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Tissue-engineered medical products — Bioactive ceramics — Method to measure cell migration in porous materials

Produits médicaux issus de l'ingénierie tissulaire — Céramiques bioactives — Méthode de mesure de la migration cellulaire dans les matériaux poreux

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

ISO 19090 was prepared by Technical Committee ISO/TC 150, *Implants for surgery*, Subcommittee SC 7, *Tissue-engineered medical products*.

Introduction

"Bioactive ceramics" are widely used in orthopaedic and dental fields due to their bioactivities and bioaffinities. Porous bioactive ceramics are designed as bone void fillers, and cell migration from tissue into their pores is an expectation for effective repair of bone defects; thus, they are one of the promising candidates for cell scaffolds for bone tissue engineering medical products.

To clarify the clinical safety and usefulness of these bioactive ceramics, physical, chemical and biological properties must be examined. In the methods used, animal tests are the ultimate and essential methods to examine biological properties of bioactive ceramics; however, numbers of both animals and animal tests must be reduced under the concept of 3R (Replacement, Reduction and Refinement)[3].

The first and most important property for porous biomaterials including bioactive ceramics is cell migration capability, because cell proliferation, differentiation, tissue formation and tissue maturation in and surroundings of porous biomaterials do not occur without cell migration.

Currently, two different cell-seeding methods are used for estimating "cell migration" property: One is dropping a cell suspension on the top surface of a porous material. This method tests the penetration ability of the "cell suspension" under gravity and estimates the number of cells that migrate into and are held within the porous material. The other method is shaking a porous material in the cell suspension. This method also tests the penetration ability of the "cell suspension" like the above method but uses shaking to drive the cells into the porous scaffolds. Both methods test the abilities of cell penetration and retention only, and do not test the intrinsic ability of the cell to migrate simulating what happens *in vivo*. Body fluid itself can sufficiently carry cells across a minor gap between the implanted material and the host bone. Accordingly, no cell migration test methods have been reported that mimic cell behaviour *in vivo*.

When porous bioceramics are implanted into bone defect, cells migrate into the pore to form new bone. In this process, migration of osteoblasts mainly plays important roles for osteoconduction. That is to say, no osteoconduction nor bone formation can occur without osteoblast migration.

Therefore, it is imperative to establish a quantifiable method to measure cell migration potential of porous bioactive ceramics in a manner similar to how cells behave *in vivo*, in order to evaluate their potential appropriately as materials for tissue-engineered medical products.

Tissue-engineered medical products — Bioactive ceramics — Method to measure cell migration in porous materials

1 Scope

The document specifies the test method to be followed for measuring and documenting the cell migration ability of porous bioactive ceramic materials.

This document is not applicable to porous materials that have low or no cell adhesion properties, for instance synthetic polymers and metals. These types of materials will require longer times to allow effective transfer and migration of cells from the cultured substrate to the test specimen. To minimize influences of cell passages, cell kinds, differences in cell culture consumables including culture medium and fetal bovine serum etc., the method uses a porous bioactive ceramics, which is clinically and widely used in each country, as a reference material for calculation of relative migration distance.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10993-5, *Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 10993-5 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
bioceramics
ceramics used that enhance biological functions when implanted in the human body

3.2
bioactive ceramics
bioceramics (3.1) with direct bone bonding property when implanted into bone defect

3.3
biomaterial
material used in or to be used in medical and dental field

3.4
full confluent
cell cultured dish is almost completely (≈95 % to 100 %) covered with a monolayer of cells

3.5
complete medium
cell culture media that is recommended for the chosen cell type by the supplier of the cells with all required supplements cell culture medium that is confirmed by the user that the cells used in the test proliferate well without any mutation

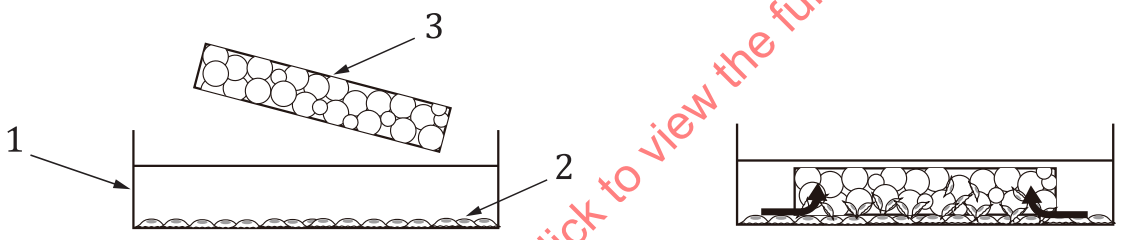
3.6
osteoblast-like cell
established cell lines which widely recognised to have “osteogenic activity”

3.7
cell group
group of cells composed of at least five cells where the distance between each cell is less than the cell width

4 Principle

The cell migration property of porous biomaterials has been estimated by measuring cell numbers that are seeded in the porous body by two methods, however, they measure penetration of cell suspension and cell attachment, not migration by cells themselves.

The present method is very simple and effective to measure cell migration by themselves. Cells confined in a confluent layer of a culture dish are able to further migrate and proliferate from this layer into the porous bioceramic materials that are placed on top of the culture dish as shown in Figure 1. To minimize influences of cell passages, cell types, differences in cell culture consumables including culture medium and serum, etc., the method uses a reference material that porous bioactive ceramics commercialised and used clinically with good clinical results in each country for calculation of relative migration distance.



a) Before: No cells exist in a test material **b) After: Cells move up to a test material through pores**

Key
1 culture dish filled with cell culture medium
2 cells adhered on a bottom of culture dish
3 test material

Figure 1 — Schematic drawing of test method

After the initial transfer of cells onto the material interface, they start to migrate into pores of the materials. This migration distance will differ in relation to the materials properties and chemical stability, surface morphologies, and pore structures similar to what is seen *in vivo* and which mimics the initial part of the bone regeneration process.

Giemsa staining is a very stable and easy method to stain cells for this method.

Linear longest migration distance of a valid cell measured from the cross section of the Giemsa stained porous bioactive ceramics is well reflected cell migration *in vivo*.

5 Test specimen

5.1 Shape and dimensions

The shape of the specimen should be a disk (10 ± 0,2) mm in diameter and (2 ± 0,1) mm in thickness.

5.2 Number of test specimens

At least five pieces are required for the comparison between two (reference and test) materials.

5.3 Reference material

Commercially available porous bioactive ceramics in each country that are confirmed as good bone void filler by basic researches and/or clinical results. The reference material available in multiple countries is recommended.

6 Procedures

6.1 Measurement of thickness and diameter of specimen

The thickness and diameter of specimen shall be measured with a calliper or a micrometer.

6.2 Sterilization of test specimens

Specimens shall be sterilized by a method which does not affect the material quality before the test.

6.3 Deaeration of test specimens

Specimens shall be immersed in 5 mL complete medium in a centrifuge tube which can be sealed tightly with a lid. After an 18 to 21 gauge needle connected with a 20 mL syringe is inserted through the lid of the tube, the specimens shall be deaerated by pulling the plunger of the syringe back completely with tapping on the tube for 2 min to 3 min to eliminate bubbles.

6.4 Cell culture

Osteoblast-like cells shall be seeded on 6-well cell culture plates or ⌀35 mm cell culture dishes and cultured to full confluency. Seeding cell number will be decided by the preliminary test to obtain confluency within 3 days. MG-63 and MC3T3-E1 are recommended for osteoblast-like cells. To obtain confluent cells within 3 days in 6-well culture plate, seeding of $6{,}0 \times 10^4$ per well for MG63 or $8{,}0 \times 10^4$ per well for MC3T3-E1 will be needed. Cells shall be observed under a phase-contrast microscope for 2 days to 3 days after seeding to check cell confluency and to avoid cell overconfluency.

NOTE The number of cells is counted by conventional methods using the hemocytometer, described in ISO 13366-1 or cell counting devices, described in ASTM F2149–01.

The number of cells to be seeded to reach full confluency within 3 days should be confirmed by the preliminary test, if the cell line other than recommended in this document will be used in this test.

When wells (dishes) are covered with confluent cells, 3 wells (dishes) shall be stained with Giemsa to record the cell confluent status before migration test as follows: After being fixed with 2 % glutaraldehyde overnight, Giemsa solution with appropriated concentration shall be prepared with PBS, and 2,5 mL of the solution shall be added to each well (dish) and incubated for 3 min at room temperature. Then the staining solution shall be removed and washed with 2,5 mL distilled water for 3 times. Stereoscopic micrograph of each well or dish shall be taken at x10 or maximum available magnification so that the bottom of plates or dishes can be observed in the same scope.

