Iterative *in vivo* cut'n'paste of chromosomal loci in *Escherichia coli* K-12 using

synthetic DNA

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Abstract

We have developed a simple and highly efficient *in vivo* method to iteratively

relocate functional chromosomal loci onto an episome in *Escherichia coli* by utilizing

synthetic DNA fragments. In this *in vivo* cut'n'paste procedure, "cutting" is executed by

the RNA-guided DNA endonuclease Cas9 and a set of guides, while "pasting" is

facilitated by the phage λ Red recombinase, which are all synthesized on easily

curable plasmids. To demonstrate the utility of this approach, we commercially

obtained synthetic DNA fragments containing locus-specific homology regions,

antibiotic marker cassettes, and standardized Cas9 target sequences. By using these

locus-specific synthetic DNA fragments, we relocated 7 functional chromosomal loci.

Scarless relocation mutants of the *trg*, *aer*, *tsr*, *malHM*, *malQT*, *macB-nadA*, and *folA*

chromosomal loci were obtained as antibiotic-resistant isolates by combining Cas9

counterselection with the restoration of an antibiotic marker cassette. The additional

antibiotic marker cassettes and standardized Cas9 target sequences present in the

synthetic DNA fragments are inherently eliminated upon completion of the procedure,

enabling iterative processing of loci. This procedure should be widely useful, especially

in genome (re-)engineering of *E. coli* and other bacteria because the procedure can

be done on wild-type cells.

<u>Introduction</u>

The ability to manipulate genomes is key for uncovering and leveraging the

potential of (micro-)biology. A milestone in our ability to edit bacterial genomes was the

development of the one-step inactivation of chromosomal genes procedure[1]. Among

many other scientific and engineering endeavours, it facilitated the creation of a

comprehensive single gene knockout library and the charting of essential genes in *E.*

coli[2], the study of bacterial motility[3,4], stringent response[5], the interplay of growth and

gene expression[6], two-component systems[7], evolution of antibiotic resistsance[8], and

chromosomal organisation[9] as well as the development of strains with reduced

genomes[10] and highly titratable induction systems[11] while also playing a crucial role in

metabolic engineering applications[12,13]. Though the one-step inactivation procedure[1]

was preceded by numerous other bacterial genome editing techniques[14–21], its ease

of use and broad applicability ensured its still continuing success. The required short

homology extensions to create genomic edits are easily attached to an antibiotic

marker cassette via PCR, the recombineering machinery is expressed from an easily

curable plasmid, and the procedure does not require any prior modification to the

bacterial genome. Furthermore, the antibiotic marker cassette can subsequently be

eliminated, resulting in an unmarked, yet not fully scarless, mutant that is compatible

with another round of genome editing.

With the rise of CRISPR-Cas technologies[22], λ Red recombineering was

successfully supplemented with sequence-specific RNA-guided counterselection[23–25].

Combining λ Red recombineering and CRISPR-Cas with intricate multi-layered

selection, counterselection, and colour screening enables large-scale genome editing

such as genome recoding[26,27], global genome rearrangement[28], and interspecies gene

transfer[29]. While these advanced genome editing techniques accomplish feats vastly

beyond the capabilities of the one-step inactivation of chromosomal genes procedure[1],

they all fall short in at least one respect. Namely, they are relatively difficult to

implement. Requiring prior assembly of DNA fragments in yeast[27], elaborate *in vitro*

DNA manipulations of helper plasmids[26,28] or episomes[29], or the presence of particular

chromosomal mutations enabling counter-selectability[26–28], and a lack of efficient

methods to eliminate helper plasmids[26–28], not only limit their adaptation to a few

specialized laboratories but furthermore impede their capacity to iteratively edit

bacterial genomes.

Here, we present *in vivo* cut'n'paste, an easy-to-use, scarless, large-scale

genome editing procedure that can be performed in wild-type cells. Leveraging

CRISPR-Cas cutting and λ Red recombineering with standardized synthetic DNA

fragments and an antibiotic marker recycling scheme completely removes the need

for *in vitro* DNA manipulations and, in conjunction with easily curable helper plasmids,

inherently enables iterative genome editing. We successfully relocated seven

functional genomic loci, including metabolic and essential genes, in *E. coli* of up to 140

kb in size.

