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(54) Title: METHODS AND KITS FOR DETECTING PROLIFERATING CELLS

(57) Abstract: The invention relates to the field of analyzing cells and cell division, in particular to means and methods for detecting low numbers of dividing immune cells in blood samples. Provided is an in vitro method for detecting proliferating cells, comprising the steps of: (i) culturing cells in the presence of a nucleoside analog comprising a first reactive unsaturated group to allow for incorporation of the nucleoside analog in the DNA of the cells; (ii) concentrating the nucleoside-labeled cells by covalent immobilization to a solid surface comprising a coating of (3-aminopropyl)triethoxysilane (APTES) or branched polyethyleneimine (PEI) that is activated by glutaraldehyde (GA); (iii) optionally fixing the concentrated and covalently immobilized cells with formaldehyde; (iv) contacting the covalently immobilized cells with a detection probe comprising a second reactive unsaturated group, such that a [3+2] or [4+2] cycloaddition occurs between the first and second reactive unsaturated groups; and (v) determining the amount of detection probe attached to the DNA of the immobilized cells to measure cellular proliferation.

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Title: Methods and kits for detecting proliferating cells.

The invention relates to the field of analyzing cells and cell division, among others in relation to immunological research. In particular, it relates to means and methods for detecting low numbers of dividing immune cells in blood samples.

Adaptive immunity, also called acquired immunity, confers specificity and immunological memory to specific pathogens. It consists of two branches: (i) The B cells that produce antibodies, which circulate through the body and bind to extracellular pathogenic structures (e.g., viral particles). Thereby, the antibodies mark these structures for destruction and prevent infection. (ii) The T cells that mediate cellular immunity. T cells recognize short peptide fragments (derived from pathogen-specific proteins) in the context of Major Histocompatibility Complexes (MHC) by cell-cell interactions with antigen presenting cells. Following the recognition of such MHC-presented peptides by T cells, they kill infected target cells (cytolytic T cells) and promote the immune response (helper T cells).

B and T cell responses are both very important in immunology research, and many studies aim to measure antigen-specific B and T cells. A good example for this are recent studies of the efficiency of SARS-CoV-2 vaccines. As has been widely documented in the press[1], multiple studies have shown that antibody levels (i.e., B cell responses) gradually decrease over time and therefore vaccines protect less well against infection[2,3]. However, despite this, it is also widely appreciated that SARS-CoV-2 vaccines still offer long-lasting protection against symptomatic disease, hospitalisation, and death. This is likely because T cell responses persist [4,5].

However, in contrast to B cell responses, T cell responses are notoriously difficult to measure in blood samples. B cell responses can

simply be measured from the levels of antigen specific antibodies in the blood using ELISA (enzyme-linked immunosorbent assay). In contrast, T cell responses are far more challenging to measure, because only a tiny fraction (<0.1%) of all T cells responds to a particular antigen.

Activated T cells can be recognized by three phenotypic changes: (i) secretion of cytokines, especially interleukin (IL)-2 and interferon (IFN)-gamma; (ii) surface expression of so-called T cell activation markers (e.g., CD25, CD69, CD154); and (iii) T cell division (activated T cells undergo cell division, whereas inactive T cells do not divide). Many techniques have been developed to identify activated T cells based on one of these parameters. However, most techniques are so-called bulk assays (e.g., qPCR, ELISA, Western blot, densitometry) that do not have the sensitivity to detect the <0.1% of dividing T cells in blood samples.

For example, two recent COVID-19 studies[4,5] showed that, in vaccinated individuals, only 0.01 – 0.1% of all peripheral blood mononuclear cells (PBMCs, the fraction of white blood cells containing 60 – 80% T cells) responded to the antigen (i.e., the SARS-CoV-2 spike protein targeted in the vaccine). These low numbers are notoriously challenging to detect, and current techniques are mostly not sensitive enough (as explained below). Moreover, the few existing techniques that are sufficiently sensitive (see below) all rely on the expression of T cell activation markers, but cannot detect one of the most important parameters of activated T cells: T cell division.

Measuring activated T cells also has clinical and diagnostic relevance. For example, a CAR-T drug product potency test based on activated T cells is an assay used to evaluate the efficacy and functional strength of Chimeric Antigen Receptor T-cell (CAR-T) therapies. This test assesses how effectively CAR-T cells can identify, bind to, and destroy target cancer cells after activation. One way to measure potency is through a cytotoxicity assay, where the activated CAR-T cells are incubated with labeled cancer cells, allowing scientists to quantify the extent of tumor cell killing. This can

be visualized using flow cytometry, imaging, or by measuring the release of specific enzymes from destroyed cancer cells. A CAR-T potency test often includes the measurement of cytokine release, as the amount and type of cytokines produced indicate the level of T cell activation and engagement with target cells. High cytokine release, for example, would typically indicate robust activation, while low cytokine levels might indicate lower potency. CAR-T potency tests also often involve checking for the proliferation of CAR-T cells, as effective CAR-T cells will expand upon activation. A potent CAR-T product should show strong proliferation in response to tumor antigens.

Currently, only a few techniques are sensitive enough to detect activated T cells in complex mixtures such as blood samples: flow cytometry and FluoroSpot/ELISpot. Each of these techniques has its limitations (see also Table 1). Flow cytometry is based on labelling of the cells with antibodies recognizing T cell activation markers (e.g., CD25, CD69, CD154). Dozens of companies sell fluorescently labelled antibodies for this purpose. Limitations are that this technique is technically complex, and that it relies on expensive antibodies and access to a flow cytometer. Although probes have been developed for detection of dividing T cells by flow cytometry (for example CFSE[6] or BrdU[7]), the sensitivity of these probes is limited by their labelling heterogeneity and experimental complexity. Therefore, they do not allow for the detection of low fractions of dividing T cells with high sensitivity.

ELISpot and FluoroSpot are established techniques for measuring the production of cytokines produced by activated T cells, typically IFN-gamma and IL-2. These techniques are generally accepted to be more sensitive and less prone to false positive cells than flow cytometry. Side-by-side comparisons of ELISpot with flow cytometry showed that the ELISpot assay is preferable for studies that require the detection of low-level responses, or the differentiation of positive and negative responses; see e.g. Karlsson et al.[8]. Therefore, ELISpot is widely regarded as a gold standard for

quantitating antigen-specific cellular responses after vaccination in clinical trials. ELISpot is robust with low intra-assay, inter-assay and inter-operator variability and can be conducted in a high-throughput manner. However, ELISpot does not allow for detection of T cell division.