6.5 Placing specimens on the cell layer

When cells have reached full confluency, one deaerated specimen shall be placed on the cell layer and the SUS316 stainless steel double ring with 10 mm in ring outer diameter and 0,8 mm to 1,0 mm in wire diameter, shown in Figure 2, shall be put on the specimen to brace in place. Both shall be placed with forceps without dropping nor agitation of the medium. There can be some roughness and unevenness on the surface of the specimen due to manufacturing process. Choose a smooth surface of the specimen to place on the cell layer.

When transferring the plates (or dishes) into the CO_2 incubator, take care not to move the specimen inadvertently since it could impair the close contact of the specimen with the cell layer. The cells shall be cultured another 3 days without medium change or any other procedure.

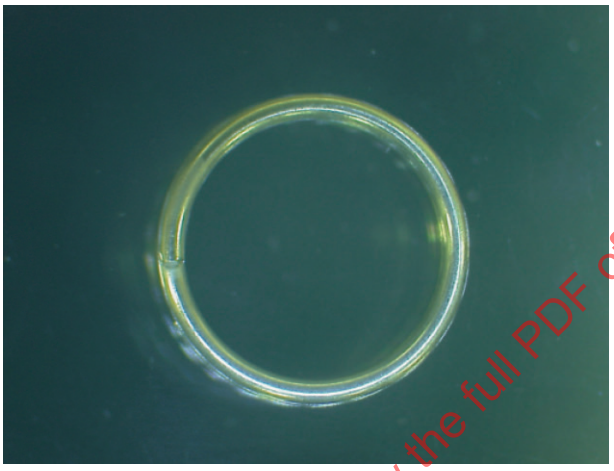


Figure 2 — Stainless steel double ring

Set the weight on the sample to ensure close contact between the sample and the confluent cell layer. However, if the influence of the extra weight on the cell is unclear, the acute cytotoxicity should be evaluated by cell proliferation assay as follows, to ensure that there is no effect.

a) wash the weight with ultrapure water with applying ultrasonic for 1 h followed by rinsing with ultrapure water 3 times.

b) dry heat sterilize the weight at 160 °C for 3 h.

c) place the weight on the full confluent cell layer as same as the cell layer to be used in the test and observed cell shape and numbers surroundings of the weight with phase-contrast microscope daily for at least 3 days.

6.6 Treatments after cell culture

The specimen shall be harvested 3 days after incubation. To prevent detachment of cells from the specimen, place the bottom side (cell contact surface) of the specimen up after harvesting. The specimen shall be washed with PBS for 3 times and fixed with 2 % glutaraldehyde overnight. After fixation, the specimen shall be washed with PBS for 3 times and immersed in 10 mL of Giemsa solution with appropriate concentration at room temperature. After 3 min, the sample shall be transferred to another vessel and washed with 10 mL of distilled water. The wash is repeated 3 times.

If this staining method does not work or is very different from the manufacturer's instruction, follow the staining protocol in the manufacturer's instruction.

Wells or dishes after harvesting specimen shall be washed with PBS for 3 times and fixed with 2 % glutaraldehyde overnight. After fixation, the wells or dishes shall be washed with PBS for 3 times and pour 2,5 mL of Giemsa solution with appropriate concentration at room temperature. After 3 min, the solution will be removed and washed with distilled water. The wash is repeated 3 times. Then,

the staining solution shall be removed and washed with 2,5 mL of distilled water for 3 times. Cell migrations from the edge of the cell transferred areas on the well or dish are observed, if the specimen and stainless steel double ring are not affected cell viability. Further, if cells in the specimen placed area showed no change in shape and numbers but did not transfer well, low "flatness" of the specimen can be the reason.

6.7 Positive and negative controls

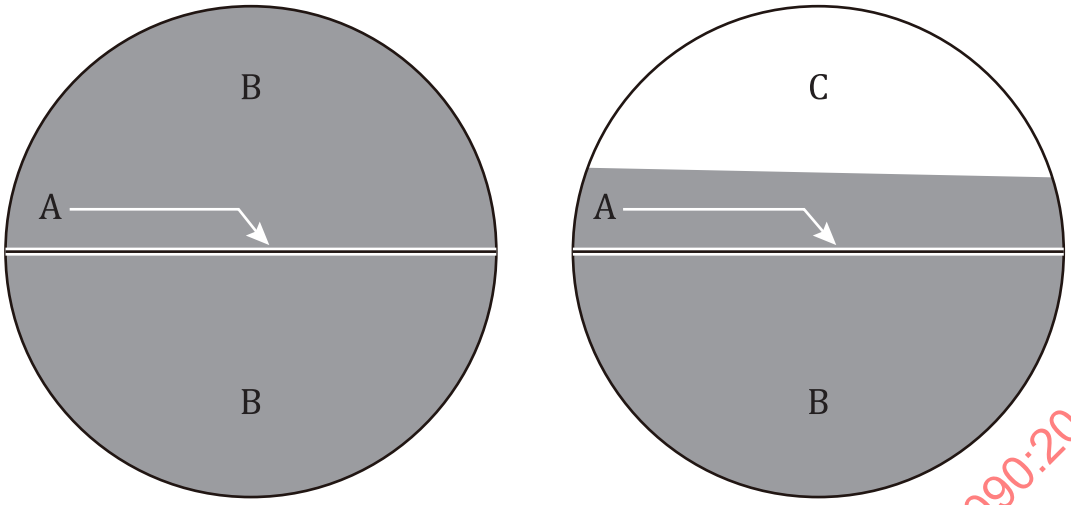
Including positive and negative controls for the assay signal can be useful for each assay. For example, an appropriate negative control can be to conduct the measurement in the absence of cells. The scaffolds can be placed in dishes without cells, incubated with culture medium, washed, stained, imaged and scored. As an example of an appropriate positive control, the scaffolds can be directly seeded with cells using a pipette, incubated in medium to let the cells adhere (possibly 4 h or 24 h), washed, stained, imaged and scored.

These controls provide assurance that the assay is working effectively and help with interpreting the results. In the case where cells migrate into the test scaffolds, it is important to demonstrate that the negative controls did not score positively for cell migration. This provides evidence that the background staining of the scaffold and the scoring procedure, among other things, are reliable. In the case where there is poor migration into the test scaffolds, it is important to demonstrate that the positive controls scored positively for cells. This provides evidence that cells were viable, the stain was effective, and the scoring procedure was reliable (among other things). Including positive and negative controls in the assay makes it possible to interpret the results and improves confidence in the conclusions.

6.8 Observations of cells that migrated into a specimen

Annexes A and B show typical results and are useful to understand practical results and procedures as referred in the following procedures.

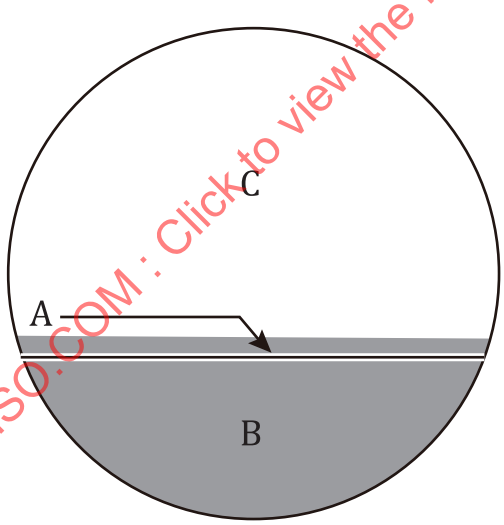
The cell-contact (bottom) side of the specimen shall be observed with a stereoscopic microscope. Stereoscopic microphotographs of the bottom side shall be taken. The specimen shall be cut into two pieces using a thin scalpel commonly used for eye surgery. Depending on the flatness of bottom surface of the specimen, stained area might have irregular distribution and shape as shown in Figures A.5 and B.3; therefore, the cutting position shall be determined from the stained specimen interface (bottom) that had been in direct contact with the cell layer; and the incision shall be made along the longest determined length through the darkest stained area of the specimen (Figures 3 to 6).