<u>Results</u>

<u>*In vivo* cut'n'paste</u>

In vivo cut'n'paste enables to iteratively relocate genomic loci from the native

chromosome onto an episome (Supp. Movie 1). In each round, a genomic locus,

defined by two pairs of tandem homology regions (HR^A-HR^B and HR^C-HR^D) is excised

from the native chromosome and subsequently integrated in between the two

homology regions HR^{OL} and HR^{TET} in an episome (Fig. 1A). The entire relocation

process occurs inside the cell without requiring any *in vitro* DNA manipulations.

Each round of *in vivo* cut'n'paste consists of two phases: (1) pre-loading and

(2) relocation. During pre-loading, two synthetic DNA fragments, PRE^{epi} and PRE^{chr}

are sequentially integrated via homologous recombination into the episome and the

native chromosome, respectively, to equip cells with the required modification for the

relocation of the genomic locus (Fig. 1B; for details see Supplementary Materials,

Supp. Fig. 1-3). PRE^{epi} is flanked by the homology regions HR^{OL} and HR^{TET}. Directly

downstream of HR^{OL} PRE^{epi} contains the inside homology region HR^B of the genomic

locus. The two outside homology regions HR^A and HR^D, flanked by the constant cut

sites CS^1 and CS^2, are located next to HR^B followed by a chloramphenicol marker

cassette and another constant cut site, CS^3. Integration of PRE^{epi} at HR^{OL} and HR^{TET}

into the episome truncates the tetracycline maker present in the episome and, thus,

renders the cells sensitive to tetracycline yet resistant to chloramphenicol. PRE^{chr} is

flanked by the homology region HR^C and HR^D, enabling its integration directly adjacent

to the genomic locus of interest in the native chromosome. In PRE^{chr} HR^C is directly

followed by 5'TET^R, encoding a short, non-functional fragment of the tetracycline

marker cassette, and HR^{TET}, a kanamycin marker cassette, flanked by the constant

cut sites CS^1 and CS^2 completes PRE^{chr}.

Once pre-loading is completed, cells contain all features required for relocation.

Firstly, the genomic locus in the native chromosome now contains HR^B on one side

and HR^{TET} on the other side. Secondly, the episome also contains both HR^B and

HR^{TET}. Thirdly, a "repair band aid", consisting of HR^A and HR^D, which can fix the

double-stranded break that will be left behind by the excision of the genomic locus

from the native chromosome, is present in the episome. Optionally, additional features

may be introduced during pre-loading, for example, a terminator in between HR^C and

5'TET^R, which would, once relocation is completed, partially insulate the relocated

locus from its new local environment on the episome.

Relocation is initiated by transforming the helper plasmid pCut. pCut expresses

guides targeting the constant cut sites CS^1, CS^2, and CS^3 from a CRISPR array, as

well as a locus-specific single guide targeting the junction between HR^A and HR^B. The

sequence encoding the locus-specific single guide can effectively be inserted into pCut

via I-CreI cutting counterselection-aided plasmid recombineering, thus completely

eliminating the need for *in vitro* DNA manipulations (Supp. Fig. 4; see Methods for

details). RNA-guide Cas9 endonuclease activity introduces double-stranded breaks

into six targeted locations across the episome and the native chromosome. These

double-stranded breaks release the "repair band aid" from the episome and the

genomic locus from the native chromosome while simultaneously exposing the

required homology regions for integration of the "repair band aid" into the native

chromosome and of the genomic locus into the episome. Importantly, integration of

the genomic locus into the episome not only fixes its double-stranded break but,

additionally, leads to the restoration of the tetracycline marker cassette, enabling

positive selection of successful relocation events in the presence of tetracycline.

Notably, the chloramphenicol and kanamycin marker cassettes, along with the

constant cut sites, which were introduced during pre-loading, are innately lost during

the relocation process. Since the helper plasmid pCut is easily curable from the cells

by inducing guides targeting its origin of replication and its antibiotic marker, relocation

mutants are inherently capable of undergoing additional rounds of *in vivo* cut'n'paste

(Fig. 1A).

