Residual antibiofilm effects of various concentrations of double antibiotic paste used during regenerative endodontics after different application times

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Abstract

Objective: We investigated the residual antibiofilm effects of different concentrations of double antibiotic paste (DAP) applied on radicular dentin for 1 or 4 weeks.

Design: Dentin samples were prepared (n=120), sterilized and pretreated for 1 or 4 weeks with the clinically used concentration of DAP (500 mg/mL), low concentrations of DAP (1, 5 or 50 mg/mL) loaded into a methylcellulose system, calcium hydroxide (Ca(OH)$_2$), or placebo paste. After the assigned treatment time, treatment pastes were rinsed off and the samples were kept independently in phosphate buffered saline for 3 weeks. Pretreated dentin samples were then inoculated with *Enterococcus faecalis* and bacterial biofilms were allowed to grow for an additional 3 weeks. Biofilms were then retrieved from dentin using biofilm disruption assays, diluted, spiral plated, and quantified. Fisher's Exact and Wilcoxon rank sum tests were used for statistical comparisons (α=0.05).

Results: Dentin pretreatment for 4 weeks with 5, 50 or 500 mg/mL of DAP demonstrated significantly higher residual antibiofilm effects and complete eradication of *E. faecalis* biofilms in comparison to a 1 week pretreatment with similar concentrations. However, dentin pretreated with 1 mg/mL of DAP or Ca(OH)$_2$ did not provide a substantial residual antibiofilm effect regardless of the application time.

Conclusions: Dentin pretreatment with 5 mg/mL of DAP or higher for 4 weeks induced significantly higher residual antibiofilm effects in comparison to a 1 week pretreatment with the same concentrations.

Keywords: Double antibiotic paste, Calcium hydroxide, Antibacterial effect, *Enterococcus faecalis*, Bacterial biofilm

1. Introduction

Root canal disinfection is an indispensable step during endodontic regeneration procedures. The disinfection is usually achieved using root canal irrigation solutions such as sodium hypochlorite (NaOCl) as well as intracanal medicaments such as calcium hydroxide $(Ca(OH)_2)$ or various antibiotic pastes. However, a plethora of recent in vitro evidence recommended the use of low concentrations of NaOCl (Martin et al., 2014) and antibiotic pastes (Althumairy, Teixeira, & Diogenes, 2014; Kim et al., 2015) in an attempt to create a balanced disinfection protocol that can eliminate root canal pathogens without damaging stem cells and dentin endogenous proteins within the root canal system (Labban, Yassen, Windsor, & Platt, 2014). On the other hand, recent studies suggested that $Ca(OH)_2$ is a stem cell friendly medicament (Althumairy, et al., 2014; Ruparel, Teixeira, Ferraz, & Diogenes, 2012) and significantly improved the attachment of apical papillae cells to dentin (Kitikuson & Srisuwan, 2016). Both double (DAP) and triple (TAP) antibiotic pastes have been clinically used in endodontic regeneration (Nagata et al., 2014; Nevins & Cymerman, 2015) and were found to have comparable antibacterial properties against different endodontic pathogens (Sabrah, Yassen, & Gregory, 2013; Sabrah et al., 2015a). A recent study suggested that a 1 week treatment with a low concentration of DAP (1 mg/mL) loaded into a methylcellulose system as well as a clinically used concentration of $Ca(OH)_2$ were efficient in eliminating 3-week old *Enterococcus faecalis* biofilm (Tagelsir, Yassen, Gomez, & Gregory, 2016).

In endodontic regeneration, the absence of obturating material in the canal during the development of newly formed tissue may enable the residual or new pathogens to multiple and initiate a new biofilm. Therefore, it is advised to provide a higher level of disinfection in comparison to traditional endodontic treatment (Fouad & Nosrat, 2013). Additionally, the

developing new tissues, regardless of their type, require adequate time to establish their structure within the root canal. Consequently, the bacteria-free environment should be maintained for an extended period of time in comparison to regular root canal therapy (Fouad & Verma, 2014). Therefore, disinfection during regenerative endodontics may require antimicrobial agents with considerable levels of substantivity (Fouad, 2011). Indeed, both TAP and DAP were suggested to have an extended residual antibiofilm effect after their removal (Sabrah et al., 2015b). Additionally, DAP was proposed to have longer residual antibiofilm properties in comparison to similar concentrations of TAP (Sabrah, et al., 2015b).

No clear consensus is available regarding the interappointment application time of intracanal medicaments during endodontic regeneration procedures. While the minimum application time clinically reported in the literature is 1 week (Ding et al., 2009; Paryani & Kim, 2013), other studies have applied these medicaments for up to 11 weeks (Shimizu et al., 2013; Thibodeau, 2009). The American Association of Endodontists (AAE) recommended an application time of 1-4 weeks with consideration of additional treatment time in cases with persistent infection (American Association of Endodontists, 2015). The aim of this study was to investigate the residual antibiofilm effects of various concentration of DAP loaded into an aqueous methylcellulose system and applied for 1 or 4 weeks. We hypothesized that DAP exerts similar residual antibiofilm effects regardless of the concentration used or application time.

2. Materials and Methods

2.1 Dentin sample preparation

Sound human permanent teeth (n=120) were collected after obtaining Institutional Review Board approval (IRB, 1408897632). The teeth were kept in 0.1% thymol solution at 4°C

and used within 6 months. The crowns were removed using a water-cooled diamond saw and the roots were used to obtain 120 standardized dentin slabs with the dimensions of $4\times4\times1.5$ mm^3. A Rotoforce 4 polishing unit (Struers, Cleveland, OH) was used to polish the pulpal side of each dentin specimen with abrasive papers (1200–4000 grit; Struers) under running water. Dentin slabs were then sonicated with 1.5% NaOCl (Value Bleach; Kroger, Cincinnati, OH) and 17% EDTA (VISTA, Racine, WI) for 4 minutes. Each sample was then wrapped with a cotton pellet saturated with sterile water, placed in Whirl-pak bags (Sigma-Aldrich, St Louis, MO), sterilized with ethylene oxide gas, and kept at 4°C until used.

2.2 Preparation of Medicaments Used in the Study

A total of 5 antimicrobials were investigated in the current study including clinically used concentrations of DAP (500 mg/mL), three low concentrations of DAP (1, 5 and 50 mg/mL) and Ca(OH)$_2$ (UltraCal XS, Ultradent, South Jordan, UT). The clinically used DAP was prepared by mixing 500 mg of equal portions of metronidazole and ciprofloxacin (Champs Pharmacy, San Antonio, TX) with 1 mL of sterile water (Kim, et al., 2015). The low concentrations of DAP (1, 5, and 50 mg/mL) were loaded into a methylcellulose system as described in previous studies (Tagelsir, et al., 2016; Yassen, Sabrah, Eckert, & Platt, 2015) to create a clinically applicable antibiotic medicament that can be injected into a root canal system (Algarni, Yassen, & Gregory, 2015). In summary, 2500, 250 and 50 mg of DAP were dissolved independently in 50 mL of sterile water. Then, 4 gm of methyl cellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) was slowly added to each DAP solution over 120 minutes under maximum stirring to obtain pastes with 1, 5, and 50 mg/mL of DAP. A DAP free placebo paste were also prepared and used as a control group. No untreated positive control group was used in the current study as our earlier pilot study has shown no difference between the untreated positive control samples

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and dentin samples treated with the placebo paste. A recent study has also found no difference between infected dentin treated with aqueous methylcellulose based paste and that treated with normal saline (Tagelsir, et al., 2016).

2.3 Treatment of dentin samples

In order to be able to precisely quantify the residual indirect antibiofilm effects of medicaments, sterilized rather than infected dentin samples were pretreated with various medicaments in the current study as described in a recent report (Sabrah, et al., 2015b). Sterilized dentin slabs were placed individually in separate wells of sterile 96 well microtiter plates (Fisherbrand, Fischer Scientific) with the pulpal side (treatment side) facing upward. Samples were then randomly divided into 5 treatment groups and 1 control group (n=20 per group). The pulpal side of each dentin slab received 200 µL of one of the treatment pastes (1, 5, 50, 500 mg/mL of DAP or $Ca(OH)_2$) or the control placebo paste. All treated samples were then incubated for 1 or 4 weeks (n=10 per group at each time point) at 37°C and 100% humidity. The two treatment times were selected based on the clinical endodontic regeneration procedure recommended by AAE (American Association of Endodontists, 2015). After the assigned treatment time, the treatment paste was rinsed off from each sample using 5 mL of sterile saline followed by 5 minutes of irrigation with 5 mL of 17% EDTA. Dentin slabs were then immersed in 200 µl phosphate buffered saline (PBS) and incubated at 37°C for 3 weeks before growing the bacterial biofilm.

2.4 Bacterial Strain and Media

E. faecalis (29212; ATCC, Manassas, VA) was grown initially on anaerobic blood agar plates (Bio-Merieux, Durham, NC). A sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) was inoculated with colonies of *E. faecalis* and incubated at 37°C with 5% CO_2 for 24 hours.

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2.5 Biofilm Growth on Treated Dentin Samples

After 3 weeks of immersion in PBS, dentin slabs were transferred independently into wells of sterile 96-well microtiter plates with the treated surface facing upward. Then, 190 μL of fresh BHI-YE and 10 μL of an overnight *E. faecalis* culture (10^6 CFU/mL) were added to each well. The slabs were incubated anaerobically for 3 weeks at 37°C and the culture media was replenished 2 times a week. After incubation, each dentin slab was gently washed with sterile saline to remove unattached bacteria. One randomly selected sample from each experimental group at each time point was processed for confocal laser scanning microscopy (CLSM) and the remaining 9 samples in each group were utilized for biofilm disruption assays. A negative untreated control group was also used in the current study to confirm the absence of any bacterial contamination from outside sources within the experimental setting of this study. Briefly, untreated sterilized dentin slabs (n=3) were individually placed in 200 μL of bacteria-free BHI-YE media and incubated for 3 weeks under the same anaerobic conditions described earlier with regular replacement of BHI-YE. After 3 weeks, biofilm disruption assays were performed to confirm the lack of any bacterial biofilm in the uninfected dentin slabs.

