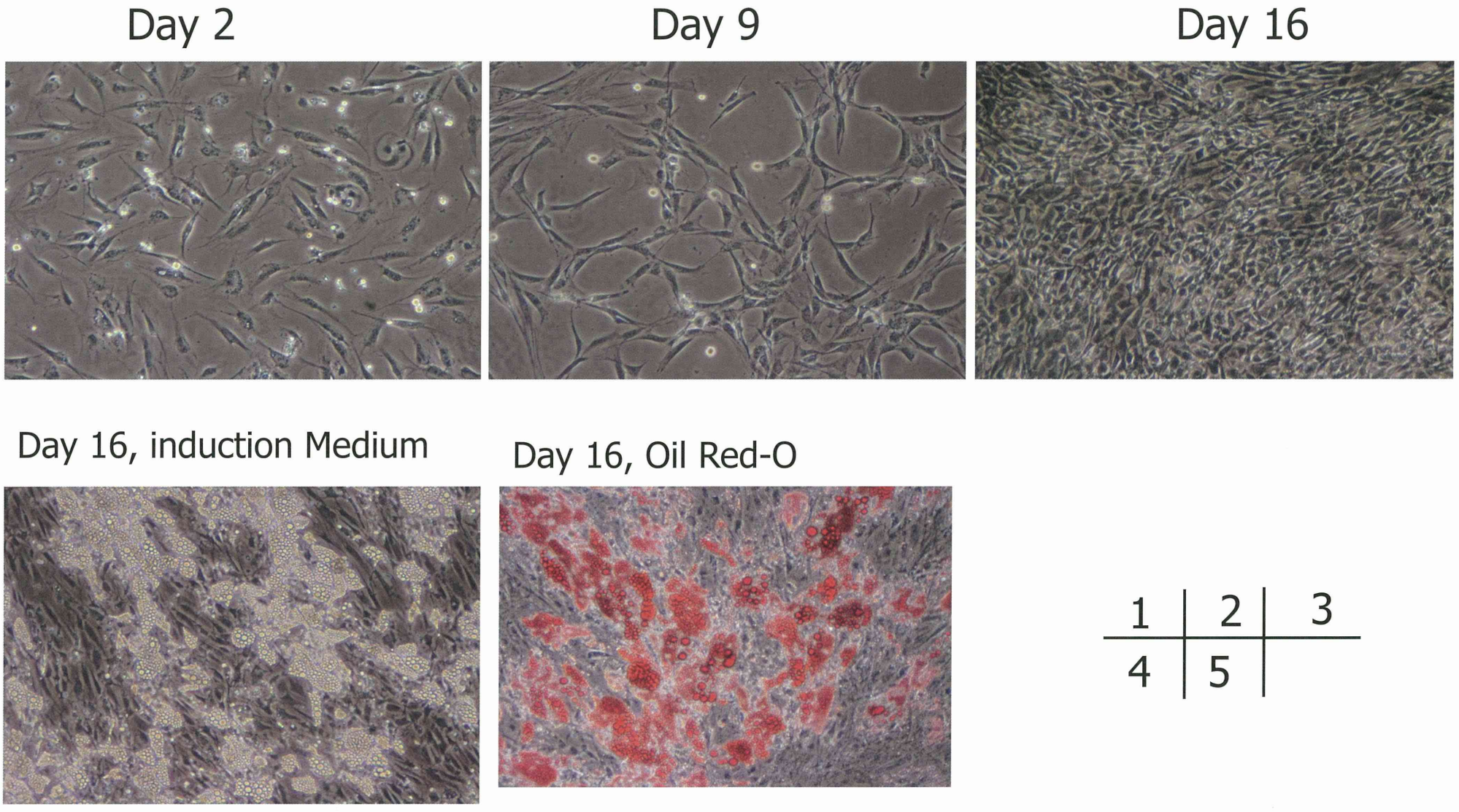

Pre-op
Intra-operative
Intra-operative, Artificial dermis
1
2
3
4
5
6
970 days
36 days
72 days

Figure 5


Day 2
Day 9
Day 16
Day 16, induction Medium
Day 16, Oil Red-O
1
2
3
4
5

S(+)-Ketamine Suppresses Desensitization of γ-Aminobutyric Acid Type B Receptor-mediated Signaling by Inhibition of the Interaction of γ-Aminobutyric Acid Type B Receptors with G Protein–coupled Receptor Kinase 4 or 5

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ABSTRACT

Background: Intrathecal baclofen therapy is an established treatment for severe spasticity. However, long-term management occasionally results in the development of tolerance. One of the mechanisms of tolerance is desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$) because of the complex formation of the $GABA_{B2}$ subunit (GB_2R) and G protein–coupled receptor kinase (GRK) 4 or 5. The current study focused on *S*(+)-ketamine, which reduces the development of morphine tolerance. This study was designed to investigate whether *S*(+)-ketamine affects the $GABA_BR$ desensitization processes by baclofen.