<u>Relocating a genomic locus</u>

In a first attempt, we employed *in vivo* cut'n'paste to relocate the *trg* locus from

its native genomic location in CNP^{empty} (*E. coli* K-12 MG1655 WT + EPI^{empty}) onto an

empty episome (EPI^{empty}). Candidates of successful relocations, CNP^{trg} (*E. coli* K-12

MG1655 Δ*trg* + EPI^{trg}), were obtained under tetracycline selection. After curing the

helper plasmids, we sent a CNP^{trg} isolate for short-read whole genome re-sequencing.

Mapping reads of the CNP^{trg} isolate to the parental CNP^{empty} reference depicted a

depth of coverage of about 400x across the native chromosome and the episome (Fig.

2A, middle row). No mutations relative to the parental strain CNP^{empty} were observed.

Yet, notably, pairs of adjacent bases at three genomic locations were not spanned by

any reads. Namely, both edges of the *trg* locus on the native chromosome (HR^A-HR^B

and HR^C-HR^D) and one edge of HR^{OL} on the episome lacked spanning reads (Fig. 2A,

middle row). No lack of spanning reads at these locations was observed when reads

of the parental CNP^{empty} strain were mapped to the CNP^{empty} reference (Fig. 2A, top

row). The lack of spanning reads in the CNP^{trg} isolates suggested that though the *trg*

locus was present, it was not located in its native genomic location, and, additionally,

that something had likely been inserted into the episome next to HR^{OL}. We updated

the reference map to include the desired relocation of the *trg* locus based on our *in*

silico design of the *in vivo* cut'n'paste process. When mapping the reads of the CNP^{trg}

isolate to this updated reference, the lack of spanning reads disappeared (Fig. 2A,

bottom row), suggesting that the *trg* locus had indeed been relocated from the native

chromosome onto the episome. Long-read sequencing of an extracted episome

further supported the successful relocation of the *trg* locus. We observed eleven reads

that spanned from the backbone of the episome across the entire, mutation-free *trg*

locus.

The *trg* locus ($\approx$1.8 kb) contains a single gene encoding the transmembrane

Trg chemoreceptor. Trg mediates chemotaxis toward D-ribose[30]. Expansion dynamics

of trg-relocated CNP^{trg} isolates in glycerol minimal medium soft agar plates in the

presence (dark colours) and absence (light colours) of D-ribose were comparable to

the behaviour of CNP^{empty} which encodes *trg* in its native genomic context (Fig. 2B,

black and blue). When the episome EPI^{trg} was removed, the expansion dynamics of

the CNP^{trg} isolates in presence of D-ribose were reduced to a slower aerotactic

behaviour[31] as observed in the absence of D-ribose, indicating that the ability to sense

D-ribose was lost along with the episome (Fig. 2B, red). As an additional test, we

separately transferred the episomes EPI^{trg} and EPI^{empty} into a strain background in

which the *trg* gene had been disrupted by a kanamycin marker cassette (*E. coli* K-12

MG1655 Δtrg::KanR). As expected, faster expansion dynamics in presence of D-

ribose were restored only when EPI^{trg}, containing the relocated *trg* locus, was present

(Fig. 2C).

<u>Iteratively relocating genomic loci</u>

Upon completion of the *trg* relocation the antibiotic marker cassettes and

constant cut sites acquired during pre-loading were inherently lost and the tetracycline

marker cassette on the episome was restored (Fig. 1A). Thus, CNP^{trg} was capable of

undergoing additional rounds of *in vivo* cut'n'paste. We iteratively applied *in vivo*

cut'n'paste to relocate two additional genomic loci using CNP^{trg} as a starting point (Fig.

3A). First, the *aer* locus (≈1.7 kb) encoding the aerotaxis receptor Aer was relocated,

resulting in CNP^{trg,aer}. Then, the *tsr* locus (≈1.8 kb) encoding the Serine and aerotaxis

receptor Tsr was relocated, resulting in CNP^{trg,aer,tsr}. The relocated loci were mutation-

free. The triple-relocated CNP^{trg,aer,tsr} qualitatively retained the colony expansion

behaviour of its ancestor CNP^{empty} (Fig. 3 B,C). Furthermore, separately transferring

the episomes EPI^{empty} (Fig. 3D), EPI^{trg} (Fig. 3 E), and EPI^{trg,aer} (Fig. 3F) into a Δ*trg* Δ*aer*

Δ*tsr* chromosomal background partially restored the colony expansion behaviour as

expected.