2.6 Biofilm Disruption Assay

The biofilm disruption assays were conducted as described in recent studies (Sabrah, et al., 2015b; Tagelsir, et al., 2016). Briefly, dentin slabs were placed into sterile test tubes containing 2 mL of sterile saline. To detach the bacterial biofilm, samples were sonicated for 20 seconds and vortexed for 30 seconds. The ability of this protocol to maintain the viability of the sonicated bacteria as well as to detach the biofilm from the surface of the dentin slabs and from dentin tubules was confirmed in a pilot study. The obtained biofilms were then diluted in sterile saline, spirally plated on blood agar plates, and incubated anaerobically for 24 hours. After that,

an automated colony counter (Synbiosis, Inc, Frederick, MD) was used to evaluate the number of colony-forming units (CFU)/mL.

2.7 Biofilm Visualization

The bacterial biofilms grown on the randomly selected dentin slabs were viewed using CLSM to characterize the viability of the formed biofilms. Briefly, a diluted mixture of equal volumes of SYTO 9 and propidium iodide dyes (Molecular Probes, Eugene, OR) was prepared according to the manufacturer's instructions. The biofilm on each dentin slab was then stained with 200 µl of the mixture, stored in the dark for 15 minutes, and viewed under CLSM (FV1000; Olympus Corp, Center Valley, PA). Dedicated software (FV10-ASW, Olympus Corp) was used to obtain images from 3 randomly scanned biofilm areas on each dentin sample. Imaris Software (version 7.7; Bitplane, South Windsor, CT) was used for live/dead bacterial quantifications and 3-dimensional biofilms construction.

2.8 Statistical analyses

Some experimental groups did not exhibit any bacterial growth. Therefore, Fisher's Exact tests were used to determine the significant differences in the presence or absence of any bacterial growth. Furthermore, Wilcoxon rank sum tests were used to compare the between antibiofilm effects of various experimental groups that demonstrated bacterial growth. The significance level was set at 0.05.

3. Results

3.1 Residual antibacterial effect of dentin

No complete eradication of bacterial biofilm was observed among any 1-week dentin treatments. Furthermore, all dentin samples demonstrated bacterial growth. Figure 1

demonstrates that dentin samples pretreated with 500 mg/mL of DAP for one week developed a significantly higher residual antibiofilm effect in comparison to all other 1-week treatments ($p<0.01$). A one week dentin pretreatment with 50 mg/mL of DAP induced a significantly higher residual antibiofilm effect in comparison to 1 week dentin pretreatment with placebo paste, $Ca(OH)_2$, 5 or 1 mg/mL of DAP ($p<0.01$). A one week pretreatment of dentin with 5 mg/mL of DAP or $Ca(OH)_2$ had a significant but limited residual antibiofilm effect in comparison to a 1 week dentin treatment with 1 mg/mL of DAP and placebo paste ($p<0.05$). However, no significant residual antibiofilm effect was observed between 1 week pretreatment of dentin with 1 mg/mL of DAP and placebo paste.

Four weeks of pretreatment of dentin with 500, 50, or 5 mg/mL of DAP demonstrated significantly higher residual antibiofilm effects in comparison to 4 weeks of dentin pretreatment with 1 mg/mL of DAP, $Ca(OH)_2$ or placebo paste ($p<0.001$). Furthermore, no significant differences in residual antibiofilm l effect were detected in dentin pretreated for 4 weeks with placebo paste, $Ca(OH)_2$, or 1 mg/mL of DAP (Figure 1).

Four weeks of dentin pretreatment with 5, 50 and 500 mg/mL of DAP demonstrated significantly higher residual antibiofilm effects and complete eradication of biofilm in comparison to a 1 week pretreatment of dentin with the same concentrations of DAP ($p<0.01$) (Figure 1). Four weeks of dentin pretreatment with 1 mg/mL of DAP or placebo paste had a significant but limited residual antibiofilm effect in comparison to a 1 week pretreatment with the same pastes ($p<0.05$). However, dentin pretreated with $Ca(OH)_2$ for 4 weeks had significantly higher biofilm formation in comparison to those that received 1 week of pretreatment with $Ca(OH)_2$ ($p<0.01$).

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3.2 Biofilm Visualization

In general, 3D images obtained through CLSM were consistent with the residual antibiofilm data obtained from biofilm disruption assays (Figure 2). The percentage of live cells (green) within the biofilm mass observed on dentin pretreated for 1 week with placebo paste, $Ca(OH)_2$, 1 and 5 mg/mL DAP were 70 ± 9 , 65 ± 7, 66 ± 13 and 60 ± 7, respectively (Figure 2). For dentin pretreated for 1 week with 50 and 500 mg/mL of DAP, very limited live cells were observed without clear biofilm structure. The percentage of live cells observed within the biofilm mass on dentin pretreated for 4 weeks with placebo paste, $Ca(OH)_2$, and 1 mg/mL DAP were 65 ± 7, 88 ± 9, and 63 ± 8, respectively (Figure 2). However, neither biofilm mass nor live cells were detected on dentin that received 4 weeks of pretreatment with 5, 50, and 500 mg/mL of DAP.

4. **Discussion**

The use of DAP as an intracanal medicament was first reported in a clinical case used contemporary principles of endodontic regeneration (Iwaya, Ikawa, & Kubota, 2001). However, DAP has been emerging lately as the antibiotic medicament of choice in endodontic regeneration due to its significant antibacterial properties against different endodontic pathogens (Sabrah, et al., 2013; Sabrah, et al., 2015a), as well as its minimum tooth discoloration potential in comparison to TAP (Akcay, Arslan, Yasa, Kavrik, & Yasa, 2014).

The current study indicates that the application time of DAP plays a significant role in determining residual antibiofilm properties of dentin as only 4 weeks of dentin pretreatment with 3 of the tested DAP concentrations (5, 50, and 500 mg/mL) completely prevented biofilm formation on dentin. The antibacterial effect of antibiotics is usually achieved during the reproductive cycle of bacterial cells (Abbott, Hume, & Pearman, 1990). Therefore, a relatively

long contact time between antibiotics and bacteria is required to maximize the beneficial use of antibiotics. It is also worth noting that both components of DAP, metronidazole and ciprofloxacin, are classified as concentration dependent antibiotics rather than time dependent antibiotics. However, it appears that longer contact time between DAP and dentin can increase the amount of DAP bonded/adsorbed to dentin and improve the residual antibacterial effect of dentin after DAP removal. A recent study found that complete DAP removal from root canal dentin is challenging even with the use of an EndoActivator system (Arslan et al., 2014). Other studies have also shown that DAP application significantly reduces the push out bond strength between the root canal and various root cements (Topcuoglu et al., 2014; Turk, Ozisik, & Aydin, 2015), which indicates the presence of a residual interaction between DAP and dentin. However, all previously mentioned studies have used the clinically used DAP concentration to create paste consistency (500-1000 mg/mL). The current study indicates that 4 weeks of dentin pretreatment with 5 mg/ml of DAP, 100-200 times less than the clinically used concentration, can maintain a residual antibiofilm effect for several weeks.

This study demonstrated that only 50 and 500 mg/mL of DAP treatment for 1 week were able to provide a significant and substantial residual antibiofilm effects. These findings suggest that in case of short term (1 week) dentin treatment with DAP, antibiotic concentration may play a significant role in the residual antibiofilm properties of dentin. Our study also demonstrated that dentin pretreated for 1 week with 1 or 5 mg/mL of DAP did not provide a substantial residual antibacterial effect (only 0.05- 0.5 $\log_{10}$ reduction). On the other hand, a recent study has indicated that dentin pretreated for 3 weeks with 1 mg/mL of DAP in liquid form provided 2 weeks of residual antibiofilm effect (Sabrah, et al., 2015b). However, the previous study used liquid forms of 1 mg/mL of DAP, allowed the bacterial growth for only 3 days and did not

provide final irrigation with EDTA after DAP removal (Sabrah, et al., 2015b). In the current

study, low concentrations of DAP were loaded into a vehicle system to create a clinically

applicable antibiotic medicament. Furthermore, the bacterial biofilm was allowed to grow for 3

weeks after samples were immersed in PBS for 3 weeks (a total of 6 weeks after DAP removal)

in order to investigate the residual antibacterial effects for longer and more challenging *in vitro*

conditions. Additionally, final EDTA irrigation was performed after DAP removal to simulate

the actual clinical scenario during endodontic regeneration.

A recent study proposed that a 1 week application of 1 mg/mL of DAP loaded into an

aqueous methylcellulose system was efficient in eliminating a substantial amount of 3-week-old

E. faecalis biofilm (Tagelsir, et al., 2016). All things considered, it seems like 1 mg/mL can

provide a direct antibiofilm effect (Algarni, et al., 2015; Tagelsir, et al., 2016). However, a

longer treatment time and/or higher concentration of DAP may be required to obtain an extended

indirect residual antibiofilm effect. In cases of necrotic immature teeth, the clinical

manifestations and the extent of preoperative infection should be always considered before

determining application time and concentration of DAP as an interappointment medicament.

Furthermore, the use of low concentrations of DAP rather than the currently used higher

concentrations (500-1000 mg/mL) should be advocated to minimize the adverse effects of DAP

on stem cells (Althumairy, et al., 2014; Kim, et al., 2015; Ruparel, et al., 2012) and dentin/root

structure (Yassen, Chu, Eckert, & Platt, 2013; Yassen, Vail, Chu, & Platt, 2013).