In ELISpot and FluoroSpot, a solid carrier such as a PVDF membrane or a 96-well plate is coated with capture antibodies that specifically bind to selected cytokines. Then, T cells are cultured on top of this antibody layer. In ELISpot, the cytokines produced by activated T cells are captured by the layer of capture antibodies and detected by second antibodies that bind to the cytokine and are conjugated to an enzyme that converts a chromogen, mostly horseradish peroxidase (HRP) which converts chromogen 3,3'-diaminobenzidine (DAB) into a dark brown precipitate. In FluoroSpot, the captured cytokines are detected by a fluorescently labelled antibody. Thereby, cytokine-producing T cells can be detected as 5 – 10 μm sized dark or fluorescent spots appearing at the sites of the cytokine-producing T cells, called SFUs (spot forming units). These SFUs can be counted by microscopy. Many companies sell ELISpot and FluoroSpot kits to detect T cell proliferation. Disadvantages of these approaches are that expensive antibodies and prolonged culturing times are needed to capture sufficient amounts of cytokines. Moreover, ELISpot and FluoroSpot require the T cells to be cultured on a surface (i.e., the layer of capture antibodies), which potentially interferes with the cell-cell interactions needed for T cell activation (antigen presentation). Finally, T cell division cannot be measured with these techniques.

Therefore, the present inventors sought to develop a new technique which does not suffer from the above limitations. In particular, they aimed at providing an improved method that allows for the simple, sensitive and reliable detection of low numbers of dividing cells in complex biological samples, and is applicable in fundamental and translational immunology research settings. Furthermore, the novel approach should not rely on

(expensive) cytokine-specific antibodies and can detect activated cells, in particular antigen-specific T cells, with high sensitivity e.g. <0.01% of dividing T cells in blood samples.

It was surprisingly observed that these goals can be met by culturing cells in the presence a nucleoside analog, such as EdU (5-ethynyl-2'-deoxyuridine), that is incorporated in the newly synthesized DNA of dividing cells, followed by concentrating the nucleoside-loaded cells by covalent immobilization to a solid support. The solid support is coated with selected polyamine compound by using a defined adhesion protocol which covalently attaches amines that are exposed on the cell surface to reactive aldehydes on the coated solid support. The concentrated and immobilized cells are then labelled (stained) with a detection probe which becomes covalently attached to the DNA of the dividing cells via the highly specific and established bio-orthogonal click chemistry reaction. The cells containing the detection probe indicative of proliferating (activated) cells can then be detected and quantified (e.g., as SFUs) similar as in ELISpot. The concept of the present invention, herein further also referred to as "ProliSpot", allows to reliably detect dividing cells within a 5-6 hours protocol.

Advantages of the new method include that it is the most sensitive detection method available for detection of proliferating cells, and that it is more cost effective than other techniques for detection of activated T cells. ProliSpot is the first technique that enables researchers to quantitively detect low numbers (<0.01%) of dividing T cells in blood samples and other complex cell mixtures. Existing techniques are either not sensitive enough or do not allow to detect dividing T cells. Importantly, in contrast to existing techniques for measuring T cell activation, no complex equipment and/or expensive antibodies are required for the ProliSpot technique. Still further, and unlike ELISpot and FluoroSpot, T cell activation can be performed

entirely in suspension culture. This is more physiological, since it does not interfere with the cell-cell interactions required for antigen presentation.

Table 1 shows a comparison of the ProliSpot method with existing techniques for assessing T cell activation. Clearly, the ProliSpot overcomes all drawbacks of existing techniques.

Table 1

Technique	*T cell division*	*Cost*	*Sensitivity*	*Suspension culture*	*Multiplex*
Flow cytometry for T cell activation markers	No	High	High	Yes	Yes
Flow cytometry for T cell activation probes	Yes	High	Low	Yes	Yes
ELISpot	No	Medium	High	No	No
FluoroSpot	No	Medium	High	No	Yes
ProliSpot	**Yes**	**Low**	**High**	**Yes**	**Yes**

Notably, whereas the invention is exemplified using T cell detection, other dividing (non-immune) cells can also readily be detected with high sensitivity using the ProliSpot technique. The ProliSpot technique therefore also has other (clinical) applications, for example for detection of rare circulating cancer cells (e.g., minimal residual disease (MRD)) or for stem cell research. Similarly, it may also be used to monitor metastases of solid tumors, such as breast cancer or lung cancer, after surgery or chemotherapy, as the number of circulating proliferating cancer cells has been reported to correlate with cancer prognosis[9].

Accordingly, the invention discloses an *in vitro* method for detecting proliferating cells, comprising the steps of:

(i) culturing cells in the presence of a nucleoside analog comprising a first reactive unsaturated group to allow for incorporation of the nucleoside analog in the DNA of the cells;

(ii) providing a solid surface coated with (3-aminopropyl)triethoxysilane (APTES) or branched polyethyleneimine (PEI);

(iii) contacting the nucleoside-labelled cells with the coated solid surface in the presence of glutaraldehyde (GA) to concentrate and covalently adhere the cells to the solid surface;

(iv) optionally fixing the adhered cells with formaldehyde;

(v) contacting the covalently adhered cells with a detection probe comprising a second reactive unsaturated group such that a cycloaddition reaction, for example a [3+2] or [4+2] cycloaddition reaction, occurs between the first and second reactive groups; and

(vi) determining the amount of first label incorporated into the DNA of the adhered cells to measure cellular proliferation.

In particular, the invention provides an *in vitro* method for detecting proliferating cells, comprising the steps of:

(i) culturing cells in the presence of a nucleoside analog comprising a first reactive unsaturated group to allow for incorporation of the nucleoside analog in the DNA of the cells;

(ii) concentrating the nucleoside-labeled cells by covalent immobilization to a solid surface comprising a coating of (3-aminopropyl)triethoxysilane (APTES) or branched polyethyleneimine (PEI) that is activated by glutaraldehyde (GA);

(iii) optionally fixing the concentrated and covalently immobilized cells with formaldehyde;

(iv) contacting the covalently immobilized cells with a detection probe comprising a second reactive unsaturated group, such that a [3+2] or [4+2] cycloaddition occurs between the first and second reactive unsaturated groups; and

(v) determining the amount of detection probe attached to the DNA of the immobilized cells to measure cellular proliferation.