Methods: The G protein–activated inwardly rectifying K^+ channel currents induced by baclofen were recorded using *Xenopus* oocytes coexpressing G protein–activated inwardly rectifying K^+ channel 1/2, $GABA_{B1a}$ receptor subunit, GB_2R, and GRK. Translocation of GRKs 4 and 5 and protein complex formation of GB_2R with GRKs were analyzed by confocal microscopy and fluorescence resonance energy transfer analysis in baby hamster kidney cells coexpressing $GABA_{B1a}$ receptor subunit, fluorescent protein–tagged GB_2R, and GRKs. The formation of protein complexes of GB_2R with GRKs was also determined by coimmunoprecipitation and Western blot analysis.

Results: Desensitization of $GABA_BR$-mediated signaling was suppressed by *S*(+)-ketamine in a concentration-dependent manner in the electrophysiologic assay. Confocal microscopy revealed that *S*(+)-ketamine inhibited translocation of GRKs 4 and 5 to the plasma membranes and protein complex formation of GB_2R with the GRKs. Western blot analysis also showed that *S*(+)-ketamine inhibited the protein complex formation of GB_2R with the GRKs.

Conclusion: *S*(+)-Ketamine suppressed the desensitization of $GABA_BR$-mediated signaling at least in part through inhibition of formation of protein complexes of GB_2R with GRK 4 or 5.

What We Already Know about This Topic:

- Tolerance to intrathecal baclofen for treatment of spasticity is produced by desensitization of the γ-aminobutyric acid type B receptor ($GABA_BR$).

What This Article Tells Us That Is New:

- In cell culture, *S*(+)-ketamine suppressed the desensitization of $GABA_BR$-mediated signaling at least in part through inhibition of formation of protein complexes of $GABA_{B2}$ subunit (GB_2R) with GRK 4 or 5.

BACLOFEN, a selective γ-aminobutyric acid type B receptor ($GABA_BR$) agonist, has been widely used as an antispasticity agent. Intrathecal baclofen (ITB) therapy is an established treatment for severe spasticity of both spinal and

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 Anesthesiology 2011; 114: 401–11

cerebral origin.[1] Recently, increasing reports have shown that ITB therapy has powerful antinociceptive effects in patients with spasticity and in patients without spasticity who experience chronic pain,[1] such as somatic pain,[2] central pain,[2,3] and complex regional pain syndrome.[4,5]

However, long-term management of ITB therapy occasionally results in the development of tolerance,[6] which makes treatment difficult with respect to both pain and spasticity. Such decreased responsiveness to baclofen, so-called baclofen tolerance, is, in part, because of the desensitization of $GABA_BR$.[7,8] In addition, the desensitization of $GABA_BR$ occurred by the formation of complexes of $GABA_BR$ and either G protein–coupled receptor kinase (GRK) 4[7,8] or 5,[7] which is a member of the GRK family consisting of GRKs 1 through 7.[9]

Until today, several agents (*e.g.*, morphine, baclofen, ketamine, clonidine, and local analgesics) have been administered intrathecally for effective chronic pain management or spinal anesthesia clinically.[10,11] Among them, intrathecal ketamine coadministration has a synergistic analgesic effect with opioids.[12] In addition, ketamine administration prevented the development of tolerance against morphine in several animal models,[13,14] although the mechanism has not yet been clearly elucidated. Regulation of tolerance of μ-opioid receptor–mediated cellular signaling, receptors to which morphine mainly act, is known to be mediated by GRKs, particularly GRK 2[15] or 3.[16,17] GRKs 2 and 3 are reported to play in desensitization processes of μ-opioid receptors[15,17] or development of tolerance to opioids in an animal model.[16] In case of $GABA_BR$, it was previously demonstrated that the desensitization of $GABA_BR$-mediated responses was associated with the formation of protein complexes of $GABA_{B2}$ receptor subunit (GB_2R) with GRK 4 or 5.[7] Our hypothesis is that ketamine would interact with GRK 4 or 5. Thus, we focused on the effects of ketamine on the modification of GRKs 4 and 5 in $GABA_BR$-mediated desensitization processes. Ketamine consists of two enantiomers, *S*(+)-ketamine and *R*(−)-ketamine, that have distinct pharmacologic properties.[18] *S*(+)-Ketamine has a three times higher anesthetic potency than that of the racemic mixture, the incidence of adverse effects is equal at the same concentration for both enantiomers,[18] and both are clinically available.[18] Thus, in the current study, we used *S*(+)-ketamine and investigated whether *S*(+)-ketamine has effects on $GABA_BR$ desensitization and the formation of complexes of $GABA_BR$ with GRK 4 or 5.