One of the interesting finding of this study is that dentin pretreated with $Ca(OH)_2$ did not

demonstrate any notable residual antibiofilm effect regardless of the treatment time. The

antimicrobial properties of $Ca(OH)_2$ are mainly achieved through the release of large amounts of

hydroxyl ions that raise the pH in the local environment and leads to denaturation of bacterial

proteins as well as destruction of microbial cytoplasmic membranes (Mohammadi & Dummer, 2011). Once $Ca(OH)_2$ is removed, the buffering capacity of dentin is expected to neutralize the pH in the root canal environment and extenuate the antibiofilm effect of $Ca(OH)_2$. Therefore, dentin treated with $Ca(OH)_2$ is not expected to have any substantial carry over residual antibiofilm effect. It is well documented that both $Ca(OH)_2$ and antibiotic pastes have direct antibiofilm effects (Sabrah, et al., 2013; Sabrah, et al., 2015b; Tagelsir, et al., 2016). However, the decision to use $Ca(OH)_2$ or DAP during endodontic regeneration should be based on the need of residual and extended antibiofilm properties within the root canal system after the removal of the intracanal medicament.

In the current study, the residual antibiofilm effect against only one strain of *E. faecalis* (commonly used as a control strain) was investigated. It is well known that some strains of *E. faecalis* have high biofilm formation capacity and can be resistant to various types of antibiotics (Al-Ahmad et al., 2014). Therefore, future studies should investigate the residual antibiofilm effect of DAP against different clinically isolated strains of *E. faecalis*. Another limitation of this study is the use of a single species *E. faecalis* biofilm rather than a multi-species biofilm, which would be more representative of the actual clinical situation. However, recent studies found that both 3 week old *E. faecalis* biofilm and a 3 week old multispecies biofilm retrieved from human dental plaque were more resistant to disinfectants used in endodontics (Du et al., 2014; Stojicic, Shen, & Haapasalo, 2013). Further studies should aim to investigate the residual antibacterial effects of DAP against multi-species biofilm as well as clinically isolated biofilms.

Our hypothesis which stated that DAP exerts similar residual antibiofilm effects regardless of the concentration used or application time was rejected. Four weeks of dentin pretreatment with 5, 50 or 500 mg/mL of DAP provided significantly higher residual antibiofilm

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effects and complete eradication of *E. faecalis* biofilms in comparison to a 1 week pretreatment

with the same concentrations. However, dentin pretreated with 1 mg/mL of DAP or the clinically

used $Ca(OH)_2$ did not provide substantial residual antibiofilm effects regardless of the

application time.

Conflict of Interest: None

References

Abbott, P. V., Hume, W. R., & Pearman, J. W. (1990). Antibiotics and endodontics. *Aust Dent J, 35*(1), 50-60.

Akcay, M., Arslan, H., Yasa, B., Kavrik, F., & Yasa, E. (2014). Spectrophotometric analysis of crown discoloration induced by various antibiotic pastes used in revascularization. *Journal of Endodontics, 40*(6), 845-848.

Al-Ahmad, A., Ameen, H., Pelz, K., Karygianni, L., Wittmer, A., Anderson, A. C., . . . Hellwig, E. (2014). Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. *Journal of Endodontics, 40*(2), 223-230.

Algarni, A. A., Yassen, G. H., & Gregory, R. L. (2015). Inhibitory effect of gels loaded with a low concentration of antibiotics against biofilm formation by Enterococcus faecalis and Porphyromonas gingivalis. *Journal of Oral Science, 57*(3), 213-218.

Althumairy, R. I., Teixeira, F. B., & Diogenes, A. (2014). Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. [Research Support, Non-U.S. Gov't]. *Journal of Endodontics, 40*(4), 521-525.

American Association of Endodontists. (2015). Considerations for Regenerative Procedures. Available at: http: www.aae.org/clinical-resources/regenerative-endodontics/considerations-for-regenerative-procedures.aspx. Accessed July 13, 2015.

Arslan, H., Akcay, M., Capar, I. D., Ertas, H., Ok, E., & Uysal, B. (2014). Efficacy of needle irrigation, EndoActivator, and photon-initiated photoacoustic streaming technique on removal of double and triple antibiotic pastes. *Journal of Endodontics, 40*(9), 1439-1442.

Ding, R. Y., Cheung, G. S., Chen, J., Yin, X. Z., Wang, Q. Q., & Zhang, C. F. (2009). Pulp revascularization of immature teeth with apical periodontitis: a clinical study. *Journal of Endodontics, 35*(5), 745-749.

Du, T., Wang, Z., Shen, Y., Ma, J., Cao, Y., & Haapasalo, M. (2014). Effect of long-term exposure to endodontic disinfecting solutions on young and old Enterococcus faecalis biofilms in dentin canals. *Journal of Endodontics, 40*(4), 509-514.

Fouad, A. F. (2011). The microbial challenge to pulp regeneration. *Advances in Dental Research, 23*(3), 285-289.

Fouad, A. F., & Nosrat, A. (2013). Pulp regeneration in previously infected root canal space. *Endodontic Topics, 28*(1), 24-37.

Fouad, A. F., & Verma, P. (2014). Healing after regenerative procedures with and without pulpal infection. *Journal of Endodontics, 40*(4 Suppl), S58-64.

Iwaya, S. I., Ikawa, M., & Kubota, M. (2001). Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. *Dental Traumatology, 17*(4), 185-187.

Kim, K. W., Yassen, G. H., Ehrlich, Y., Spolnik, K., Platt, J. A., & Windsor, L. J. (2015). The effects of radicular dentine treated with double antibiotic paste and ethylenediaminetetraacetic acid on the attachment and proliferation of dental pulp stem cells. *Dental Traumatology, 31*(5), 374-379.

Kitikuson, P., & Srisuwan, T. (2016). Attachment Ability of Human Apical Papilla Cells to Root Dentin Surfaces Treated with Either 3Mix or Calcium Hydroxide. *Journal of Endodontics, 42*(1), 89-94.

Labban, N., Yassen, G. H., Windsor, L. J., & Platt, J. A. (2014). The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. *Dental Traumatology, 30*(6), 429-434.

Martin, D. E., De Almeida, J. F., Henry, M. A., Khaing, Z. Z., Schmidt, C. E., Teixeira, F. B., & Diogenes, A. (2014). Concentration-dependent effect of sodium hypochlorite on stem cells of apical papilla survival and differentiation. *Journal of Endodontics, 40*(1), 51-55.

Mohammadi, Z., & Dummer, P. M. (2011). Properties and applications of calcium hydroxide in endodontics and dental traumatology. *International Endodontic Journal, 44*(8), 697-730.

Nagata, J. Y., Gomes, B. P., Rocha Lima, T. F., Murakami, L. S., de Faria, D. E., Campos, G. R., . . . Soares Ade, J. (2014). Traumatized immature teeth treated with 2 protocols of pulp revascularization. *Journal of Endodontics, 40*(5), 606-612.

Nevins, A. J., & Cymerman, J. J. (2015). Revitalization of open apex teeth with apical periodontitis using a collagen-hydroxyapatite scaffold. *Journal of Endodontics, 41*(6), 966-973.

Paryani, K., & Kim, S. G. (2013). Regenerative endodontic treatment of permanent teeth after completion of root development: a report of 2 cases. *Journal of Endodontics, 39*(7), 929-934.

Ruparel, N. B., Teixeira, F. B., Ferraz, C. C., & Diogenes, A. (2012). Direct effect of intracanal medicaments on survival of stem cells of the apical papilla. *Journal of Endodontics, 38*(10), 1372-1375.

Sabrah, A. H., Yassen, G. H., & Gregory, R. L. (2013). Effectiveness of antibiotic medicaments against biofilm formation of Enterococcus faecalis and Porphyromonas gingivalis. *Journal of Endodontics, 39*(11), 1385-1389.

Sabrah, A. H., Yassen, G. H., Liu, W. C., Goebel, W. S., Gregory, R. L., & Platt, J. A. (2015a). The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established Enterococcus faecalis biofilm. *Clinical Oral Investigations, 19*(8), 2059-2066.

Sabrah, A. H., Yassen, G. H., Spolnik, K. J., Hara, A. T., Platt, J. A., & Gregory, R. L. (2015b). Evaluation of Residual Antibacterial Effect of Human Radicular Dentin Treated with Triple and Double Antibiotic Pastes. *Journal of Endodontics, 41*(7), 1081-1084.

Shimizu, E., Ricucci, D., Albert, J., Alobaid, A. S., Gibbs, J. L., Huang, G. T., & Lin, L. M. (2013). Clinical, radiographic, and histological observation of a human immature permanent tooth with chronic apical abscess after revitalization treatment. *Journal of Endodontics, 39*(8), 1078-1083.

Stojicic, S., Shen, Y., & Haapasalo, M. (2013). Effect of the source of biofilm bacteria, level of biofilm maturation, and type of disinfecting agent on the susceptibility of biofilm bacteria to antibacterial agents. *Journal of Endodontics, 39*(4), 473-477.

Tagelsir, A., Yassen, G. H., Gomez, G. F., & Gregory, R. L. (2016). Effect of Antimicrobials Used in Regenerative Endodontic Procedures on 3-week-old Enterococcus faecalis Biofilm. *Journal of Endodontics, 42*(2), 258-262.

Thibodeau, B. (2009). Case report: pulp revascularization of a necrotic, infected, immature, permanent tooth. *Pediatric Dentistry, 31*(2), 145-148.

Topcuoglu, H. S., Arslan, H., Akcay, M., Saygili, G., Cakici, F., & Topcuoglu, G. (2014). The effect of medicaments used in endodontic regeneration technique on the dislocation resistance of mineral trioxide aggregate to root canal dentin. *Journal of Endodontics, 40*(12), 2041-2044.