Key

A cutting position
B stained area
C non-stained area

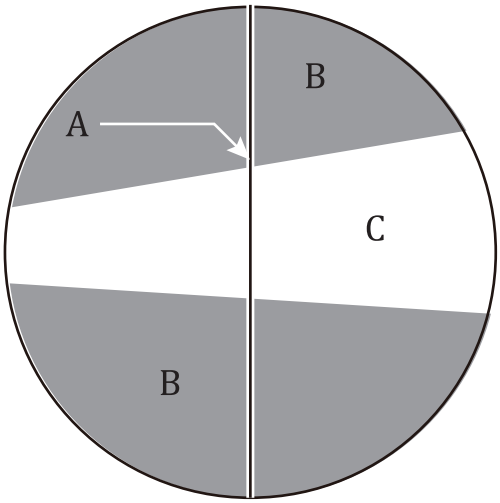
Figure 3 — Schematic drawing to determine cutting line for specimen surface of which stained area is more than a half



Key

A cutting position
B stained area
C non-stained area

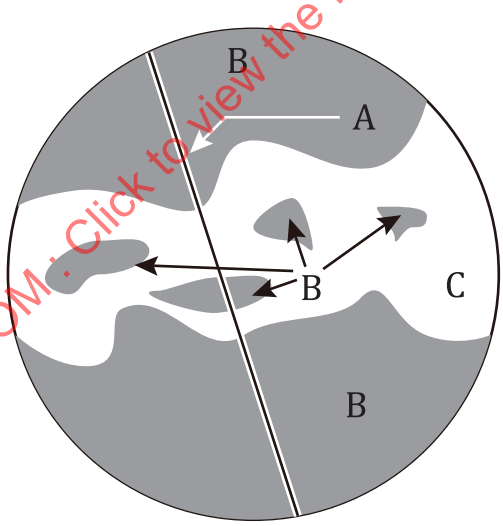
Figure 4 — Schematic drawing to determine cutting line for specimen surface of which stained area is less than a half



Key

A cutting position
B stained area
C non-stained area

Figure 5 — Schematic drawing to determine cutting line for specimen surface of which stained areas are partitioned



Key

A cutting position
B stained area
C non-stained area

Figure 6 — Schematic drawing to determine cutting line for specimen surface of which stained areas are distributed

The cross section should be observed preferably with a stereoscopic microscope. Stereoscopic microphotographs of the cross section of the specimen and micrometre at the same magnification shall be taken.

NOTE 1 Specimens are cleaved with a scalpel that is normally used for eye surgery. If it is impossible to cleave specimens with a scalpel, the specimen is cut with a diamond cutter or similar apparatus using phosphate buffered saline as lubricant.

NOTE 2 The same specimen stained with the same Giemsa staining solution after soaking in the same cell culture medium without cells for the same period can be used as a control to confirm background staining level of the specimen to clarify the stained parts of the test specimens are cell(s) or not.

NOTE 3 Fluorescent labelling of cells and observation with a confocal microscope can be used for the following longest migration distance measurement as well, if confocal microscope is available in the laboratory.

6.9 Measurement of migration distance of cells in a specimen

6.9.1 General

All measurements should be performed preferably under a stereoscopic microscope at x10 and x20 (objective x1 and x2, eye-piece x10) magnification.

6.9.2 Maximum migration distance of cell

The longest linear distance from the bottom side of the specimen to the furthest stained cell or cell group shall be measured for each specimen. Before measurement, the following shall be checked.

a) Using a stereoscopic microscope at high magnification (at least x40), the cellular nature of the staining shall be validated using general cell criteria including nuclear staining and morphological features showing cellular process and attachment to the surface of the porous structures.

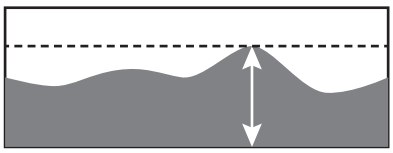
b) Validation of cell and cell group for measurement described below is based on the results from multiple inter-laboratory tests. The following empirical validation avoids inclusion of a cell or cell group that can have lodged non-specifically within the larger pore sites as a result of cell culture media changes and washings prior to fixation.

The followings are the protocol to validate cell and cell group in detail.

Longest linear length from bottom side to top end of a cell or cell group, which observed most far from bottom side, shall be measured.

6.9.2.1 Determination of linear longest migration distance for cell group

As shown in Figure 7, the top of a cell group is continued from the bottom side; thus, the white line is measured as the linear longest distance for a cell group.

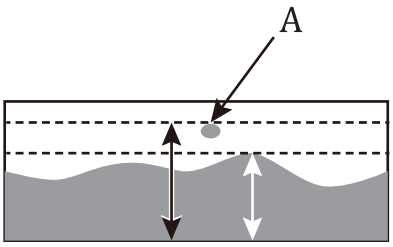


Key

grey area stained area with many cells

NOTE The white line is the longest linear distance.

Figure 7 — Measurement point of migrated distance in a simple stained area

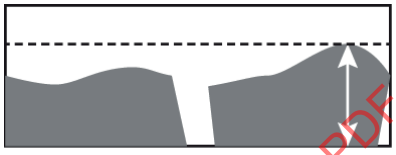


Key

grey area	stained area with many cells
A	single cell

NOTE The white line is the longest linear distance.

Figure 8 — Measurement point of migrated distance in a simple stained area with a cell but not a cell group



Key

grey area	stained area with many cells

NOTE The white line is the longest linear distance.

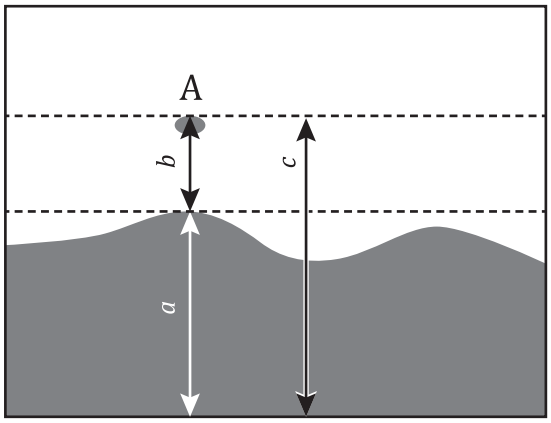
Figure 9 — Measurement point of migrated distance in partitioned stained area

In case 1, the cell is divided from the top of the cell group as shown in Figure 8, the dark line indicates linear longest distance to a "cell", but not to a cell group; thus, the linear longest distance to a "cell group" is the white line.

Even stained area is divided as shown in Figure 9, the higher stained part is measured as a linear longest distance for a cell group as indicated as the white line. Thus, if no cell group divided from bottom stained area are observed as in Figure 7, the linear longest distance of the "cell group" is defined as white lines in Figure 7.

When cell groups are divided from bottom stained area, the following validation shall be performed.

In case 1 (Figure 10), the closest distance from the cell group A to the cell group that already is confirmed as a valid cell group, length of line *b*, is shorter than that of line *a*, migration distance of valid cell group; thus, A is confirmed as a valid cell group. In this case, no divided cell group with longer distance from bottom side. Accordingly, the linear longest distance to cell group is a length of line *c*.



Key

A cell group

a migration distance of a valid cell group

b distance between cell group A and a valid cell group

c linear longest distance of cell group

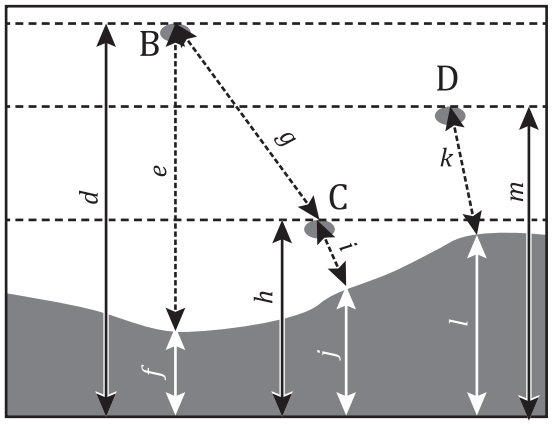
Figure 10 — The measurement point of migration distance in stained area when a cell group migrate irregularly in specimen at very near to the stained area

In case 2 (Figure 11), start from the cell group C, which is the closest cell group from bottom side. Length of line *i*, distance to the closest valid cell group from the C, is shorter than that of line *j*; thus the C is a valid cell group and the length of line *h* is valid migration distance as the same as the cell group A and line *c* in the case one. However, much longer distances from the other cell group D to bottom, length of line *m*, are also valid due to the same reason, *i.e.*, length of line *k* is shorter than that of line *l*. Contrarily, cell group B is not valid, because the length of line *g*, the closest distance to the valid cell group, is longer than that of line *h*.

6.9.2.2 Determination of linear longest migration distance for cell

After determined linear longest migration distance for the "cell group," the same validation will be performed for a cell, using the closest "cell group" not "cell." For instance, in Figure 11, when all circles, B, C and D, are "cells", not "cell groups", the cell B shall be validated using lines *e* and *f* instead of lines *g* and *h*. Therefore, even if length of line *g* is shorter than that of line *h*, cell B is not valid because length of line *e* is longer than that of line *f*. When C is a "cell group", using lines *g* and *h* for validation of the cell B.