Thus, according to the invention nucleoside-labelled cells are subjected to a concentration and immobilization step wherein cells are covalently adhered to a (aldehyde-functionalized) solid surface comprising a coating with a specific polyamine (APTES or branched PEI) that is activated by GA. In one aspect, cells are covalently adhered to a solid surface comprising a coating with APTES that is activated by GA. In another aspect, cells are covalently adhered to a solid surface comprising a coating with branched PEI that is activated by GA. Mixtures of PEI and APTES can also be used. It was surprisingly found that these selected polyamine compounds in combination with GA allow for a very dense (up to at least 250,000 cells/cm^2) and homogeneous attachment of the cells. More specifically, other cell adhesives used in the art for cell adhesion or immobilization, including paraformaldehyde (PFA), concanavalin A (conA) and poly-L-Lysine (PLL), or structurally related compounds such as poly(allylamine) or MPTES, failed to give the desired degree of cell concentration.

Unexpectedly, it was observed that the present approach for concentrating of nucleoside-loaded cells allows for the exposure of cells to rather harsh labeling (staining) conditions without cells becoming detached or being washed away. For example, a method of the invention is advantageously used with reagents / reactive groups that rely on a [3+2] cycloaddition reaction in the presence of Cu(I). However, copper-free cycloadditions using other reagents such as bicyclo[6.1.0]nonyne (BCN) are also within the scope of the present invention.

A method of the invention is not taught or suggested in the art. EdU is commercially available, and many biotechnology companies sell EdU-based kits for measuring cell division. However, these kits rely on bulk measurements (i.e., of the complete cell culture by bulk fluorescence or flow

cytometry) and therefore have too low sensitivity to detect the <0.01% dividing T cells in complex blood samples. The newly developed ProliSpot approach overcomes this limitation by concentrating the T cells covalently to a surface e.g. up to a density of at least 80,000 cells per well of a 96-well plate. The challenge in adhering the cells was that the chemicals and wash steps of the (copper-based) click reaction resulted in substantial loss of cells. This challenge was overcome by concentrating the cells by covalent immobilization to PEI- or APTES-functionalized supports using glutaraldehyde prior to the click reaction.

Covalent cell attachment hence allows for an optics-based readout similar as in ELISpot and FluoroSpot, which is less prone to false negatives compared to flow cytometry, thereby substantially increasing the sensitivity of the detection of nucleoside-containing cells.

An EdU-based assay for the identification of proliferating T cells was previously described in several studies. See for example Stempels *et al.*[10] or Sun *et al.*[11]. However, these protocols involved either flow cytometric detection or immobilization of (activated) cells to a poly-L-Lysine coated glass plate prior to loading with EdU. As explained above, flow cytometry is less sensitive and more prone to detection of false positive cells than ELISpot/FluoroSpot type readouts. Whereas in principle it would be possible to use an ELISpot/FluoroSpot type readout on non-covalently adhered cells (for instance to poly-L-lysine coated glass), this has only a very low sensitivity as the harsh chemicals and wash steps required for the copper-based labeling reaction result in a substantial loss of the cells.

EP1937849B1 relates to methods for the labeling of nucleic acid polymers that involve a [3+2] cycloaddition between a nucleotide analogue, such as EdU, incorporated into a nucleic acid polymer, and an azide-coupled detection probe. Disclosed is a protocol for the EdU-labeling, fixation,

staining with fluorophore-azide, and imaging of stained cells, wherein cells in suspension are fixed by an aldehyde (paraformaldehyde or glutaraldehyde), with or without permeabilization. It is generally stated that the protocol can be used to detect cellular proliferation in normal, diseased and injured tissues. However, EP1937849B1 is silent about concentrating EdU-labelled cells by covalent attachment to a solid substrate prior to subjecting to the [3+2] cycloaddition reaction, as disclosed in the present invention. Without this step, too many cells are lost and the sensitivity is far too low for detecting very low populations of (activated) cells.

Hence, in a method of the invention the cells are covalently attached onto the GA-activated PEI- or APTES coating. This is distinct from known methods wherein a surface is activated with APTES and GA to immobilize a matrix onto which cells are subsequently seeded and cultured.

For example, the APTES /GA chemistry was previously used to provide a biocompatible platform for the enhanced adhesion and proliferation of mesenchymal stem cells (MSCs). Because of the inherent high hydrophobicity of a poly(dimethylsiloxane) (PDMS) surface, cell culture on these surfaces is unfavorable, causing cells to eventually dislodge from the surface. Kuddannaya *et al.*[12] reported that the hydrophobicity of native PDMS decreased significantly upon surface functionalization using APTES/GA to immobilize either fibronectin (FN) or collagen type 1 (C1) protein on PDMS. Adhesion of MSCs was mostly favorable on chemically modified PDMS surfaces with APTES + glutaraldehyde + protein.

The study of Price et al. (Biomaterials 2016, Vol. 121, pp. 179-192) aims at providing a soft, ligand-rich microenvironment for pluripotent stem cells. Disclosed is the use of aldehyde-functionalized glass coverslips using APTES and GA in order to provide a glass support onto which soft

Matrigel/acrylamide matrices can be immobilized by a polymerisation reaction to provide a polymerized composite substrate. This composite substrate is then used for the seeding (i.e. noncovalent attachment) of cells. Dividing cells cultured on the matrices can be identified using conventional EdU staining. In contrast to the present invention, these cells adhere passively through the matrices and the cells are not covalently adhered to the support.

Similarly, Riau et al., (Applied Materials & Interfaces, vol. 7, no. 39, 2015, pp. (21690-21702) describes a method using APTES with a carbodiimide crosslinker chemistry for linking a collagen matrix to PMMA substrates onto which corneal fibroblasts are seeded. Like in Price et al., dividing cells cultured on the matrices are identified using EdU. Riau et al., is also silent about first labelling cells with nucleoside and subsequently concentrating the nucleoside-labeled cells to a solid support by covalent immobilization to an APTES/GA coating.

While aldehyde groups are well known to be capable of reacting with amine groups on the cell surface proteins, the present approach involving a direct covalent linkage of cells to an aldehyde-functionalized surface is against the widespread view that this is not ideal for several reasons. First, the reaction between aldehyde groups and cell surface proteins can be too strong and potentially harmful to cells, affecting cell viability and function. Second, aldehyde groups might react with various cell surface molecules in a non-specific manner, leading to undesirable cell behavior.