Turk, T., Ozisik, B., & Aydin, B. (2015). Time-dependent effectiveness of the intracanal medicaments used for pulp revascularization on the dislocation resistance of MTA. *BMC Oral Health, 15*(1), 130.

Yassen, G. H., Chu, T. M., Eckert, G., & Platt, J. A. (2013). Effect of medicaments used in endodontic regeneration technique on the chemical structure of human immature radicular dentin: an in vitro study. *Journal of Endodontics, 39*(2), 269-273.

Yassen, G. H., Sabrah, A. H., Eckert, G. J., & Platt, J. A. (2015). Effect of different endodontic regeneration protocols on wettability, roughness, and chemical composition of surface dentin. *Journal of Endodontics, 41*(6), 956-960.

Yassen, G. H., Vail, M. M., Chu, T. G., & Platt, J. A. (2013). The effect of medicaments used in endodontic regeneration on root fracture and microhardness of radicular dentine. *International Endodontic Journal, 46*(7), 688-695.

Figure 1. The residual antibacterial effect of dentin pretreated for 1 or 4 weeks with different concentrations of DAP (1, 5, 50 and 500 mg/mL), calcium hydroxide and placebo paste represented as the mean (±SD) of the log CFU/mL. Different upper case letters represent statistical significance between different treatments within the same application time. Different lower case letters represent statistical significance between similar treatments within the two application times.

Figure 2. CLSM 3D images showing live (green) and dead (red) cells of 3 week old *E. faecalis* biofilms formed on dentin samples previously treated with a 1 week placebo paste (A1), 1 week calcium hydroxide (A2), 1 week 1 mg/mL DAP (A3), 1 week 5 mg/mL DAP (A4), 1 week 50 mg/mL DAP (A5), 1 week 500 mg/mL DAP (A6), 4 week placebo paste (B1), 4 week calcium hydroxide (B2), 4 week 1 mg/mL DAP (B3), 4 week 5 mg/mL DAP (B4), 4 week 50 mg/mL DAP (B5), or 4 week 500 mg/mL DAP (B6).

Figure 1
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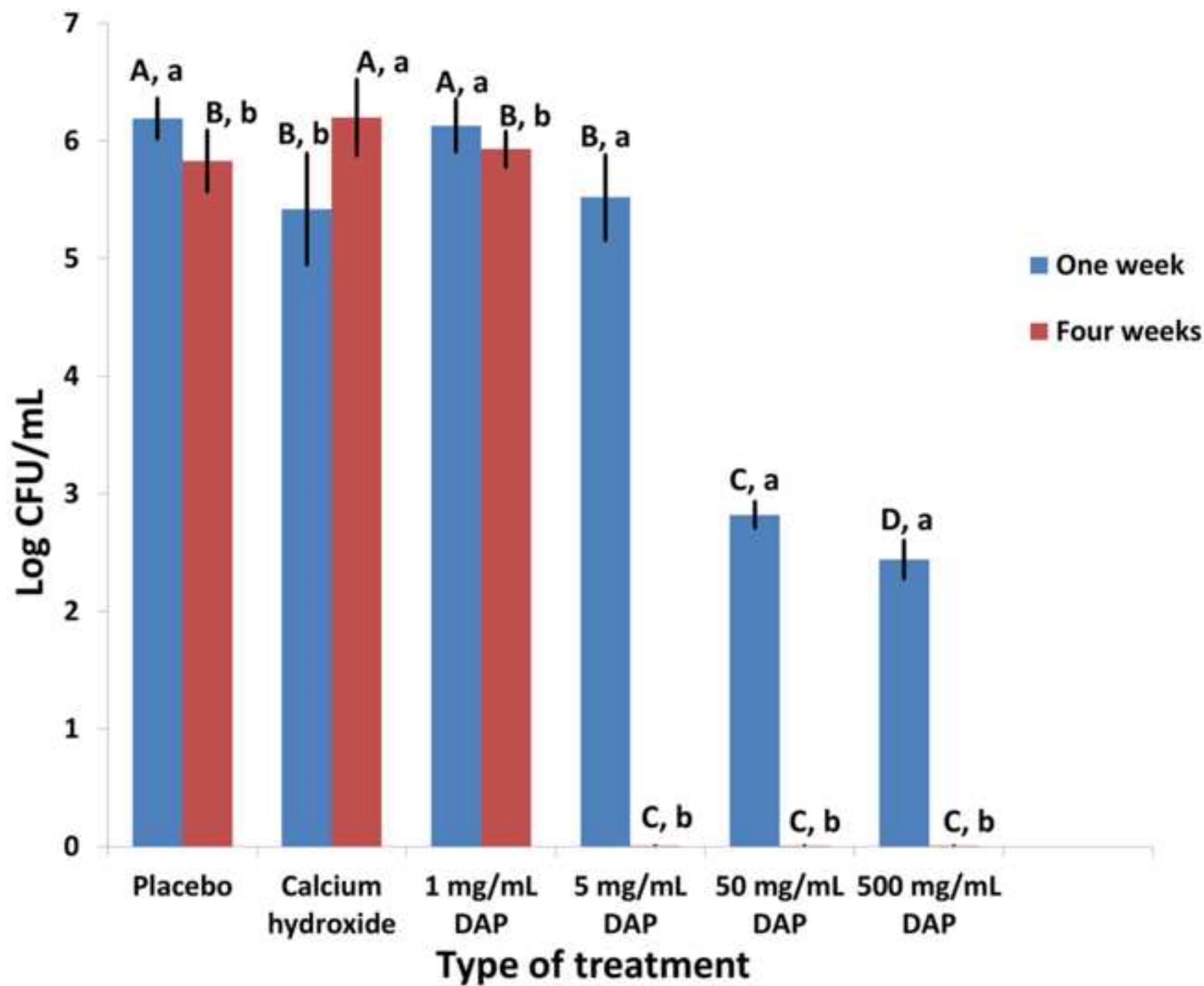
Figure 1