In an independent attempt, using CNP^{empty} as a starting point, we successfully

iteratively relocated two genomic loci (≈8.1 kb and ≈7.9 kb) encoding 10 genes

involved in maltose utilisation (Fig. 4A). Again, the relocated loci were mutation-free.

The relocation mutants CNP^{malHM} and CNP^{malHM,malQT} retained their ability to uptake

and ferment maltose (Fig. 4B).

<u>Relocating a large genomic locus and an essential gene</u>

To illustrate the utility of *in vivo* cut'n'paste to process very large genomic loci,

we attempted to relocate the *macB-nadA* locus ($\approx$140 kb) containing 135 genes from

its native genomic location onto the episome. The relocation was successful and

mutation-free.

As opposed to traditional sequential deletion and complementation procedures,

all genes, including those located inside the genomic locus that is edited, remain

present throughout the entire *in vivo* cut'n'paste process. Hence, *in vivo* cut'n'paste

should be well suited for manipulating hard-to-edit genomic features such as essential

genes. Indeed, we successfully relocated the essential gene *folA* onto an episome,

resulting in CNP^{folA}. An episome containing an essential gene that is simultaneously

absent from the native chromosome, as is the case in CNP^{folA}, is expected to be stably

maintained even in the absence of antibiotic selection. As a direct test, we replaced

the tetracycline antibiotic marker cassette of the episome in CNP^{folA} by a green

fluorescent reporter (GFP), resulting in CNP$^{folA,TetR::gfp}$. During serial culturing of

CNP$^{folA,TetR::gfp}$ in LB medium without any antibiotic supplementation 100% of the

bacterial population depicted green fluorescence (Fig. 5, Supp. Fig. 5), clearly

confirming the stable maintenance of the antibiotic marker-free episome as an extra-

chromosomal element (see Methods for details).

<u>Unintended side effects</u>

All relocated genomic loci were mutation-free. Yet, some of the relocation

mutants acquired background mutations in their native chromosome while undergoing

in vivo cut'n'paste (Supp. Table 7). In general, it remains unclear whether these

background mutations have any functional relevance or are merely coincidental

genetic drift.

Discussion

We developed *in vivo* cut'n'paste - an easy-to-use procedure for large-scale

genome editing in *E. coli* that does not rely on any *in vitro* DNA manipulations. We

successfully relocated multiple genomic loci encoding metabolic and essential genes,

generated a genomic alteration >100 kb, and performed up to three consecutive edits

in a single strain. All relocated loci, including the 140 kb *macB-nadA* relocation, were

mutation-free. Due to its robustness, as each construction step is accompanied by

positive antibiotic selection, *in vivo* cut'n'paste is well-suited for implementation via lab

automation.

In vivo cut'n'paste can be adapted to precisely characterise gene function. One

round of *in vivo* cut'n'paste effectively creates a complementation mutant in which the

gene of interest is located on a single-copy extra-chromosomal replicon. Thus, *in vivo*

cut'n'paste inherently overcomes the limitations of traditional sequential "delete and

complement" procedures hampering the investigation of essential genes while also

circumventing any convoluting copy number effects. By iterating *in vivo* cut'n'paste

arbitrary modules of genes, like those obtained here (Fig. 3 & 4), can be created. For

example, genes that are co-modulated or functionally connected but natively

dispersed across the chromosome can easily be clustered onto a single extra-

chromosomal replicon[32]. Utilising the origin of transfer (oriT) encoded on the episome,

such modules can be transferred to other strains or species via conjugation (oriT)

without the size-limitations imposed by transformation[33], and, hence, potential future

adaptation of *in vivo* cut'n'paste to other bacterial species will only broaden its utility

(see Supplementary Text). Moreover, relocations of essential genes, such as

demonstrated here in CNP[folA], are highly desirable in the context of large-scale

fermentation applications in green biotechnology as they allow to stably maintain

transferable extra-chromosomal replicons in production strains without incurring the

metabolic costs and legal requirements associated with continual antibiotic selection[34].