Materials and Methods

Drugs and Chemicals

Baclofen was purchased from Tocris Cookson, Bristol, United Kingdom; and *S*(+)-ketamine, gentamicin, and sodium pyruvate were obtained from Sigma, St Louis, MO. All other chemicals used were of analytic grade and were obtained from Nacalai Tesque, Kyoto, Japan.

Construction of Complementary DNA and Preparation for Complementary RNAs

Complementary DNA (cDNA) for rat G protein–activated inwardly rectifying K^+ channel (GIRK) 1 and mouse GIRK2 were provided by Henry A. Lester, Ph.D. (Professor of Biology, Caltech, Pasadena, CA). $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), GB_2R, and anti-hemagglutinin (HA)–tagged GB_2R were provided by Niall. J. Fraser, Ph.D. (Glaxo Wellcome, Stevenage, United Kingdom). Cerulean, a brighter variant of cyan fluorescent protein, was obtained from David W. Piston, Ph.D. (Professor of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN); and Venus, a brighter variant of yellow fluorescent protein, was obtained from Takeharu Nagai, Ph.D. (Professor of Nanosystems Physiology, Hokkaido University, Sapporo, Japan). Human GRK4 was provided by Antonio De Blasi, Ph.D. (Professor of Istituto Neurologico Mediterraneo Neuromed, Pozzilli, Italy); and rat GRK5 was obtained from Yuji Nagayama, M.D., Ph.D. (Professor of Medical Gene Technology at Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan). For receptor construction, the N-DYKDDDDK-C (FLAG) epitope tag (5′-GAACAAAAACTCATCTCAGAAGAGGATGTG-3′) was engineered to ligate the N-terminus of GRK 4 or 5 by using standard molecular approaches that use polymerase chain reaction. Venus-fused GB_2R was created by ligating the receptor cDNA into *Hin*dIII sites into the corresponding sites of Venus cDNA. Venus- or Cerulean-fused GRKs 4 and 5 were created by ligating the GRK cDNA sequences into the *Not*I or *Bam*HI sites of corresponding Venus or Cerulean sites. All cDNAs for transfection in baby hamster kidney (BHK) cells were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA). For expression in *Xenopus* oocytes, all cDNAs for the synthesis of complementary RNAs (cRNAs) were subcloned into the pGEMHJ vector, which provides 5′- and 3′-untranslated regions of the *Xenopus* β-globin RNA, ensuring a high concentration of protein expression in the oocytes.[19] Each of the cRNAs was synthesized with a messenger RNA kit (mCAP messenger RNA Capping Kit; Ambion, Austin, TX) and with a T7 RNA polymerase *in vitro* transcription kit (Ambion) from the respective linearized cDNAs.[20]

Oocyte Preparation and Injection

Immature V and VI oocytes from *Xenopus* were enzymatically dissociated, as previously described.[21,22] Isolated oocytes were incubated at 18°C in ND-96 medium (containing 96-mM NaCl, 2-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, and 5-mM HEPES, pH 7.4) containing 2.5-mM sodium pyruvate and 50-μg/ml gentamicin. For measurement of GIRK currents induced by baclofen, cRNAs of GIRKs 1 and 2 (0.2 ng each) and $GB_{1a}R$ and GB_2R (5 ng each) were coinjected into the oocytes, together with or without GRKs (4 or 5) or FLAG-tagged GRKs (FLAG-GRK4 or FLAG-GRK5) (3 ng each). The final injection volume was less than 50 nl in all

cases. Oocytes were incubated in ND-96 medium and used 3–8 days after injection, as previously reported.[21]

Electrophysiologic Recordings

Electrophysiologic recordings were performed using the two-electrode voltage clamp method with an amplifier (Geneclamp 500; Axon Instruments, Foster City, CA) at room temperature. Oocytes were clamped at −60 mV and continuously superfused with ND-96 medium or 49 mM K^+ (high potassium) solution, in which tonicity was adjusted to reduce concentrations of NaCl (48-mM NaCl, 49-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, and 5-mM HEPES, pH 7.4) in a 0.25-ml chamber at a flow rate of 5 ml/min. Then, baclofen alone or *S*(+)-ketamine and baclofen were added to the superfusion solution. Voltage recording microelectrodes were filled with 3 M potassium chloride, and their tip resistance was 1.0–2.5 MΩ. Currents were continuously recorded and stored with a data acquisition system (PowerLab 2/26; AD Instruments, Castle Hill, Australia) and a computer (Macintosh; Apple, Cupertino, CA), as previously described.[21,22] All test compounds applied to oocytes were dissolved into the ND-96 medium or 49-mM K^+ media.