Step (i) of a method of the present invention comprises culturing cells in the presence of a nucleoside analog comprising a first reactive unsaturated group to allow for incorporation of the nucleoside analog in the DNA of the cells.

In one embodiment of the present invention, the first reactive unsaturated group comprises an azide group and the second reactive unsaturated group

comprises an alkynyl group. An another embodiment, the first reactive unsaturated group comprises an alkynyl group and the second reactive unsaturated group comprises an azide group.

Suitably, the nucleoside analog is selected from the group consisting of 5-ethynyl-2'deoxyuracil (EdU), 5-ethynyl-2'-deoxycytidine (5-EdC), 5-azido-2'-deoxyuracil (AdU), 5-Azidomethyl-2'-deoxyuridine (5-AmdU) and 5-vinyl-2'-deoxyuridine (5-VdU), as well as their triphosphate and phosphoramidite forms. Whereas it is technically also possible to perform the method with BrdU and related analogs, these assays are antibody-based and therefore require DNA denaturation for detection of the incorporated nucleoside. In contrast, the above nucleoside analogs are incorporated during DNA synthesis and detected using a click reaction—a copper(I)-catalyzed reaction between an azide and an alkyne.

The nucleoside labeling step may be performed for at least 30 minutes, preferably at least 45 minutes. Very good results are obtained when cells are cultured in the presence of nucleoside analog for about 60 to 180 minutes, such as 90 to 120, 100 to 150 or 90 to 180 minutes. The nucleoside analog may be used at conventional concentrations that allow for sufficient incorporation into the DNA in proliferating cells, e.g. 100 nM to 20 μM, preferably 1 to 10 μM, such as 2-5 μM, 3-8 μM or 5-10 μM.

In a specific aspect, the nucleoside analog is EdU. Accordingly, in one embodiment the invention provides a method comprising (i) culturing cells in the presence of EdU to obtain EdU-labelled cells; (ii) concentrating the EdU-labeled cells by covalent immobilization to an aldehyde-functionalized solid surface, wherein said aldehyde-functionalized solid surface comprises a coating with APTES or branched PEI that is activated by GA; optionally followed by fixing the immobilized cells with paraformaldehyde, e.g. as a formaldehyde solution; (iii) contacting the concentrated covalently immobilized cells with a detection probe comprising an azide-coupled detection probe such that a [3+2] cycloaddition occurs between the EdU and

the detection probe; and (iv) determining the amount of EdU incorporated into the DNA of the immobilized cells to measure cellular proliferation.

The cells may be of any origin but typically a method of the invention is advantageously used for detecting (low frequency) mammalian cells in a biological sample, like a blood sample of a mammalian subject or laboratory animal. In one embodiment, the cells are obtained from a healthy human subject or from a human patient. Cells can be taken at the stage of diagnosis, during therapy and/or after therapy. Cells may comprise T cells, cancer cells or stem cells. In one aspect, the cells comprise cancer cells such as MRD (minimal residual disease) cells. In another embodiment, cells comprise stem cells. In a preferred embodiment, the cells comprise T cells. In a specific embodiment, the cells are modified to express a CAR (chimeric antigen receptor) construct e.g. CAR-T cells.

Suitably, step (i) comprises culturing a blood sample in the presence of nucleoside and antigen. The blood sample may comprise peripheral blood mononuclear cells (PBMCs) isolated using methods known in the art. For example, to isolate PBMCs from the buffy coat, density gradient centrifugation can be used. PBMCs may be removed from the buffy coat using manual or automated methods, with or without density-gradient materials such as albumin, Ficoll, and Percoll. PBMCs include lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells. In humans, the frequencies of these populations vary across individuals, but typically, lymphocytes are in the range of 70–90 %.

Cells are preferably cultured in suspension to allow for (near) physiological intercellular interactions. In one embodiment, step (i) comprises culturing a suspension of T cells in the presence of an antigen of interest. Exemplary antigens include tumor antigens and pathogenic antigens, such as viral antigens. For example, the antigen is a cytomegalovirus (CMV), SARS-CoV-2 or influenza antigen.

In another embodiment, the antigen is an antigen recognized by a CAR-T cell. For example, provided is a method for determining the potency of a CAR-T cell drug product wherein (patient) T cells genetically engineered to express a CAR construct are labeled with nucleoside in the presence of the relevant CAR antigen(s) and subsequently concentrated and immobilized to a solid surface using APTES or branched PEI. CAR T-cells may be manufactured either from the patient's own blood, known as an autologous treatment, or from the blood of a healthy donor, known as an allogeneic treatment, or from a cell line.

In a specific aspect, cells are cultured in the presence of an antigen (fragment) used for vaccination. Other embodiments relate to culturing cells in the presence of a peptide library, for example a T cell activating peptide library. In a specific aspect, cells are cultured in the presence of a CMV or SARS-CoV spike-protein library.

Control conditions may be included, such as negative control samples without antigen or with random peptides, and positive control samples e.g. phytohemagglutinin (PHA) or beads conjugated with antibodies directed against (CAR) T cell receptors for strong T cell activation.

In other embodiments, cells are cultured in the presence of nucleoside in the absence of antigen. For example, provided is a method for detecting MRD wherein a blood sample e.g., PBMCs, are labeled with nucleoside and subsequently covalently immobilized to a solid surface using GA-activated APTES or branched PEI. Dividing cancer cells can be further identified in by antibody labeling (see immunolabeling protocol of Example 2), for example for the myeloid antigens CD13, CD15, CD33 and CD65 in MRD of acute myeloid leukemia.

Following the labelling with nucleoside analog, cells may be washed with buffer, such as PBS, prior to step (ii) wherein labelled cells are fixed with glutaraldehyde (GA) or paraformaldehyde (PFA) according to methods

known in the art. PFA/GA has been widely used as a cross-linking fixation agent. PFA causes covalent cross-links between molecules, effectively gluing them together into an insoluble meshwork that alters the mechanical properties of the cell surface. Typically, cells are contacted with an aqueous solution of formaldehyde, for example a 4% formaldehyde solution in PBS obtained by dissolving paraformaldehyde powder in a heated PBS solution.