In vivo cut'n'paste empowers experimenters to refactor genomes, akin to the

seven genomic edits demonstrated here, without requiring expert knowledge in

advanced bacterial genome engineering methodologies. Consequently, *in vivo*

cut'n'paste can contribute to diverse areas of research, from studying bacterial

motility[3,4], to examining stringent response[5], to dissecting the interplay of growth and

gene expression[6], to investigating two-component systems[7], to elucidating the

evolution of antibiotic resistance[8], to characterizing chromosomal organisation[9], and

beyond. We envision that *in vivo* cut'n'paste can also enable novel experimental

evolution approaches by enabling the "freezing" of parts of the bacterial genome

during adaptation. By relocating sets of genes onto an episome that is periodically

"reset", i.e., replaced by its unevolved ancestor, evolutionary space can thus be

restricted as chosen by the experimenter. The ability to relocate genomic loci makes

in vivo cut'n'paste particularly well-suited for studying how genome architecture

impacts cell functioning, a topic of which we are only beginning to scratch the

surface[35,36]. Ultimately, *in vivo* cut'n'paste opens up novel avenues to obtain minimal

genomes via bottom-up approaches to complement traditional, ineffectual top-down

genome reduction attempts.

Materials and Methods

See "Methods" document.

Data availability

All data is publicly available (see Supp. Table 8 for details). All custom-written

scripts are supplied in the Supplementary Materials. Sequencing data was deposited

at NCBI (PRJNA1271273; see Supp. Table 1, 2, and 3 for assession numbers).

Sequence-verified, annotated maps of episomes and helper plasmids are supplied in

the Supplementary Materials. Strains are available for non-commercial use through

the Belgian Coordinated Collections of Microorganisms (https://bccm.belspo.be; see

Supp. Table 1 for assession numbers). Helper plasmids are available for non-

commercial use through Addgene (https://addgene.org; see Supp. Table 3 for

assession numbers).

Authors' contributions

SG, ABB, and MHHN developed the initial concept of *in vivo* cut'n'paste. SG

implemented, tested, and refined *in vivo* cut'n'paste iteratively. SG performed all

experiments, analysed the data, and wrote the manuscript. All authors read the final
version of the manuscript.

Conflicts of interest

The authors do not have any conflicts of interest to disclose.

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<u>Figures and figure legends</u>

See "Figures" document.

<u>Supplementary Materials</u>

422 See "SupplementaryMaterials" file.

1 **Iterative *in vivo* cut'n'paste of chromosomal loci in *Escherichia coli* K-12 using**

2 **synthetic DNA**

5 <u>**Figures and figure legends**</u>

Figure 1: Iterative *in vivo* cut'n'paste

a) Overview of the iterative *in vivo* cut'n'paste workflow. **b)** Illustration of the synthetic

pre-loading DNA fragments and their features. A more detailed description of the

design choices is provided in the Supplementary Material. Half-arrows indicate the

relative orientations of features. Solid, downwards arrows mark cut sites. pRedCas9

and pCut: easily curable helper plasmids, HR: homology region, CS: cut site, TET^R:

tetracycline marker cassette, CM^R: chloramphenicol marker cassette, KAN^R:

kanamycin marker cassette, 5'TET^R: non-functional fragment of TET^R, Tet:

tetracycline, Cm: chloramphenicol, Kan: kanamycin, R: resistant, S: susceptible, and

bp: base pairs. Not to scale.

Figure 2: *In vivo* cut'n'paste of the trg locus

a) Number of mapped reads in 3.2 kb segments of the native chromosome and the episome. Only reads mapping to a position and to both of its direct neighbors are counted. Strain identities and reference maps as indicated within the panel. The trg locus is highlighted in yellow. Triangles mark locations of homology regions. **b-c)** Colony expansion as function of time in glycerol minimal medium with (dark) or without (light) 100 μM D-ribose. **b)** CNP^{trg} (blue, solid), CNP^{trg} without EPI^{trg} (red, solid), and CNP^{empty} (black, solid). Three independent CNP^{trg} isolates and a single replicate of CNP^{empty}. **c)** *E. coli* K-12 MG1655 Δtrg::KanR with EPI^{trg} (blue, dashed), *E. coli* K-12 MG1655 Δtrg::KanR with EPI^{empty} (red, dashed), and CNP^{empty} (black, solid). A single replicate each.

Figure 3: Iterative *in vivo* cut'n'paste of three chemoreceptor loci

a) Illustration of iterative *in vivo* cut'n'paste of the trg, aer, and tsr loci. **b-f)** Colony

expansion as function of time in glycerol minimal medium with 100 μM D-ribose (blue),

with 100 μM L-Serine (red), or without any additions (black). Strain identities as defined

within the individual panels.