After validations mentioned above, the longer distance between linear longest distances of the cell and cell group shall be chosen as the maximum cell migration distance for the specimen.



Key

B, C, D irregularly migrated cell groups or cells
d longest linear distance of cell group/cell B when C is a valid "cell group"
e closest distance between cell group/cell B and a valid cell group when C is not a valid "cell group"
f migration distance of a valid cell group closest to cell group/cell B when C is not a valid "cell group"
g closest distance between cell group/cell B and a valid cell group when C is a valid "cell group"
h longest linear distance of cell group/cell C
i closest distance between cell group/cell C and a valid cell group
j migration distance of a valid cell group closest to cell group/cell C
k closest distance between cell group/cell D and a valid cell group
l migration distance of a valid cell group closest to cell group/cell D
m longest linear distance of cell group/cell D

Figure 11 — The measurement point of migration distance in stained area when many cell groups migrate irregularly in specimen

6.10 Cells remaining on the well or dish after harvesting specimen

To confirm the cell's behaviour to move to the specimen from the bottom of wells or dishes, the wells (or the dish) will be stained with Giemsa after harvesting the specimen as follows.

— A 2,5 mL of Giemsa solution with appropriate concentration in the 50 mL sized centrifugal tube at room temperature will be added to each well or dish after fixation treatment for 3 min.

— the staining solution will removed and washed with 2,5 mL of distilled water for 3 times.

— Stereoscopic micrograph of each well or dish will be taken at x10 or maximum available magnification that bottom of plate or dish will be able to be observed in one photo.

7 Calculation

7.1 Mean and standard deviation

The mean migration distance and its standard deviation shall be calculated for each specimen group by using Formula (1) and (2).

Mean value

$$\bar{\sigma} = \frac{\sum_{i=1}^{n} \sigma_i}{n} \quad (1)$$

Standard deviation

$$s = \left[\frac{\sum_{i=1}^{n} (\sigma i - \bar{\sigma})^2}{n-1} \right]^{\frac{1}{2}} \quad (2)$$

where

σ_i is the maximum validated measured distance;

n is the total number of test piece.

7.2 Relative mean and standard deviation by normalization

The mean migration distance and its standard deviation calculated in 7.1 shall be used for calculation of the relative mean and standard deviation using Formula [3] and [4].

Relative mean value

$$\bar{\sigma}_R = \frac{\sigma_B}{\sigma_A} \quad (3)$$

Relative standard deviation

$$S_R = \bar{\sigma}_R \times \left[\left(\frac{S_A}{\sigma_A} \right)^2 + \left(\frac{S_B}{\sigma_B} \right)^2 \right]^{\frac{1}{2}} \quad (4)$$

Relative distance and standard deviation for reference sample can be calculated using Formula [5].

$$\bar{\sigma}_R \pm S_R = 1 \pm \frac{S_A}{\sigma_A} \quad (5)$$

where

$\bar{\sigma}_A$ is the mean distance for reference sample;

$\bar{\sigma}_B$ is the mean distance for test sample;

$\bar{\sigma}_R$ is the relative distance;

S_A is the standard deviation of the mean distance for reference sample;

S_B is the standard deviation for the mean distance for test sample;

S_R is the standard deviation for the relative distance.

8 Test report

The result of evaluation of cell migration in porous bioceramic scaffolds shall be reported in terms of the following data:

a) reference to this document, i.e. ISO 19090;

b) name, product number, lot number, main component, size, pore size, pore size distribution, pore shape and porosity with their measurement methods, and manufacturer of the test specimen;

c) year/month/day of experiment, name, affiliation and position of experiment executor. The quality management system of the Laboratory also be mentioned if applicable;

d) name and origin (distributor) of cell line used, the total monolayer cell number prior to placing ceramics on the monolayer, cell counting method and percent cell confluency;

e) name, type, catalogue number, lot number of culture medium, serum and antibiotics (concentration of serum and antibiotics in culture medium, if applicable);

f) name, catalogue number, lot number and manufacturer (or seller) of culture plate, pipet tips, pipettes, sterile filters, cell culture flasks, etc. for cell culture;

g) name, catalogue number, lot number and manufacturer (or seller) of reference porous ceramics sample;

h) test results;

   1) mean longest linear distance as a “cell migration distance” and its standard deviation.

   2) stereoscopic microphotographs of cross section and bottom side of specimen(s).

   3) stereoscopic micrographs of Giemsa stained cells on the well of 6-well plate prior to the migration test.

   4) stereoscopic micrographs of Giemsa stained cells on the well of 6-well plate after the migration test.

   5) relative cell migration distance and its standard deviation normalized by reference sample data.

i) comments.

Annex A
(informative)

Results of tests for determination of test conditions

A.1 General

As one of the indices of "bone regeneration property," cell migration ability, which is closely related to bone tissue ingrowth to scaffold biomaterials, will be evaluated *in vitro*. The preliminary tests performed in Japan revealed that "flatness" of the porous ceramics specimens is quite important for cell transfer from cell layer on the culture dish to the specimen. The "flatness" can be interpreted that the ratio of the total length of pore wall edges on the surface of the specimen contact with cell layer to that can be observed by naked-eye. It can be measured but control of the "flatness" is difficult. In fact, Japanese manufacturers and researchers have tried to prepare "flat" surface by machining; however, no methods were enough to control it. For instance, polished specimens showed worse results than as-cut specimens. Thus, methods to stabilize cell transfer from a culture dish to specimen had to be investigated. One is weight application with a SUS 316 stainless steel double ring and collagen coating on the surface of a culture dish. The weight application can increase deformation of cell layer to allow increasing cell-specimen contact. The collagen coating also increases deformation of cell layer through increasing softness of cell-coated collagen composite layer to allow increasing cell-specimen contact. In addition, results of *in vitro* test were compared with *in vivo* bone formation test to evaluate the reflection of the *in vitro* test result to the bone formation.

This test will not supply any information about osteoblastic activities or bone formation abilities. Those will be evaluated by other methods provided in the future.

A.2 Materials and method

A.2.1 *in vitro* test

Materials for test were provided by 4 Japanese manufacturers and 2 Japanese research institutes (university and national institute). Materials provided from manufacturers were clinically used ones and those from institutes were not used clinically. This round robin test is mainly performed to confirm the influences of the weight and/or collagen coating. In addition, we cannot be provided enough numbers of specimens from several providers, number of specimens for the test was reduced to 3 in each condition. Materials used for the first test are summarized in Table A.1.

Table A.1 — Specimens for the first test

Abbreviation	Composition and pore structure	Size mm		Porosity vol %	Mean pore diameter µm	Sterilization
		Diameter	Height			
A	HAp (random, spherical)	10	2	73	170	Autoclave
B	HAp+β-TCP (random, spherical)			50	170	
C	α-TCP (random, spherical)			78,5	266,9	Ethylene oxide gas
E	HAp/Col composite (random)			95	140	
G	β-TCP (random, irregular)	5		75	120	Autoclave
H	HAp (Unidirectional, oval)	10		75	50	

HAp: hydroxyapatite

TCP: tricalcium phosphate

HAp/Col: hydroxyapatite/collagen

Table A.2 — Test conditions for the first test

Institute	Cells	Conditions 1		Conditions 2	
		Weight	Collagen coat	Weight	Collagen coat
NIMS	MG63	Yes	Yes	No	Yes
AIST	MC3T3-E1	Yes	No	No	No
U-Tokyo	MG63	No	Yes	No	No

NIMS: National Institute of Material Sciences

AIST: Advanced Institute of Sciences and Technology

U-Tokyo: University of Tokyo

Cell lines used in the first test were chosen from MG63 and MC3T3-E1. The weight was SUS 316 stainless steel double ring (10 mm in diameter for A, B, C, E and H, and 5 mm in diameter for G, diameter of wire construct ring was 0.8 mm in diameter,) that was preliminarily confirmed its non-cytotoxicity. Collagen coating was performed by manufacturer's instruction. The test conditions are summarized in Table A.2. Due to the limitation of the sample provided, 3 specimens were used for each condition, except for AIST (n = 2 for condition 1 and n = 1 for condition 2).

A.2.2 *in vivo* tests as a control of the test

The same materials were implanted into mouse cranium hole as the same size as that of the specimen diameter. Three-dimensional image was observed by *in situ* micro X-ray computer tomography at 0, 2 and 4 weeks after implantation. The images at 2 and 4 weeks post-implantation were compared with that of at 0 weeks. X-ray denser and lighter regions were defined as newly formed bone and resorbed regions respectively, and their volumes were calculated. Finally, subtraction of the lighter from the denser region volumes was calculated to be a bone formation volume (index).