Figure 2 (A1)
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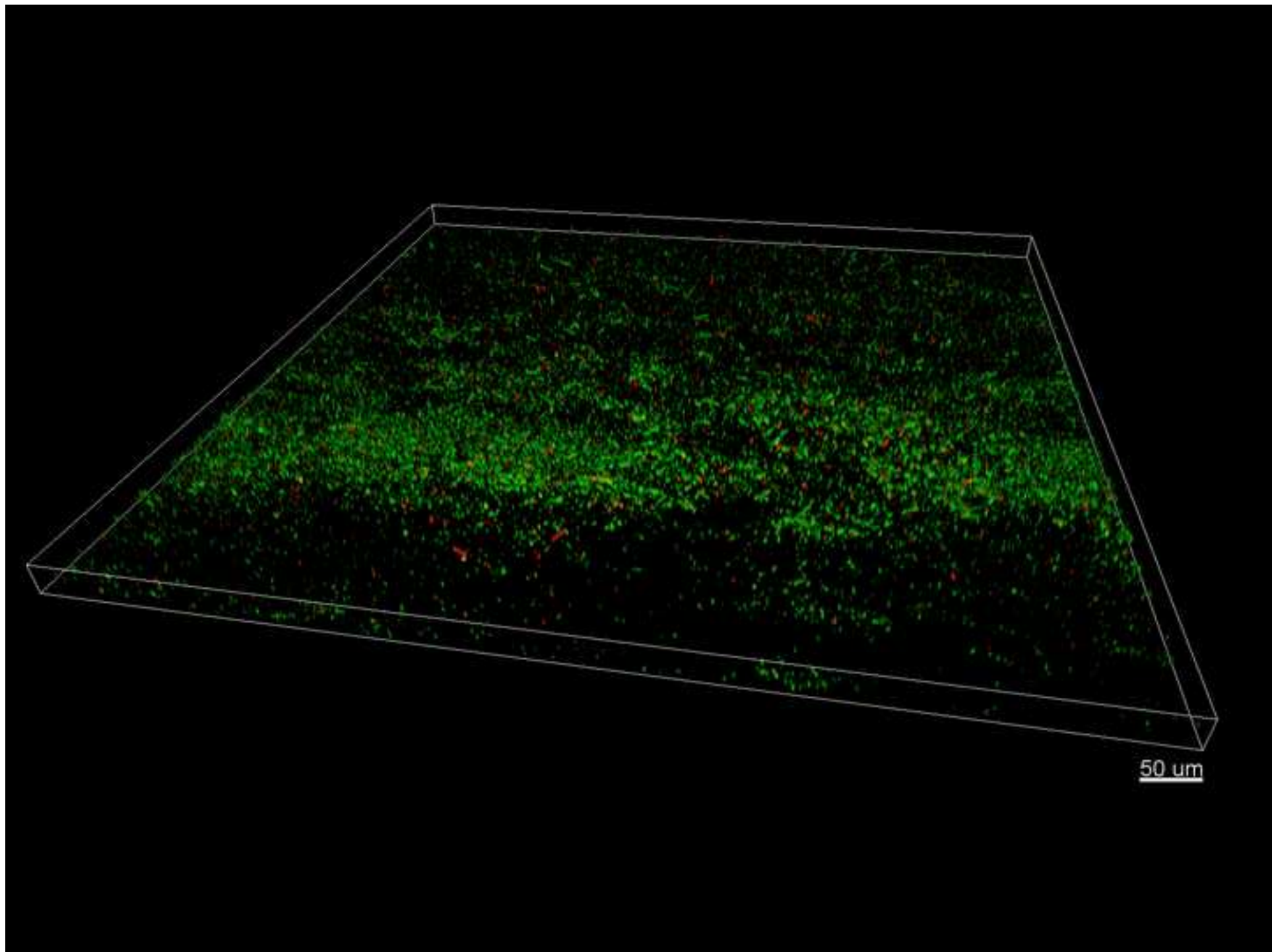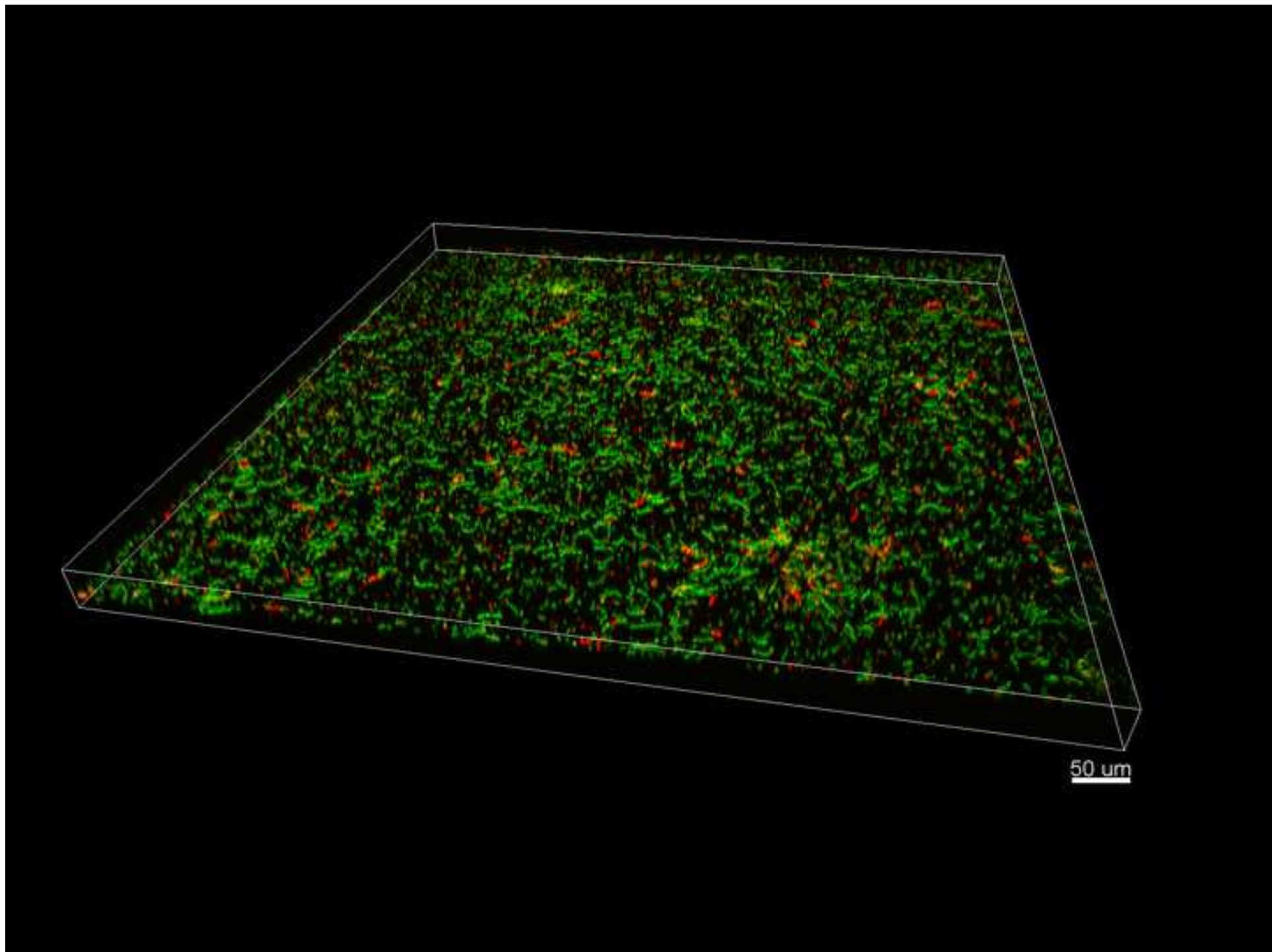
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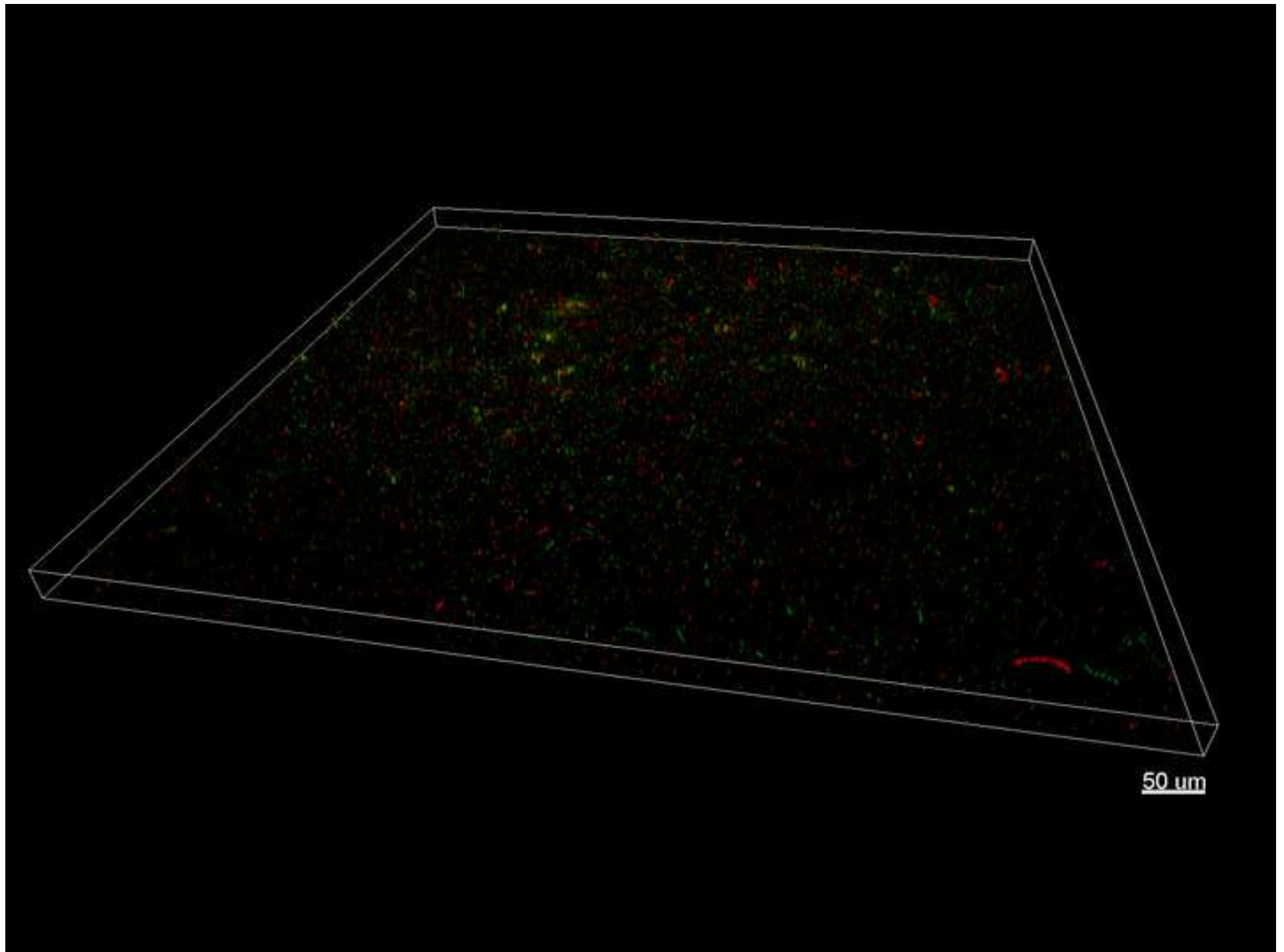