A method of the invention may comprise providing a solid surface coated with one or both of the selected polyamines APTES and PEI. To allow for optics-based readout similar as in ELISpot and FluoroSpot, the solid surface is preferably a flat-bottom surface of an optically transparent multi-well plate. In one embodiment, the solid surface is the surface of a glass or plastic support, such as a microscope glass slide or a multi-well (e.g., 6-, 12- or 96-well) plate.

In one aspect, a cleaned glass surface is coated with APTES. This is readily achieved by a cleaning solution or plasma cleaning for 10 minutes and immediately afterwards applying 2% APTES (Sigma-440140) in acetone for 10 seconds. This is followed by washing once with acetone. Just before use, the chamber is filled with a 1 % glutaraldehyde (Sigma-Aldrich, G7651) in PBS solution and incubated for 30 minutes at room temperature (RT) and washed five times with bidest H_2O to remove excess glutaraldehyde.

In another aspect, a solid (glass or plastic) surface is coated with branched PEI. As used herein, the term 'PEI' refers to polyethylenimine (also known as polyaziridine) which is a polymer with repeating units composed of the amine group and two carbon aliphatic CH_2CH_2 spacers. In contrast to linear polyethyleneimines that contain all secondary amines, branched PEIs contain primary, secondary and tertiary amino groups in a ratio of 1:2:1, respectively and hence can be fixed with glutaraldehyde. Any branched PEI with a general backbone of (CH_2CH_2NH) can be used. The branched PEIs

are liquids at all molecular weights. The branched PEI can have different molecular weights e.g. 800, 2000, 25,000 or 750,000 MW PEI. In one aspect, branched PEI with an average Mn of ~60,000 and an average Mw of ~750,000 (available from Sigma-Aldrich) is used. In another aspect, the branched PEI has an average Mw of ~25,000 (by LS) and an average Mn of ~10,000 (by GPC).

Coating of glass cover slips or glass or plastic bottom e.g. 96-well plates is readily achieved by incubating with 0.2% (w/v) PEI (Sigma Aldrich p3143) in bidest H_2O for 24 hours. Solutions were removed and air dried prior to use. Just before use, the chamber is filled with a 1 % glutaraldehyde (Sigma-Aldrich, G7651) in PBS solution and incubated for 30 minutes at RT and washed five times with bidest H_2O to remove excess glutaraldehyde.

Concentrating nucleoside-labeled cells by covalent immobilization to the activated PEI or APTES coating substantially increases detection sensitivity, as indicated by a loss of ~75% of the cells in the absence of glutaraldehyde (GA) (Figure 2B). This loss could be largely prevented by GA fixation for PEI and APTES. However, GA fixation could not prevent cell loss with PLL and PAA, likely because of a lower density on primary amines and/or lower cross-linking efficiency. Furthermore, the effect of GA could not be mimicked by formaldehyde (FA) (see Figure 11).

Preferably, a method of the invention comprises concentrating an covalently immobilizing the cells to the polyamine-coated solid surface to reach at least about 60% confluency, more preferably at least about 70% confluency. For example, cells, such as T cells, are attached at a density of at least 40 cells per 100 μm^2, preferably at least 50, 60, 70 or 80 cells per 100 μm^2. In one embodiment, cells, such as T cells, are covalently immobilized at a density of at least 20,000 cells per well of a 96-well plate, preferably at least 40,000, more preferably at least 60,000 cells, most preferably at least 80,000 cells

per well. As will be understood, there is a physical limitation to the maximum number of cells that can be attached to a polyamine-coated well surface. Typically, up to about 250,000 cells can be immobilized in a well of a 96-well plate.

In some embodiments, one or more centrifugation steps may be performed to concentrate cells to a high density on an activated coated surface. Suitable centrifugation speed, time and temperature are well known in the art.

In one embodiment, the step of concentrating cells comprises introducing a suspension of 50,000, 100,000 or 200,000 nucleoside-labeled cells in a well of a polyamine-coated 96-well plate, followed by centrifuging the multi-well plate for 5 min at 300g to concentrate the cells on the support at a high (>80%) confluency (Figure 11). For covalent immobilization, the polyamine coating can be either activated before the centrifugation step with GA or the GA can be added afterwards (Figure 10).

As is shown herein below (see Example 8), the order of polyamine activation by GA is not critical. Hence, nucleoside labeled cells can be contacted with APTES/PEI coating that is either preactivated with GA (e.g. including washing away excess GA) or GA can be added to the APTES/PEI coated surface together with addition of the cells. In both cases, the reactive aldehydic groups of GA covalently link the cells to the APTES/PEI coating, which allows for concentrating cells to very high densities while ensuring cell stability throughout the nucleoside staining procedure. Hence, in one embodiment step (ii) comprises concentrating the nucleoside-labeled cells by covalent immobilization to a solid surface comprising a coating of APTES or branched PEI that is pre-activated by GA. In another embodiment, nucleoside-labeled cells are contacted in the presence of GA with a solid surface coated with APTES or branched PEI to concentrate and covalently immobilize the cells to they solid surface.

Once the nucleoside-labeled cells have been concentrated and covalently adhered to a solid surface, they are subjected to a staining protocol using a detection probe which becomes covalently attached to the DNA of dividing cells via the highly specific bio-orthogonal reaction product (known in the art as a cycloaddition or "click chemistry") described herein above. In some embodiments, the detection probe comprises a detectable label attached to a second reactive unsaturated group that can undergo a cycloaddition reaction, for example a [3+2] or [4+2] cycloaddition reaction, with the first reactive group of the nucleoside analog.

The staining reaction can involve a [3+2] or a [4+2] cycloaddition. The Diels-Alder [4+2]-cycloaddition of a conjugated diene and a dienophile (an alkene or alkyne), is an electrocyclic reaction that involves the 4 π-electrons of the diene and 2 π-electrons of the dienophile. In one aspect, a method of the invention utilizes a fluorogenic intercalating agent capable of undergoing inverse electron-demand Diels-Alder (IEDDA) reactions with a vinyl-conjugated nucleoside analog such as 5-vinyl-2'-deoxyuridine (5-VdU). In contrast to a normal electron demand Diels-Alder reaction, where an electron-rich diene reacts with an electron-poor dienophile, in an inverse-electron-demand Diels-Alder reaction (IEDDA), an electron-rich dienophile reacts with an electron- poor diene.