A.3 Results

A.3.1 *in vitro* test

A.3.1.1 Effect of stabilization method on migration distance

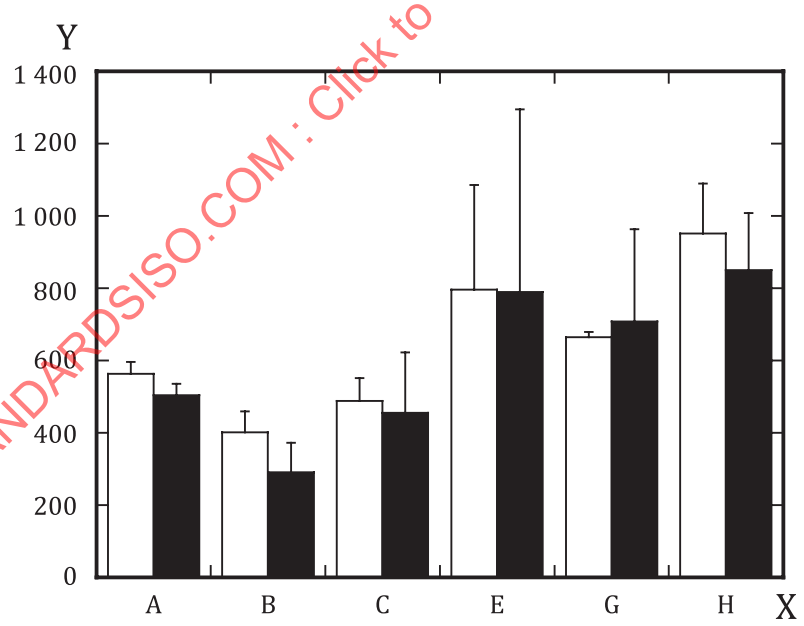
The migration distances for each specimen tested in NIMS, ASIT and U-Tokyo are summarized in Figures A.1 to A.3, respectively.

From one-way ANOVA statistical analysis, no significant differences were observed in the data from the same institute. However, the application of stabilization methods (the weight application with the stainless steel double ring, NIMS and AIST, and the collagen coating, U Tokyo) showed better cell migration distance tendency than that of without stabilization methods except for sample C in NIMS and sample E in AIST. Therefore, both methods were effective to increase stability of cell-specimen contact. Cost effectiveness and easy operability, the weight application is better for the primary method and the collagen coating is the optional one.

A.3.1.2 Migration distances among samples

The differences among institutes can be due to differences in cell lineages, cell types (MG63 and MC3T3-E1), lot of media (cell culture media, fetal bovine serum, antibodies and others) and other deviations by operators as well as cell culture devices. Normalization by the same sample in each institute can be useful to ignore these differences. Therefore, normalized values by A in each institute were calculated as shown in Figure A.4.

Tendency of maximum migration distance is very similar among the institutes, B demonstrated the shortest migration distance; A, C, E and G indicated similar and moderate migration distances; and H showed the longest migration distance. In addition, cell types did not affect the results in comparison to other factors.