Cell Culture and Transfection

The BHK cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. For confocal microscopic assay, BHK cells were seeded at a density of 1×10^5 cells/35-mm glass-bottomed culture dish (World Precision Instruments, Sarasota, FL) and cultured for 24 h. Transient transfection was then performed with a transfection reagent (Effectene; Qiagen, Tokyo, Japan) in 0.2 μg each cDNA, as previously described,[7,20] and according to the protocol provided by the manufacturer. Cells were used in confocal microscopy and fluorescence resonance energy transfer (FRET) analysis 16–24 h after transfection.

Confocal Fluorescence Microscopy

For translocation studies of GRKs and protein complex formation of $GABA_BR$ with each GRK (4 or 5) using confocal microscopy and the FRET assay, GB_2R and each of the GRKs (4 and 5) were fused through the carboxyl terminus to Cerulean or Venus. The BHK cells cultured in 35-mm glass-bottomed dishes were cotransfected with 0.2 μg Venus-fused $GABA_BR$ and Venus- or Cerulean-fused GRKs. A ×63 magnification 1.25–numerical aperture oil immersion objective was used with the pinhole for visualization. Both Venus and Cerulean were excited by a 458-nm laser, and images were obtained by placing the dish onto a stage in a confocal microscope (Zeiss LSM510 META; Carl Zeiss, Jena, Germany).

Photobleaching and Calculation of FRET Efficiency

To confirm FRET between Venus and Cerulean, we monitored acceptor photobleaching analysis in BHK cells that coexpressed $GB_{1a}R$, Venus-fused GB_2R, and Cerulean-fused GRKs. FRET was measured by imaging Cerulean before and after photobleaching Venus with the 100% intensity of a 514-nm argon laser for 1 min, a duration that efficiently bleached Venus with little effect on Cerulean. An increase of donor fluorescence (Cerulean) was interpreted as the evidence of FRET from Cerulean to Venus. All experiments were analyzed from at least six cells with three independent regions of interest. As a control, we examined the FRET efficiency of the unbleached area of membrane in the same cells in at least three areas. In some cases, we performed a photobleaching assay using fixed BHK cells. Cells were fixed as previously described.[23]

FRET efficiency was calculated using emission spectra before and after acceptor photobleaching of Venus.[24] According to this procedure, if FRET is occurring, then photobleaching of the acceptor (Venus) should yield a significant increase in fluorescence of the donor (Cerulean). Increase of donor spectra because of desensitized acceptor was measured by taking the Cerulean emission (at 488 nm) from spectra before and after acceptor photobleaching. FRET efficiency was then calculated using the following equation: $E = 1 - I_{DA}/I_D$, where I_{DA} is the peak of donor (Cerulean) emission in the presence of the acceptor, and I_D is the peak in the presence of the sensitized acceptor, as previously described.[25] Before and after this bleaching, Cerulean images were collected to assess changes in donor fluorescence.

Coimmunoprecipitation and Western Blotting

Monoclonal anti–FLAG M2 was obtained from Sigma; monoclonal anti-HA (12CA5), from Roche, Mannheim, Germany; and polyclonal anti-HA (Y-11), from Santa Cruz Biotechnology, Santa Cruz, CA. The BHK cells were transiently cotransfected with each of the FLAG-tagged GRK cDNAs, HA-tagged GB_2R (HA-GB_2R), and nontagged $GB_{1a}R$ cDNAs. Twenty-four hours later, the cells were harvested, sonicated, and solubilized in a protein extraction buffer containing a combination of protease inhibitor cocktail (PRO-PREP; iNtRON Biotechnology, Sungnam, Korea) for 1 h at 4°C. The mixture was centrifuged (at 15,000 rpm for 30 min), and the supernatants were incubated with FLAG or HA (12CA5) antibody at 5 μg/ml overnight at 4°C. The mixture was centrifuged, and the pellets were washed five times by centrifugation and resuspension. Immunoprecipitated materials were dissolved in sample buffer (Lammeli) containing 0.1-M dithiothreitol subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and subjected to immunoblotting using monoclonal antibodies against FLAG (1:10,000) and polyclonal HA (Y-11) (1:10,000); then, bovine mouse or goat rabbit anti-IgG was conjugated with horseradish peroxidase at 1:5,000 and reacted with chemiluminescence Western blot detection reagents (Nacalai Tesque).