In a preferred embodiment, a method comprises a [3+2] cycloaddition reaction. For example, it comprises a [3+2] cycloaddition reaction between the ethynyl group of the incorporated nucleoside analog carrying an alkynyl group, e.g. EdU, and the azide group of the probe. To this end, the concentrated and covalently immobilized cells are contacted with a solution of the azide-coupled detection probe to allow for the [3+2] cycloaddition reaction using methods known *per se* in the art. In one embodiment, it comprises the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC). See for

example EP1937849 for exemplary staining solutions comprising azide-coupled detection probe and $CuSO_4$. Alternatively, it comprises the use of a Cu-chelating agent that can mobilize copper (I) ions that are naturally present in the cells in the vicinity of the cycloaddition reaction.

Suitably, the nucleoside-labeled cells are concentrated and immobilized to the polyamine coated surface by incubation for about 2 hours at 4 °C. Following wash steps with cold PBS, the cells can be fixed for 15 minutes with 4% formaldehyde in PBS at 4 °C. Afterwards, cells may be washed extensively with PBS and stained e.g. with dye-azide e.g. Eterneon2 GREEN 488. Staining protocols using labeled dyes are well known in the art. The staining solution comprising the detection probe is preferably prepared fresh just prior to use. Suitably, an antioxidant such as a salt of L-ascorbate e.g. sodium or potassium L-ascorbate, is included in the staining solution.

For example, staining with an azide dye is suitably performed for 1 h with Hepes buffered saline (HBS; 0.01 M HEPES pH 7.4 and 0.15 M NaCl) containing 1 mM $CuSO_4$, 0.5 mM THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine), 5 mM sodium L-ascorbate; 0.01% (w/v) BSA, and aminoguanidine 2.2% (w/v).

As will be appreciated by a person skilled in the art, a method of the invention may involve any type of azide-coupled detection probe to stain for cells that have incorporated nucleoside analog during their proliferation. In one aspect, the probe is an azide-coupled fluorophore wherein the fluorophore can be detected by an optics-based readout system, such as an FluoroSpot apparatus. Suitable fluorophores include the fluorescent dyes FITC, Cy3, Cy5, Alexa fluor series, Eterneon GREEN, Eterneon RED, fluorescein (6-FAM), carboxytetramethylrhodamine (5-TAMRA-azide), and 5-6-sulforhodamine.

In another embodiment, the azide-coupled probe is an azide-coupled enzyme, preferably an enzyme serving as reporter enzyme by generating a detectable product. Suitably, azide-coupled horse radish peroxidase (HRP) is used which produces H_2O_2 which can in turn oxidize a chromogen e.g. DAB (3, 3'- diaminobenzidine) into a coloured product that precipitates at the position of dividing cells, and can be detected by an ELISpot apparatus. Azido-HRP is readily obtained by conventional methods including for instance diazo-transfer of imidazole-1-sulfonyl azide to HRP.

Still further, the detection probe itself does not give rise to a detectable signal but instead contains a reactive group that specifically binds to cognate binding partner that is conjugated to a detectable (fluorescent) label or reporter enzyme. For example, the streptavidin-biotin complex is suitably used to mediate binding of a detectable label or enzyme to the [3+2] cycloaddition product. In one embodiment, the detection probe is azide-PEG-biotin and a streptavidin-conjugated label or enzyme e.g. HRP is used.

Since a method of the invention is compatible with multiplex analysis, it may further comprise contacting the immobilized cells with one or more additional detection reagent(s), preferably one or more spectrally-shifted reagent(s). For example, the immobilized cells are also stained with a DNA-specific probe such as DAPI (4′,6-diamidino-2-phenylindole) which is a blue-fluorescent DNA stain. This allows for the segmentation of a desired number of cells to be analyzed (e.g. 10,000 to 100,000 cells per condition) on the basis of nuclear staining, and subsequent scoring of EDU-positive cells.

Alternatively or additionally, one or more (fluorochrome-conjugated) antibodie(s) may be used e.g. for phenotyping cells. For example, in case of MRD detection in acute myeloid leukemia, the identification of dividing cancer cells can involve antibody labeling for the myeloid antigens CD13, CD15, CD33 and CD65, or any other known panel of MRD markers.

In step (v) of a method of the invention the presence of a detection probe that is covalently linked to (the DNA of) the immobilized cells, and optional additional detection reagent(s), is/are detected. Thus, cellular proliferation is measured indirectly by detecting the amount of detection probe attached to nucleoside analog incorporated into the DNA of the immobilized cells.

This step may involve the use of conventional detection equipment, depending on the type of probe(s) used. For example, it comprises the use of brightfield microscopy, fluorescence microscopy or an ELISpot or FluoroSpot reader. In a preferred embodiment, step (v) comprises the use of an ELISpot reader, a FluoroSpot reader or a similar specialized reader system designed to analyze and count spots on (standardized) assay plates. In some embodiments, the reader can detect multiple fluorescent signals, typically between two to four colors. These readers are preferably equipped with high-resolution cameras and optical filters to capture sharp images of the fluorescent spots. The imaging capability is essential for distinguishing between closely spaced spots. Preferably, the reader is paired with software that automates the detection and counting of fluorescent spots. The software allows users to fine-tune detection parameters, such as sensitivity and threshold settings, to optimize spot detection based on specific assay conditions and reduce background noise. Data from a reader can be exported in formats suitable for further analysis, such as CSV, Excel, or PDF files, and may integrate with laboratory information management systems (LIMS).

Preferably, the reader provides adjustable sensitivity and detection thresholds. Since spot sizes and intensities can vary based on cell type, incubation time, and assay conditions, readers typically offer adjustable thresholds and sensitivity settings to ensure reliable detection. The ability to differentiate small or faint spots from background noise is

crucial for quantitative accuracy, especially in assays with low frequencies of cytokine-producing cells.

Suitably, the reader comprises automated data analysis software to reduce human error and improve reproducibility. This software enables automated data acquisition, analysis, and export, and often includes statistical tools and options for analyzing spot size, intensity, and count per well, thus providing a comprehensive dataset for further analysis.

Preferably, the reader features color and multi-spot detection capabilities. For example, the reader can detect multiple colors or substrates, enabling multiplexed assays where multiple signals are measured in the same well. For instance, dual-color or multi-color detection allows researchers to measure various cell populations simultaneously. For example, differentiate CD4+ helper T cells from CD8+ cytolytic T cells, or differentiate among different subtypes of helper T cells. These readers are also compatible with various plate formats, from 96-well to 384-well plates, which adds flexibility, especially in high-throughput settings.