Key

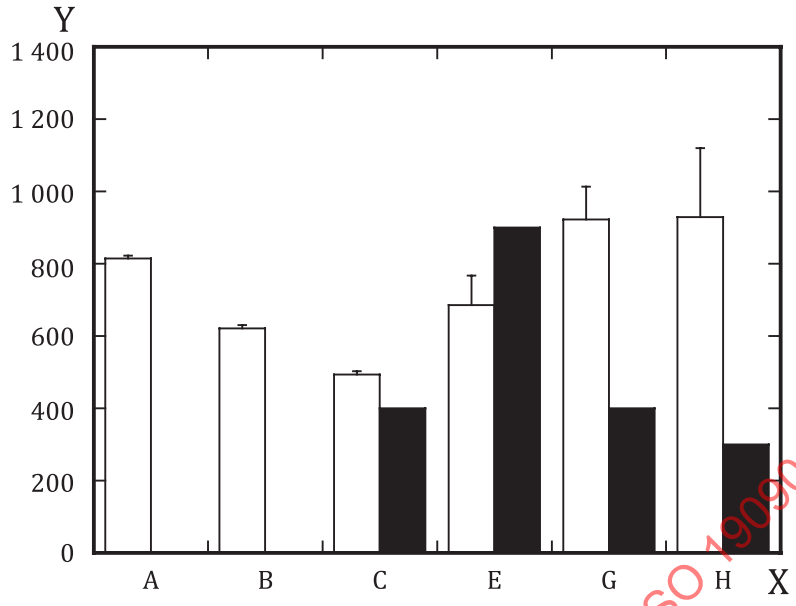
X specimen abbreviation

Y migration distance of cells, in micrometre

□ data from specimen with weight application and collagen coating

■ data from specimen with collagen coating

Figure A.1 — Maximum cell migration distances for each specimen in NIMS



Key

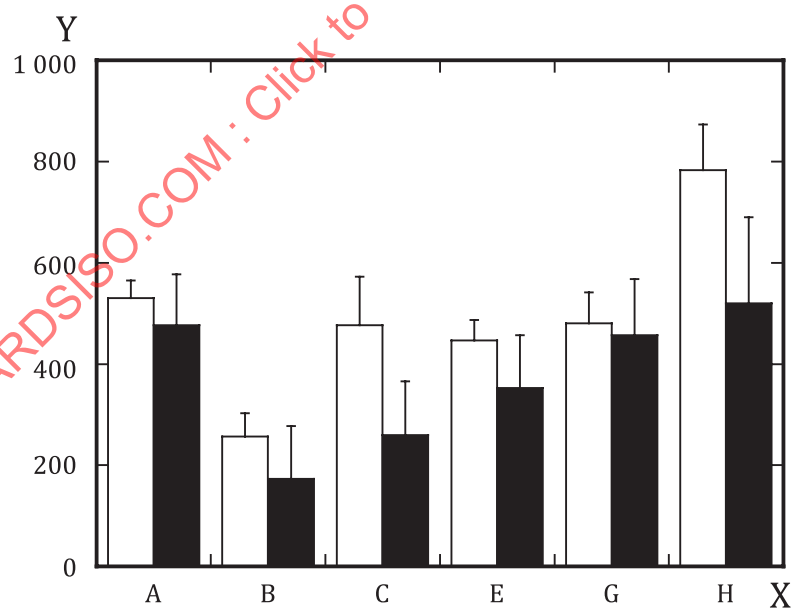
X specimen abbreviation

Y migration distance of cells, in micrometre

□ data from specimen with weight application

■ data from specimen without additional treatments

Figure A.2 — Maximum cell migration distances for each specimen in AIST



Key

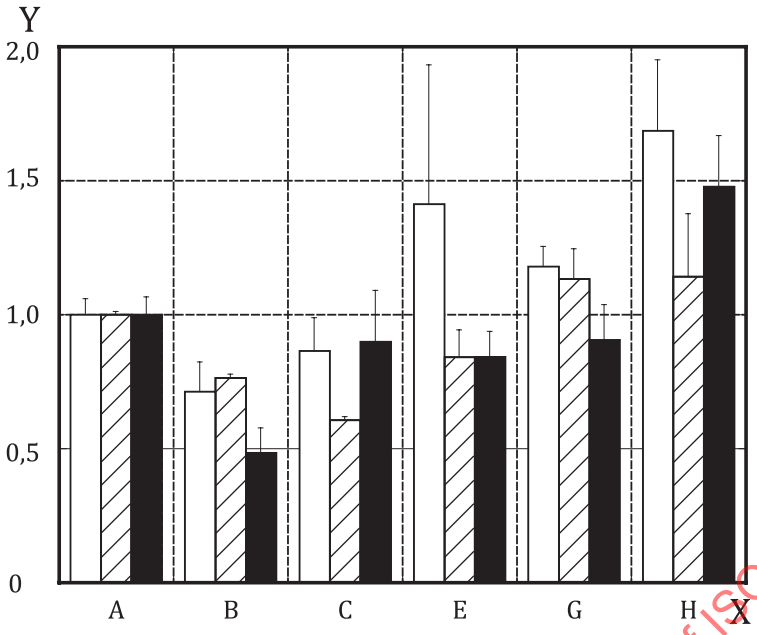
X specimen abbreviation

Y migration distance of cells, in micrometre

□ data from specimen with collagen coating

■ data from specimen without additional treatments

Figure A.3 — Maximum cell migration distances for each specimen in U-Tokyo



Key

X specimen abbreviation

Y maximum migration distance value of cells, normalized by distance of A

□ data obtained by NIMS with weight application and collagen coating

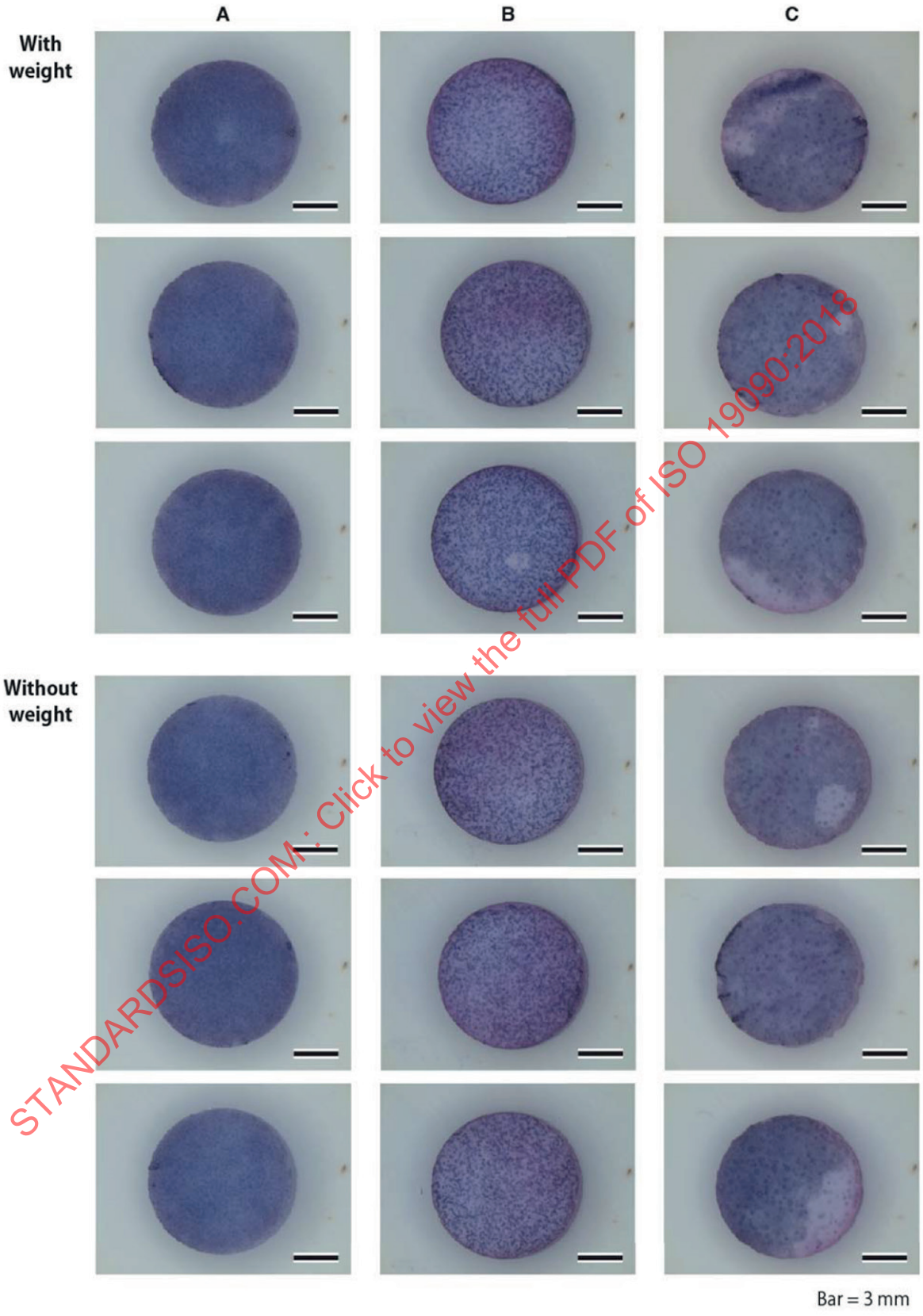
■ data obtained by AIST with weight application

▨ data obtained by U-Tokyo with collagen coating

Figure A.4 — Maximum cell migration distances normalized by A for each specimen in three different institutes

A.3.1.3 Stereoscopic microphotographs of bottom side and cross section of specimen

Bottom (cell contact side) and cross section photos of samples stained are shown in Figure A.5 and Figure A.6, respectively. Specimen E showed higher background than other specimens due to collagen contained in the materials. Even though, the measurement was as easy as the other ones at 10x magnification.



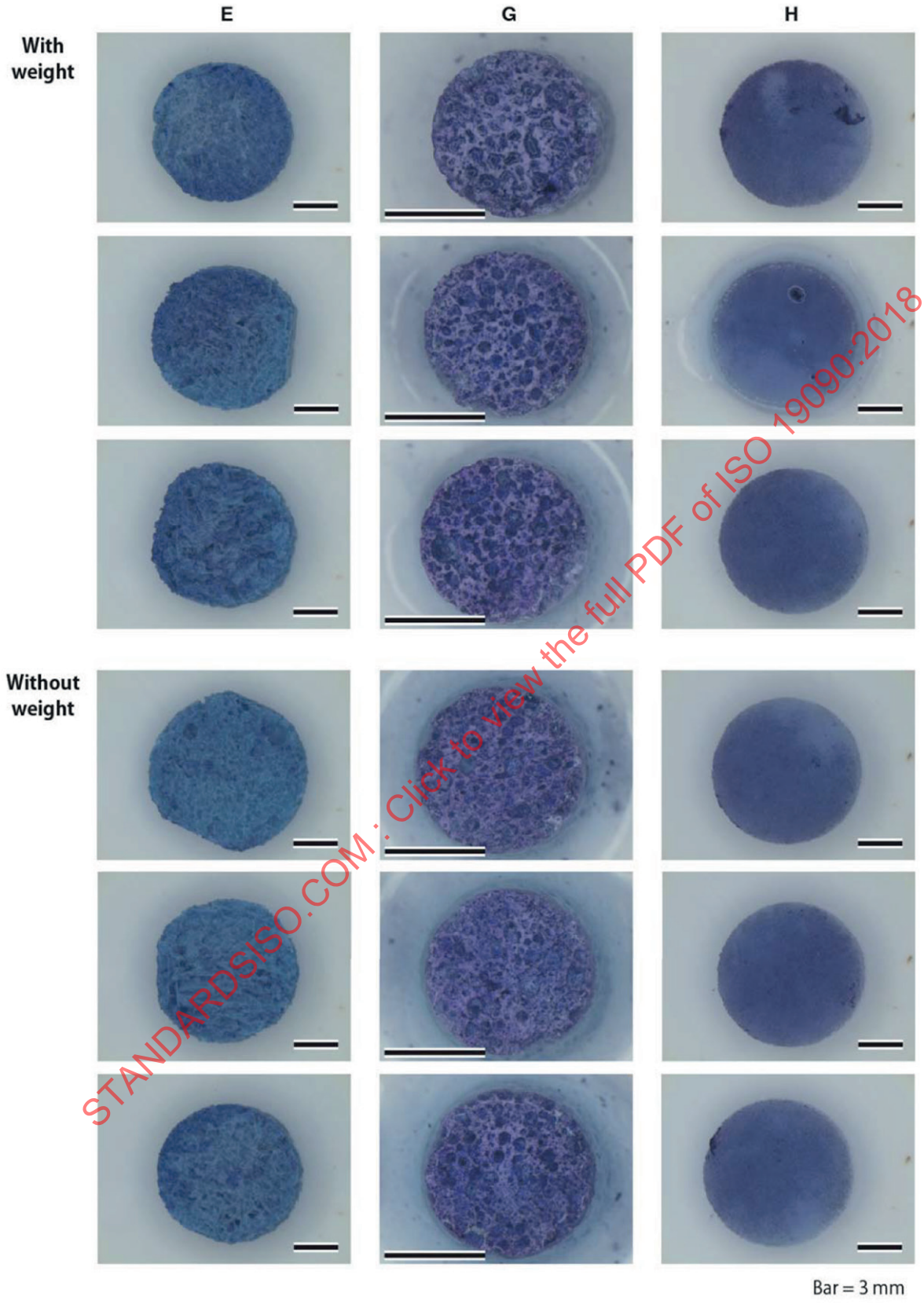
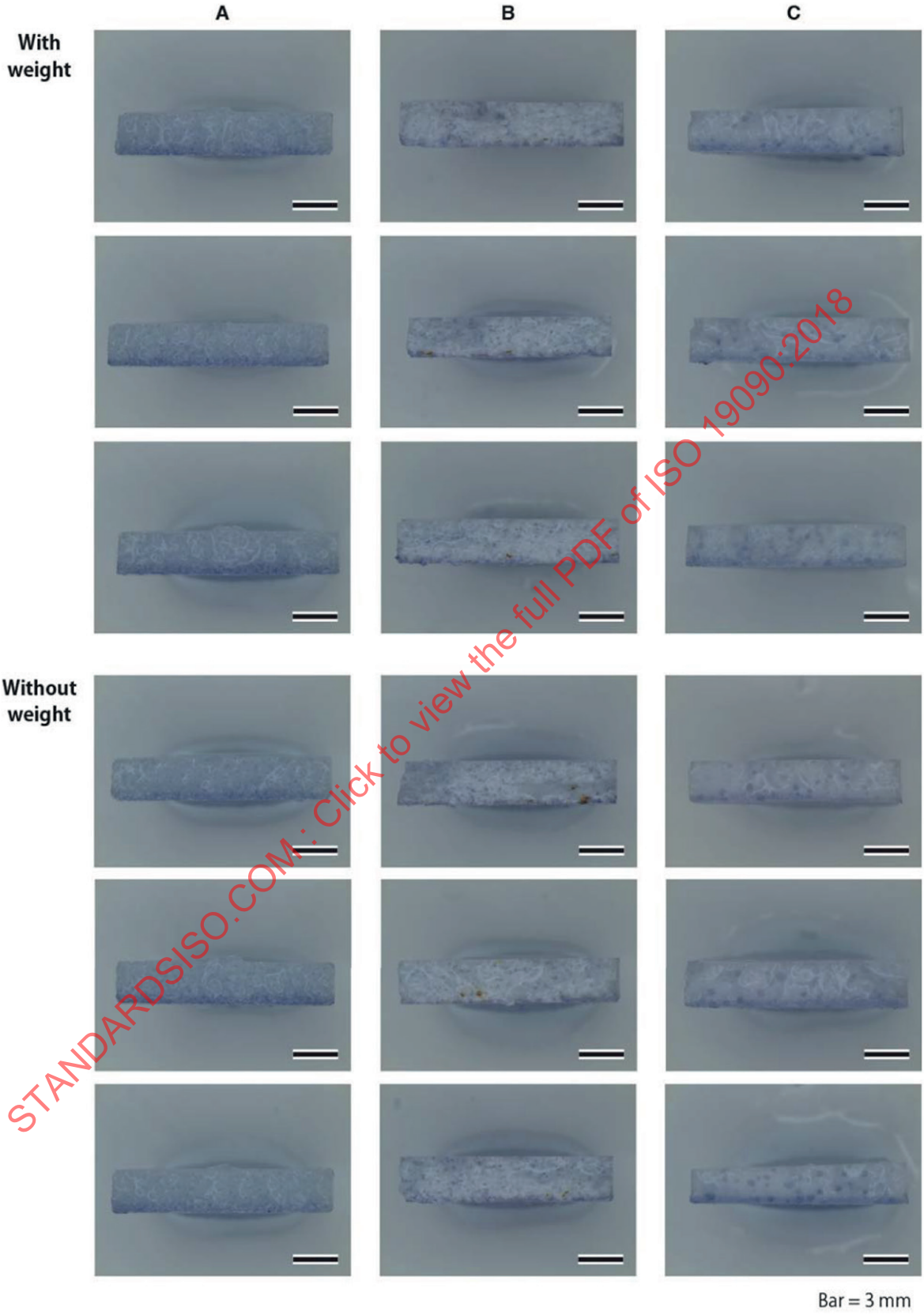