Figure 4: Iterative *in vivo* cut'n'paste of two maltose-utilization loci

a) Illustration of iterative *in vivo* cut'n'paste of the malHM (i.e., *malEFGH* and

malKlamBmalM) and malQT (i.e., *malPQ* and *malT*) loci. **b)** Maltose utilization

phenotypes on maltose-MacConkey+Tet/agar at 37 °C after 9 hours. Dark red: cells

can utilize maltose, pale yellow: cells cannot utilize maltose. Strain identities as

defined within the panel. Scale bar: 10 mm.

Figure 5: Maintenance of an antibiotic marker-free episome during serial culturing

a) False-colored fluorescence (GFP; excitation: 455-485 nm, emission: 508-557 nm, exposure time: 15 msec) and **b)** brightfield images of CNP$^{folA,TetR::gfp}$ plated onto LB agar at transfers during serial culturing in LB without any antibiotic supplementation. Numerals within the panels indicate transfer numbers. Cultures reached comparable densities of OD$_{600}$ ≈4 at each transfer. Scale bar: 30 mm.

Supplementary Figure 1: Merging of synthetic DNA fragments during simultaneous

pre-loading

Illustration of merged dimers of the synthetic DNA fragments PRE[epi] and PRE[chr] due

to sequence homology at shared features. Merging of PRE[epi] and PRE[chr] causes

feature loss. Locations of merging are highlighted in bold. Definitions as defined in Fig.

1. Not to scale.

Supplementary Figure 2: Integration of synthetic DNA fragment PRE^{epi} during sequential pre-loading

Illustration of integration events of the synthetic DNA fragment PRE^{epi} due to homology at pairs of shared features into A) the episome, B) the native chromosome, or C) a native chromosome containing PRE^{chr}. Integration events may cause feature loss. Many pairs of shared features cannot undergo successful integration due to their

76   relative orientations (feature orientation mismatch) or locations (feature order

77   mismatch). Homologies used for integration are highlighted in bold. Definitions as

78   defined in Fig. 1. Not to scale.

A)

PRE^chr → chromosome

HR^C & HR^D

B)

PRE^chr → episome

no shared pairs of features

C)

PRE^chr → pre-loaded episome

CS¹ & HR^D	feature order mismatch
CS¹ & CS²	feature orientation mismatch
CS¹ & HR^TET	feature orientation mismatch
HR^D & CS²	feature orientation mismatch
HR^D & HR^TET	feature orientation mismatch
CS² & HR^TET	feature order mismatch

Supplementary Figure 3: Integration of synthetic DNA fragment PRE^chr during

sequential pre-loading

Illustration of integration events of the synthetic DNA fragment PRE^chr due to homology

at pairs of shared features into A) the native chromosome, B) the episome, or C) an

episome containing PRE^epi. Many pairs of shared features cannot undergo successful

integration due to their relative orientations (feature orientation mismatch) or locations

(feature order mismatch). Homologies used for integration are highlighted in bold.

Definitions as defined in Fig. 1. Not to scale.

Supplementary Figure 4: Insertion of locus-specific guide sequence into pCut_GFP

via I-CreI cutting counterselection-aided plasmid recombineering

a) Overview of I-CreI cutting counterselection-aided plasmid recombineering. **b)**

Image of transformants (1:10^4 dilution) plated onto LB-agar containing ampicillin [100

mg/L] (plate diameter: 90 mm). **c)** Agarose gel electrophoresis of diagnostic PCRs

across the engineering location in pCut (primers: diag_guide_A, diag_guide_B). Non-

green transformants (lanes 2-7), green transformants (lanes 8 and 9), and "gene ruler

[1 kb]" DNA ladder (lanes 1 and 10). Expected band sizes are indicated by black

triangles.

Supplementary Figure 5: *E. coli* K-12 MG1655 WT plated on LB agar

a) False-colored fluorescence (GFP; excitation: 455-485 nm, emission: 508-557 nm,

exposure time: 15 msec) and **b)** brightfield images of *E. coli* K-12 MG1655 WT plated

onto LB agar. Identical contrast settings as in Fig. 5. Scale bar: 30 mm.