The invention also provides a kit-of-parts for use in a method as herein disclosed. The kit may comprise the following components:

- a polyamine-coated carrier for receiving cells, the carrier comprising a surface coated with (3-aminopropyl)triethoxysilane (APTES) or branched polyethyleneimine (PEI)
- a first container comprising a nucleoside analog comprising a first reactive unsaturated group; and
- a second container comprising a detection probe comprising a second reactive unsaturated group which can undergo a [3+2] or [4+2] cycloaddition reaction with the first reactive group.

Optionally, the kit may comprise an additional (third) container comprising glutaraldehyde (GA) and/or a further (fourth) container containing an

antioxidant, preferably a salt of L-ascorbate such as sodium or potassium L-ascorbate. The antioxidant may be included in the staining solution comprising the detection probe just prior to use.

In a specific embodiment, the invention provides a kit-of-parts e.g. for detecting antigen-specific T cells, comprising -

- a first container comprising APTES or branched PEI, preferably branched PEI;
- a second container comprising a nucleoside analog comprising a first reactive unsaturated group, preferably 5-ethynyl-2′-deoxyuridine (EdU);
- a third container comprising a detection probe comprising a second reactive unsaturated group which can undergo a [3+2] or [4+2] cycloaddition reaction with the first reactive group;
- a fourth container comprising glutaraldehyde (GA); and
- at least one antigen of interest.

Suitably, the kit comprises at least one antigen of interest that is coupled to a bead. Suitable beads for T cell activation are known in the art. For example, an antigen of interest may be conjugated to beads having a diameter of between 1-4.5 μm, which can be made of polystyrene of polystyrene covered with a layer of magnetite (so-called Dynabeads). The antigen may be present in a separate (i.e. fifth) container. Alternatively, the invention provides a kit wherein the antigen of interest is comprised in the container also comprising the nucleoside analog comprising a first reactive unsaturated group, preferably EdU. This container is suitably used for collecting (sampling) a (patient) cell sample and for subsequent culturing and labeling the cells in the presence of an antigen of interest. The sampling container may be a conventional blood collection tube, such as a sterile glass or plastic test tube with a colored rubber stopper creating a vacuum seal inside of the tube, facilitating the drawing of a predetermined volume of

liquid. The blood collection tube may contain one or more conventional additives such as anticoagulants (EDTA, sodium citrate, heparin) or a gel with density between those of blood cells and blood plasma. In one aspect, the kit comprises a sampling container is a conventional tube for diagnostic venous blood specimen collection, comprising at least one antigen of interest, a nucleoside analog comprising a first reactive unsaturated group, preferably EdU, and heparin.

Exemplary antigens of interest include clinically relevant antigens, such as a tumor antigen, pathogenic antigen or disease-specific antigen. Preferably, the kit comprises a tumor antigen or a pathogenic antigen, more preferably a viral antigen.

In a specific embodiment, the kit comprises one or more (bead-bound) antigen(s) that is targeted by a CAR expressed on e.g. a CAR-T cell, CAR-NK cell or CAR-macrophage. Exemplary CAR antigens include CD19, CD20, CD22, CD7, BCMA (B-cell maturation antigen), CD30, CD123, CLL1 (human C-type lectin-like molecule-1), Mesothelin, HER2 (Human Epidermal Growth Factor Receptor 2), EGFRvIII (Epidermal Growth Factor Receptor variant III), Glypican-3 (GPC3), PSMA (Prostate-Specific Membrane Antigen), and any combination thereof.

Optionally, the kit also comprises a carrier for receiving cells. The carrier can be a polyamine-coated carrier comprising a surface coated with (3-aminopropyl)triethoxysilane (APTES) or branched polyethyleneimine (PEI). Alternatively, the kit comprises an uncoated carrier for receiving cells that can be provided with an aldehyde-activated coating by the end user using APTES/PEI and GA comprised in the kit.

Carriers for receiving cells are known in the art. Preferred carriers have an optically clear (transparent) bottom section for receiving and imaging cells. In one aspect, the carrier comprises a glass or plastic surface. Preferred carriers include multiwell plates having a plastic or glass bottom section. In one embodiment, the carrier is a multiwell plate, preferably having footprint dimensions in line with the proposed SBS

industry standard. This guarantees compatibility with all microplate-based instrumentation. Examples of standard formats include 8x12 (96)-, 16x24 (384)-, 32x84 (1536)-, and 48x72 (3456)-well assay plates, with the wells configured in two-dimensional arrays. The wells can have a conical or flat bottom. In a preferred embodiment, a plate of the invention is a 96-well flat bottom assay plate, e.g. of optically clear polystyrene or glass, having footprint dimensions in line with the proposed SBS industry standard. The glass or plastic carrier may be coated with APTES, branched PEI or a combination thereof. In a preferred embodiment, the kit comprises a multiwell plate (to be) coated with PEI.

The kit may comprise a nucleoside analog comprising an azide group and a detection probe comprising an alkynyl group. Alternatively, the kit may comprise a nucleoside analog comprising an alkynyl group and a detection probe comprising an azide group, such as an azide-coupled fluorophore or an azide-coupled enzyme, preferably azide-coupled HRP.

The nucleoside analog is suitably selected from the group consisting of 5-ethynyl-2'deoxyuracil (EdU), 5-ethynyl-2'-deoxycytidine (5-EdC), 5-azido-2'-deoxyuracil (AdU), 5-Azidomethyl-2'-deoxyuridine (5-AmdU) and 5-vinyl-2'-deoxyuridine (5-VdU), as well as their triphosphate and phosphoramidite forms. In one aspect, the nucleoside analog is 5-ethynyl-2'deoxyuracil (EdU) or 5-ethynyl-2'-deoxycytidine (5-EdC).

The kit may further comprise one or more additional spectrally-shifted reagent(s), such as a DNA-specific probe and/or a fluorochrome-conjugated antibody. In one embodiment, the kit comprises DAPI to facilitate the automatic counting and segmentation of the individual cells by image processing. Preferred T cell kits comprise anti-CD3 antibody conjugated to a spectrally shifted fluorophore for validation of cell type.

Also provided is the use of a method or kit as herein disclosed in a research or clinical (diagnostic) setting. For example, it allows for identifying dividing (activated) T cells in blood samples and other complex cell mixtures. Given

the importance of T cell responses in health and disease, there is a very large potential market for such a technique. T cell proliferation is one of the key outcomes of adaptive immune responses, and therefore a relevant parameter in studies addressing many diseases and disorders, including autoimmune diseases, cancer, metabolic diseases, neurodegenerative diseases.