Figure A.5 — Bottom side of specimens with or without weight



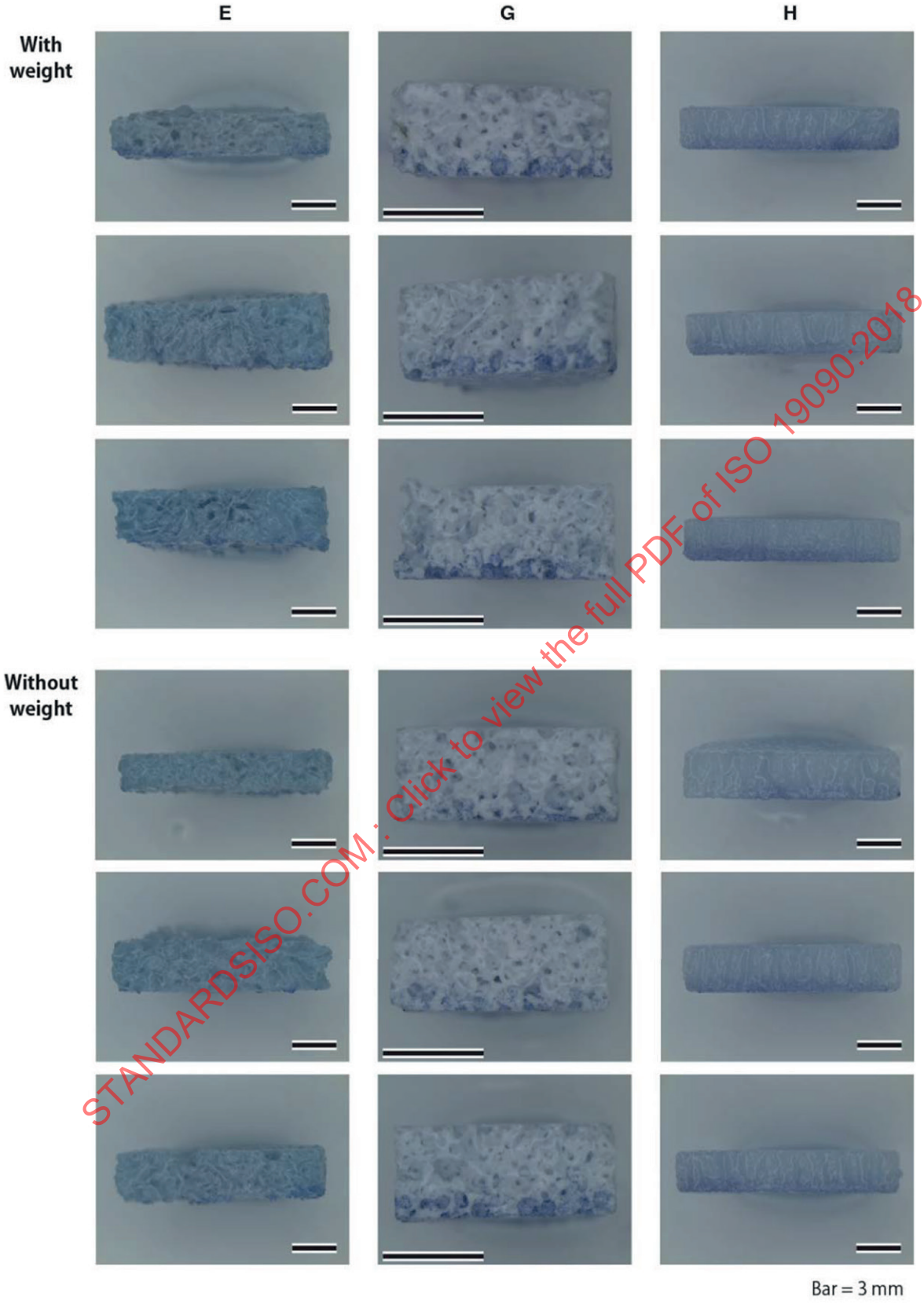


Figure A.6 — Cross section of specimens with or without weight

A.3.2 Results of *in vivo* test as a control of first test

A.3.2.1 Micro X-ray computer tomographs

Micro X-ray computer tomographs are summarized in Figure A.7. Image for A and B seemed to be a typical bone formation in the random pored bioactive and non-bioresorbable ceramics. For bioresorbable ceramic materials, images for C, α-TCP showed material resorption in early (2W) stage then large amount of bone formation were observed at later (4W) stage. Contrarily, images for G, β-TCP, seemed to be resorbed continuously in comparison to C, and increase of bone mass seemed smaller than C, even clinical results of C are considered to be the same as the porous HAp materials. Images for E, HAp/Col nanocomposite material had less X-ray absorption properties due to high porosity and the presence of collagen.