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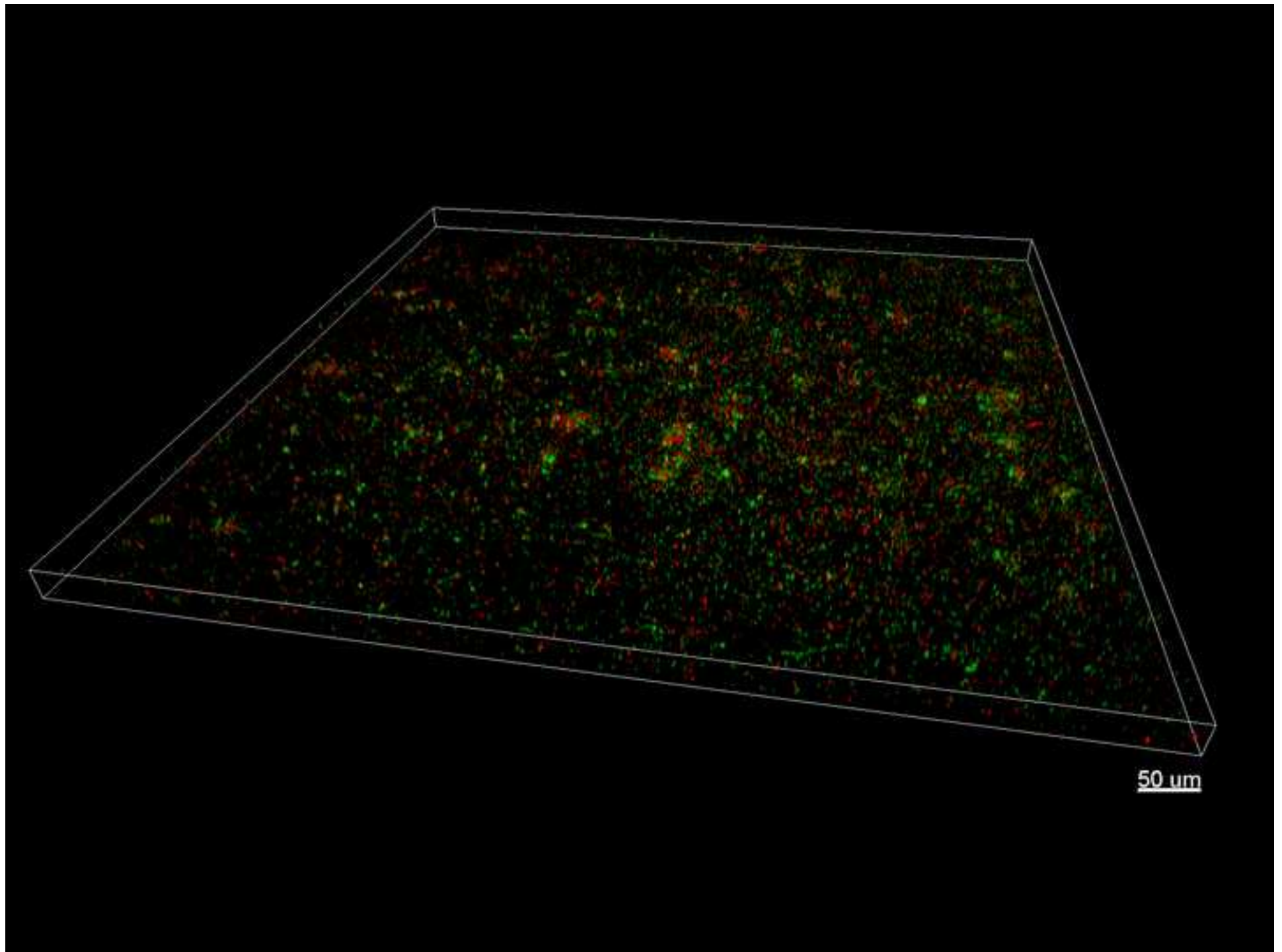
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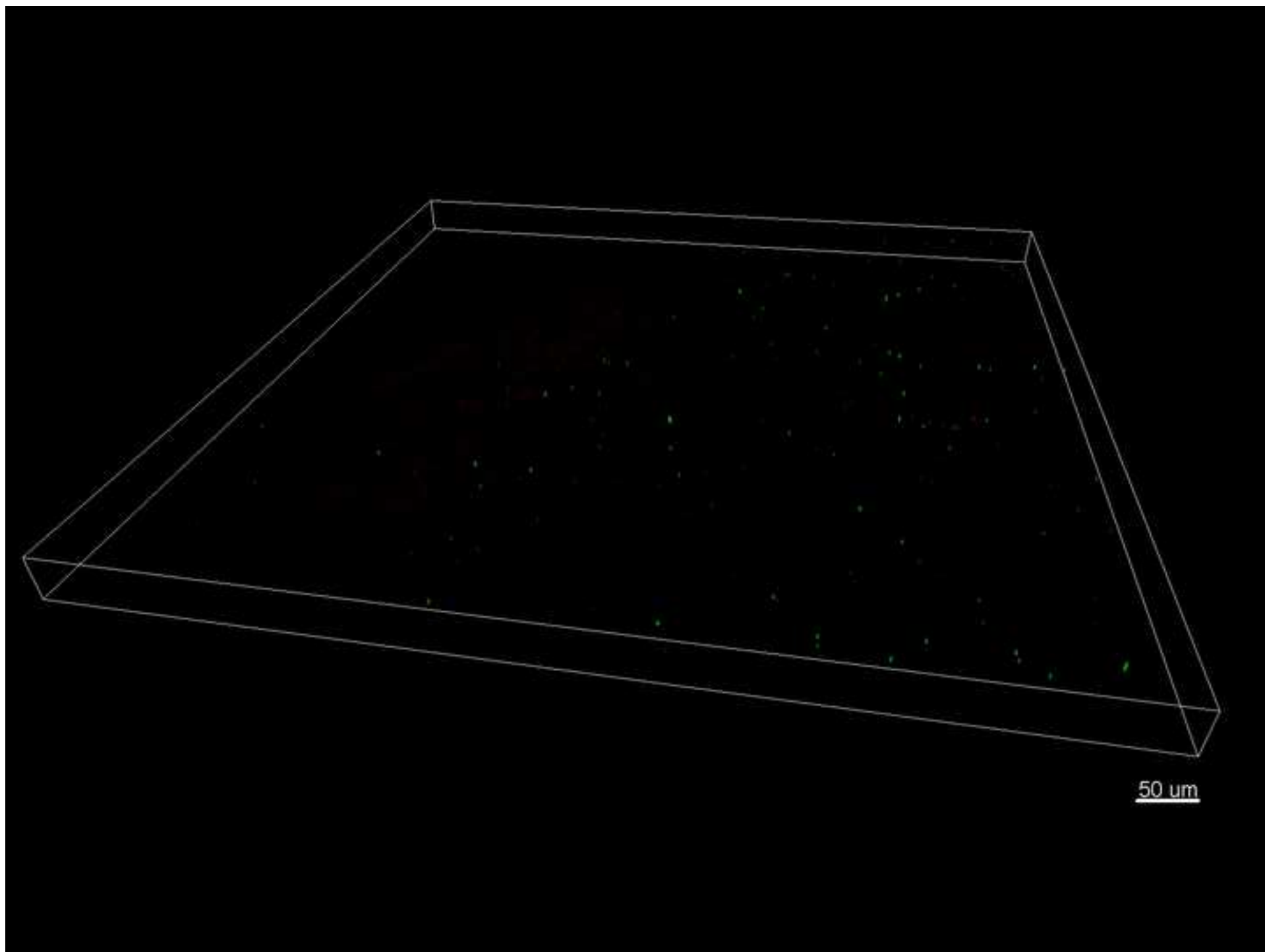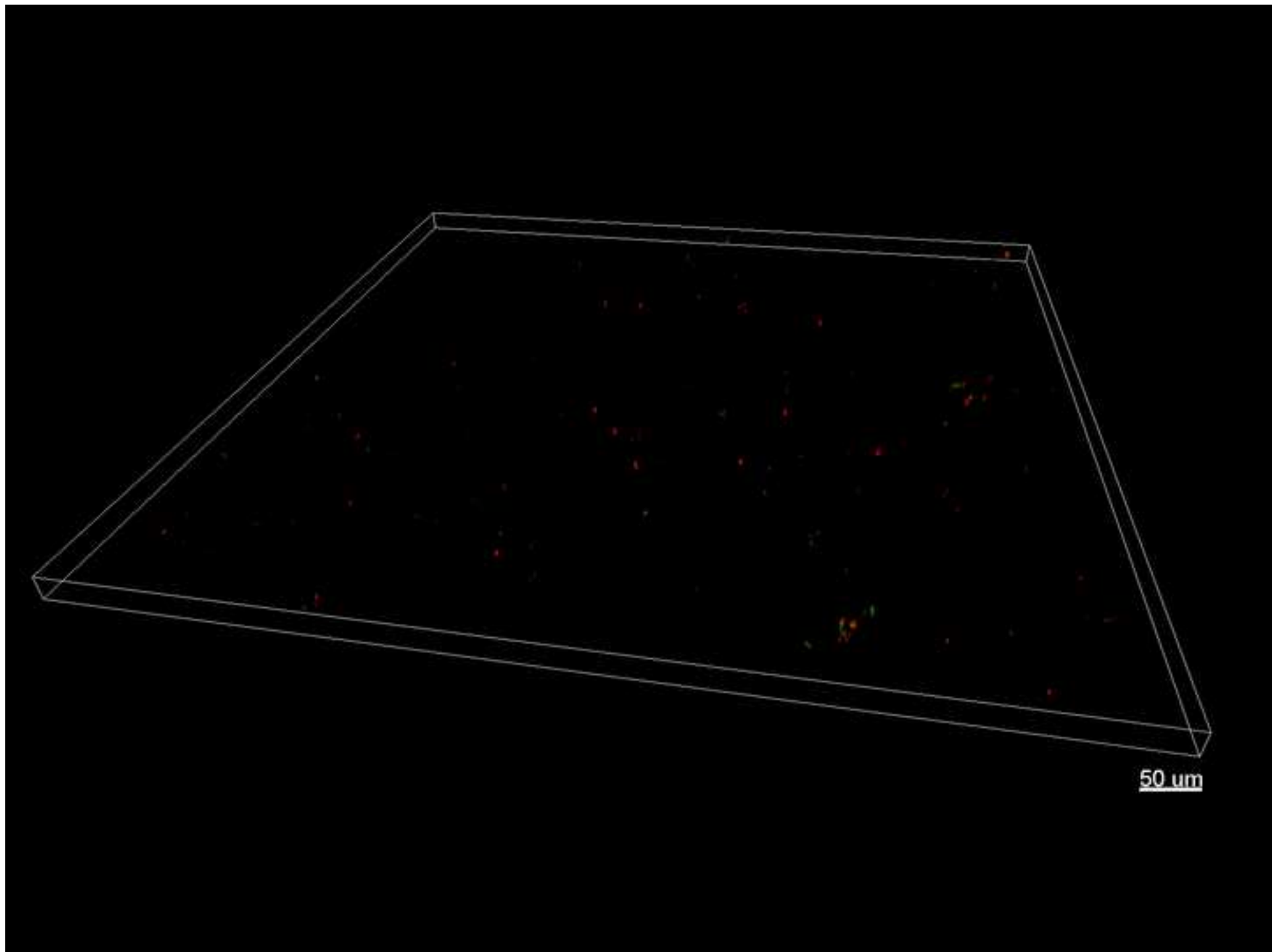
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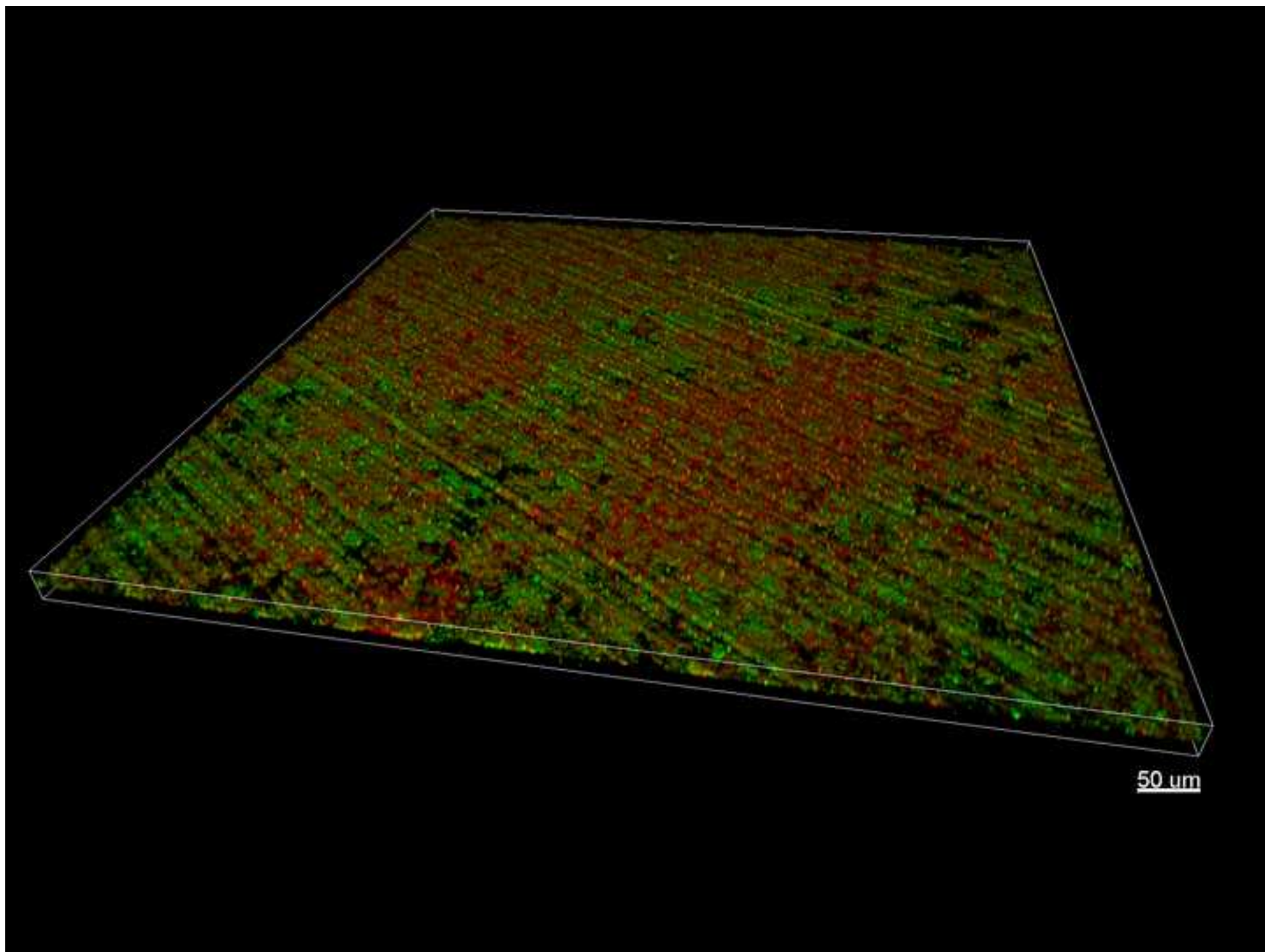