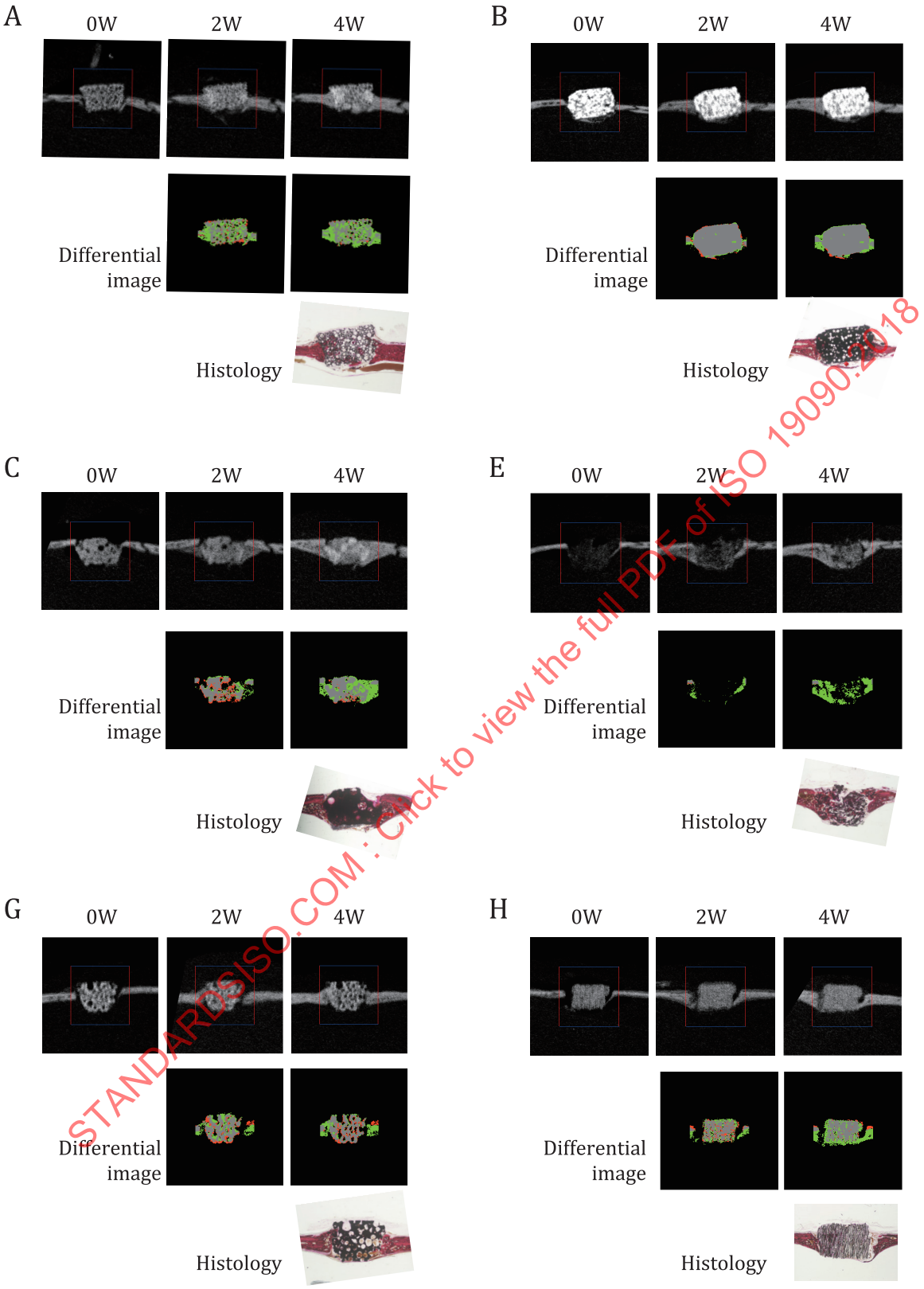
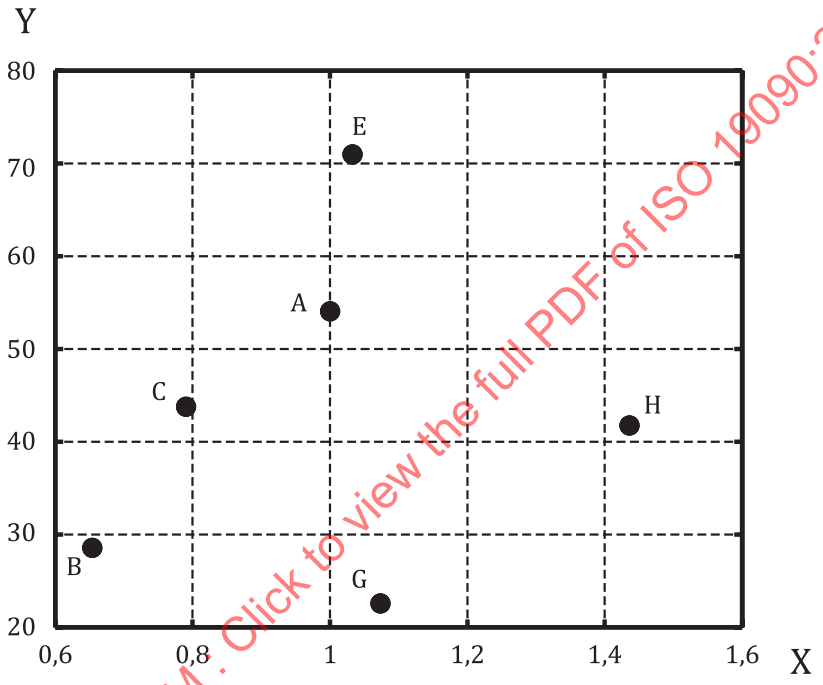


Figure A.7 — Micro X-ray computer tomographs, their differential images and histological sections of specimens tested

Accordingly, threshold for ceramics materials did not suit for E and less bone formation was observed at 2 weeks after implantation; however, large amount of bone formation was observed at 4 weeks due to its high osteoconductivity, which has already confirmed by animal [1] and clinical tests. [2] Images

for H, unidirectional porous HAp, seemed lower new bone mass in comparison to A. The reason was that cells needed detour to migrate into H's pore because its pore direction is completely perpendicular to cell source area, bone edges. In other animal experiments, H with preferable direction, pores were parallel to the cell source area, showed better cell and bone tissue migrations than randomly pored HAp. Comparison between cell migration distance *in vitro* and bone formation index at 4W are shown in Figure A.8. For the reasons mentioned above, sample G, bioresorbable β-TCP ceramics with random and irregular pores, and H, unidirectional porous HAp, did not show good relation between *in vitro* migration distance and bone formation index. These results suggested that materials having unusual porous structure, such as unidirectional one that has unique orientation for cell and tissue migration, need careful treatment of implantation for optimal cell and tissue migration. In addition, bioresorption is sometimes a negative factor to examine bone formation with a micro X-ray computer tomography, because X-ray opaqueness decreases with time by materials resorption.



Key

X maximum migration distance of cells normalized by distance of A

Y bone formation index

NOTE Specimens are shown as abbreviation (see Table A.1).

Figure A.8 — Averaged and normalized maximum migration distance *vs* bone formation index at 4 weeks after operation

Annex B
(informative)

Results of international round robin test

B.1 General

This annex provides results of the international round robin test.

B.2 Materials and method

Materials for test were provided by 4 Japanese manufacturers and 2 Japanese research institutes (university and national institute. Materials provided from manufacturers were clinically used ones and those from institutes were not used clinically. Materials used for the first test are summarized in Table B.1. Five specimens were used for each test.

Table B.1 — Specimens for the first test

Abbreviation	Composition and pore structure	Size (mm)		Porosity vol %	Pore size µm	Sterilization
		Diameter	Height			
A	HAp (random, spherical)	10	2	73	170	Autoclave
B	HAp+β-TCP (random, spherical)			50	170	
C	α-TCP (random, spherical)			78,5	266,9	Ethylene oxide gas
E	HAp/Col composite (random, spherical)			95	140	
G	β-TCP (random, irregular)			75	120	Autoclave
H	HAp (unidirectional, oval)			75	50	

Participant institutes were, NIMS, AIST, U-Tokyo (UT), in Japan, University of Cambridge in UK (UK) and University of Gothenburg in Sweden (SWE).

Cell lines used in the second test were chosen from MG63 and MC3T3-E1. The MC3T3-E1 cell line was only used in AIST. Weight was SUS 316 stainless steel double ring (10 mm in diameter and diameter of wire construct ring was 0,8 mm in diameter,) that was preliminarily confirmed its non-cytotoxicity.

B.3 Results of round robin Test

B.3.1 Migration distance

The round robin test was performed internationally by six different institutes with application of SUS-316 double ring weight. The migration distances for each specimen are summarized in Figure B.1.