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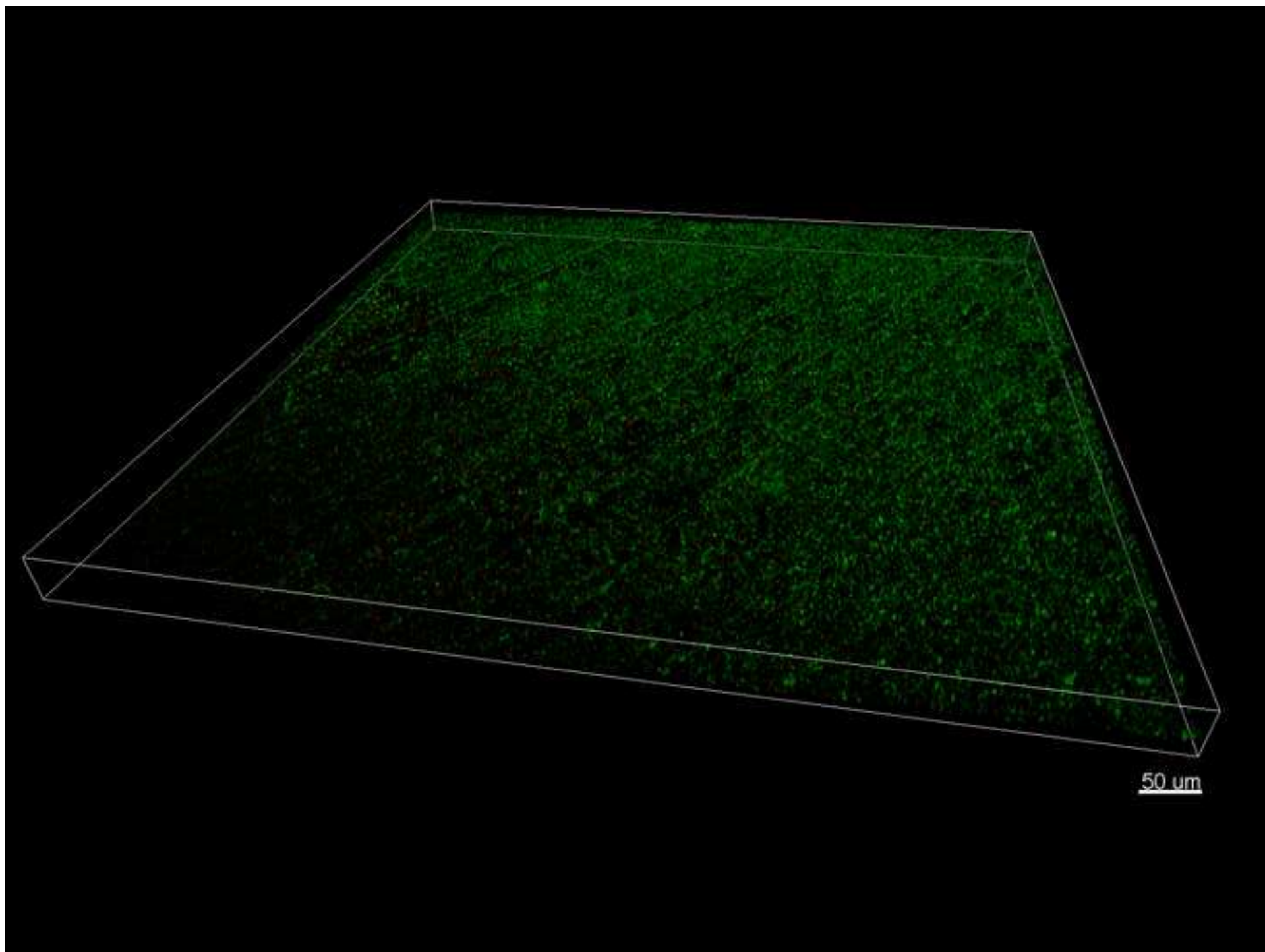
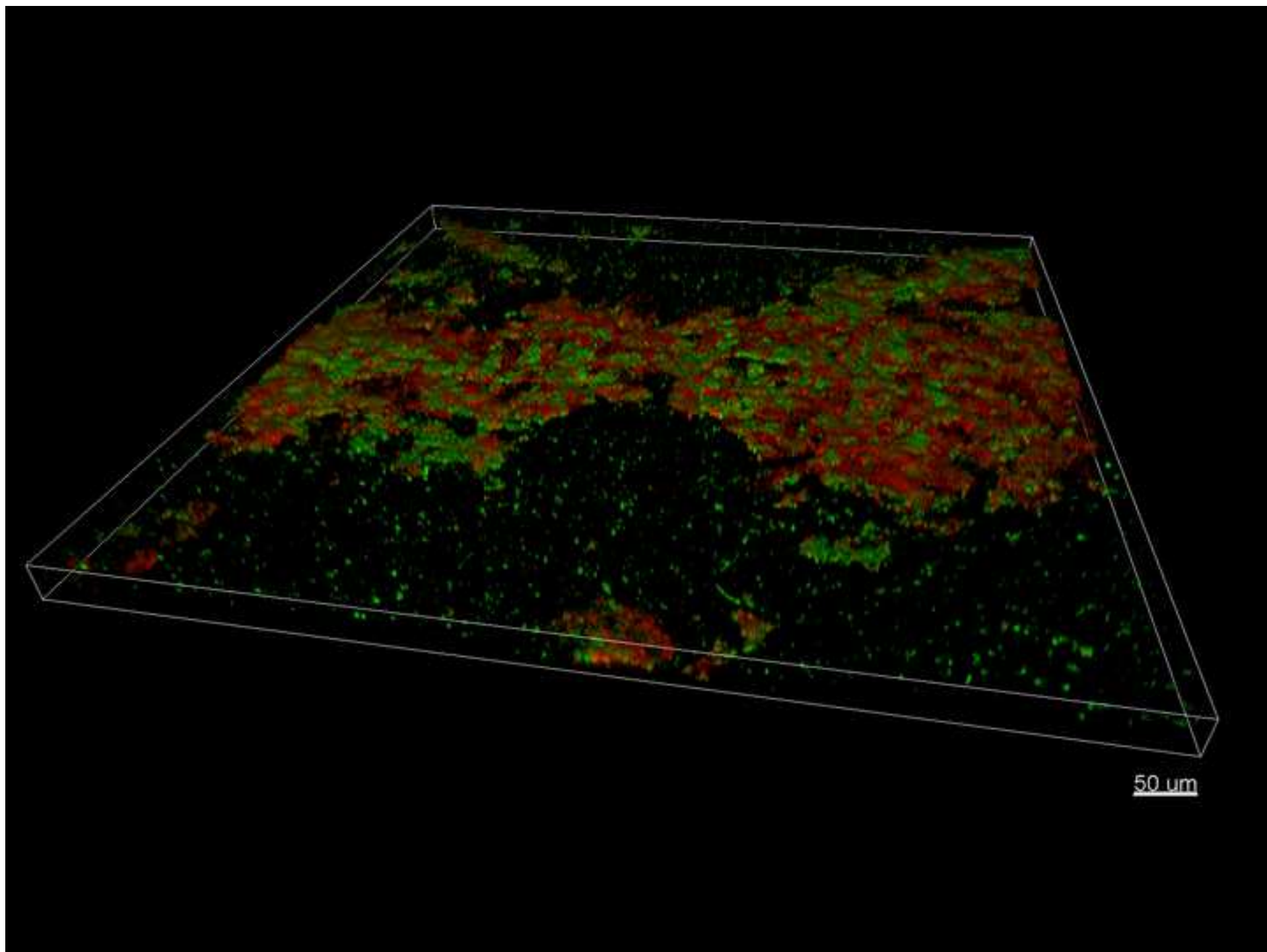**Figure 2 (B3)**
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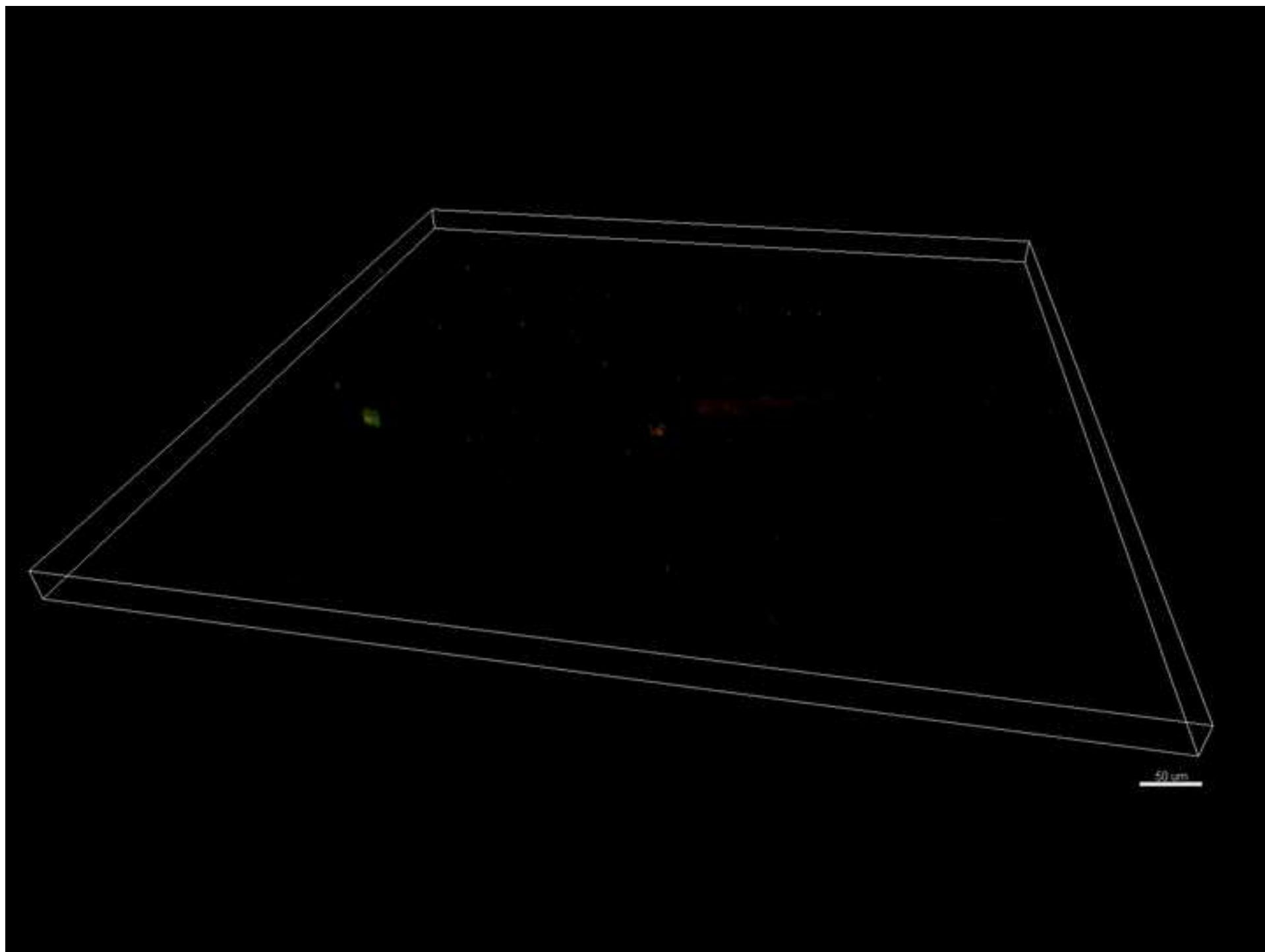

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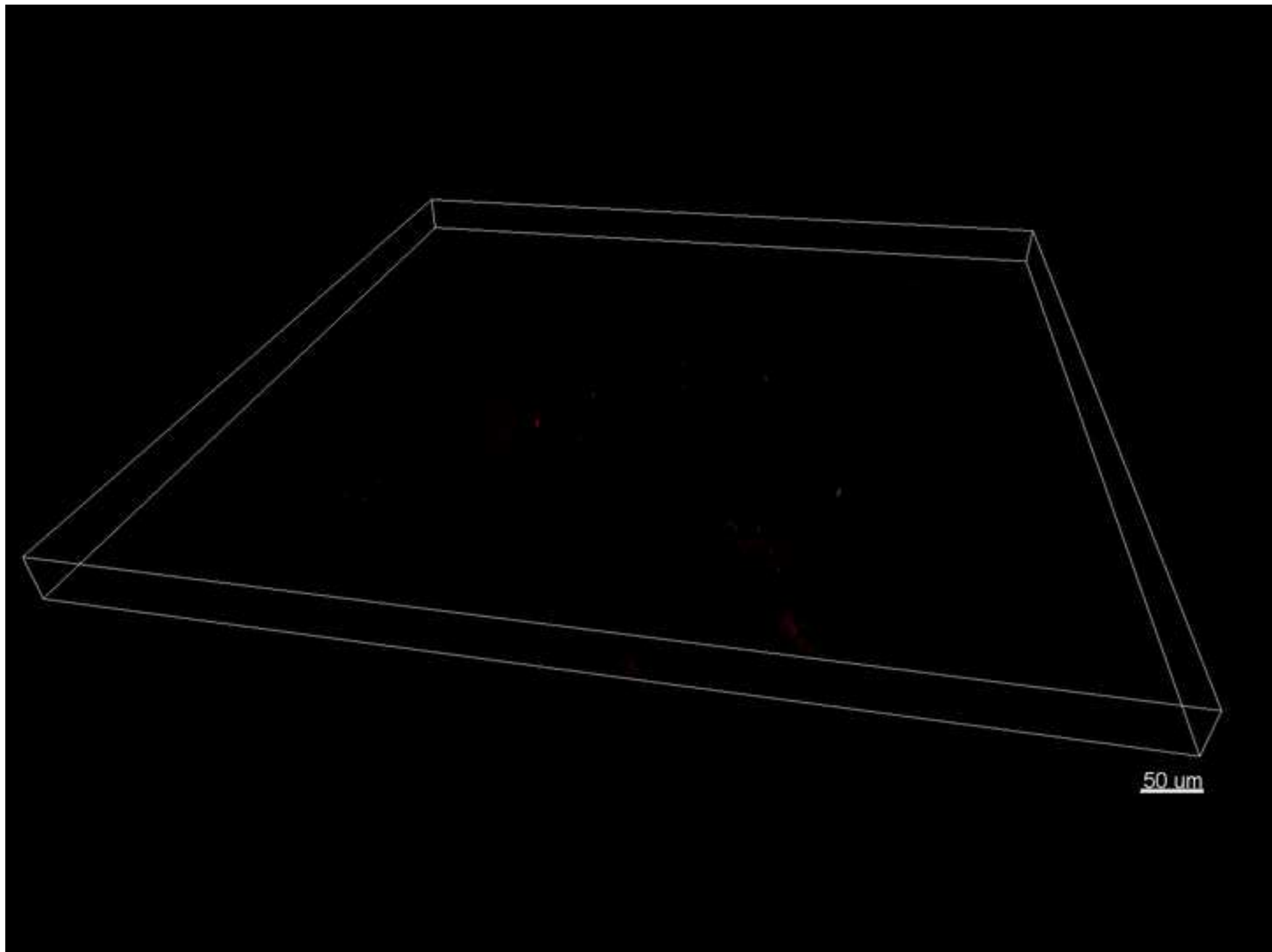**Figure 2 (B4)**
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Figure 2 (B5)
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