For example, a method and/or kit allows for monitoring the efficacy of a vaccine, including SARS-CoV-2 vaccines. As another example, one of the key readouts in clinical trials for immunotherapies is T cell activation. In addition, by monitoring patient-specific immune responses, cell proliferation assays could aid in predicting therapy outcomes and thereby play a central role in the development of personalized medicine. For example, in PDL1-expressing non-small-cell lung carcinoma, T cell proliferation has been used to predict patient-specific anti-PD1 responses.

In addition, the use of the approach provided herein is not limited to the division of T cells, but also other dividing cells can be detected with high sensitivity. The ProliSpot technique will therefore also have other applications, for example for detection of rare circulating (MRD) cancer cells or for stem cell research.

The ProliSpot concept is also advantageously used in the clinic as diagnostic assay, for example for determining T cell responses in allergies, or as a readout for vaccination responses e.g. as alternative to measuring antibody titers.

In another specific aspect, the invention finds its use in the field of CAR (Chimeric Antigen Receptor) cell therapy. CAR cell therapy is an advanced cancer treatment where a patient's T cells (immune cells) are genetically engineered to recognize and attack cancer cells. T cells are collected, modified to express synthetic receptors (CARs) targeting one or more specific cancer antigens, and then infused back into the patient. These CAR-

T cells can identify and destroy cancer cells more effectively, even those that evade normal immune responses. Originally developed for blood cancers like leukemia and lymphoma, CAR cell therapy is expanding to treat other cancer types and autoimmune diseases, offering hope for durable remissions and advancing personalized immunotherapies.

Measuring T cell activation in a clinical context is essential for multiple reasons, especially when evaluating immunotherapies such as CAR-T cell therapy. One significant aspect is predicting treatment efficacy. Activated T cells indicate that the immune system is responding to a target, such as cancer or infection. For CAR-T cells, effective activation upon contact with cancer cells often correlates with treatment success, as these cells become primed to recognize and kill tumor cells. By assessing the extent of proliferation upon exposure to one or more specific antigens, clinicians can gauge the potency of T cell-based therapies, with higher activation levels generally suggesting a more effective immune response and improved outcomes.

Measuring T cell activation also plays a role in determining the optimal dosage of CAR-T cells or other T cell therapies. By analyzing the dose-response relationship, clinicians can identify the dose needed to achieve ideal activation without excessive risk, balancing therapeutic efficacy with safety. Activation monitoring is also useful in estimating the duration of therapeutic effects since higher activation can correlate with more sustained tumor clearance. This information can help in adjusting dosage frequency to maintain an effective immune response over time.

As is demonstrated herein below, a method of the invention is advantageously used to assesses the CAR-T cells' ability to expand (proliferate) in response to antigen exposure, which is an essential property for effective treatment. Provided herein is a method and kit-of-parts making use of a (bead-bound) antigen that is targeted by a CAR expressed on e.g. a CAR-T cell, CAR-NK or CAR-macrophage. Exemplary CAR antigens include CD19, CD20, CD22, CD7, CD123, CLL1 (human C-type lectin-like molecule-

1), BCMA (B-cell maturation antigen), CD30, Mesothelin, HER2 (Human Epidermal Growth Factor Receptor 2), EGFRvIII (Epidermal Growth Factor Receptor variant III), Glypican-3 (GPC3), PSMA (Prostate-Specific Membrane Antigen), and any combination thereof. Herewith, the invention provides novel means and methods to measure the potency of a CAR-T drug product, e.g. as an alternative to the conventionally used (IFNγ) cytokine release and target cell killing assays as potency assay for T cell activation.

There is currently no standardized test available to assess CAR-T drug product potency. This issue is significant because each CAR-T cell receptor has a unique recognition domain, which means that developing a new potency assay is required for each product. These assays rely on specific individualized parameters, such as cytotoxicity or cytokine production when co-cultured with cancer cell lines. As a result, the variability between assays limits the ability to standardize and harmonize this essential quality control test, creating a major challenge for decentralized manufacturing of the same CAR-T cell product across multiple sites. Additionally, current T cell potency assays, which depend on cytokine production or cytolytic activity, are typically developed in-house and require labor-intensive co-culture activation steps. This makes them time-consuming, less sensitive, labor-intensive, and subject to high variability. Consequently, potency testing for the drug product (DP) can take over a day, with results often available only after CAR-T cells have already been infused into patients. To address this, a portion of the CAR-T cells is usually tested earlier in the production process, with potency determined on this sample—a practice known as drug substance testing. However, drug formulation buffers can affect CAR-T cell activity, and regulatory authorities strongly recommend potency testing on the final DP. Therefore, current potency testing for CAR-T therapies represents a suboptimal compromise given current technical constraints.

The ProliSpot assay for CAR-T cells (also referred to as ProliCART assay) according to the invention can overcome these drawbacks and can be standardized and validated according to Good Manufacturing Practices (GMP). Importantly, it is readily transferable to a Contract Manufacturing Organization (CMO) involved with the production and validation of CAR-T cells under GMP conditions, ensuring that products are consistently high-quality, safe, and effective. In addition, it is readily transferable to hospitals were the CAR-T drug product potency can be determined prior to administration to the patient. Whereas proliferation-based assays indicating CAR-T cell expansion have been used in the art (e.g. flow Cytometry-Based Tracking using proliferation dyes (e.g., CFSE) to track cell divisions after CAR-T cells encounter target cells; or mere BrdU or EdU incorporation to measure DNA synthesis), these assays are technically more complex and/or require access to a flow cytometer. Moreover, known assays do not reach the 0.01% or lower sensitivity of the ProliCART assay herein disclosed. Whereas this level of sensitivity may typically not be required for CAR-T drug product potency testing, it does allow for monitoring therapy efficacy using blood samples of patients that have undergone CAR-T therapy.

Safety monitoring is another critical reason to measure T cell activation. Overactive T cells can lead to cytokine release syndrome (CRS), a potentially severe inflammatory reaction due to excess cytokine production, such as IL-6 and IFN-γ. This overactivation can also cause immune-related toxicity by attacking healthy, non-cancerous cells, potentially leading to tissue damage and organ dysfunction. Monitoring activation markers helps clinicians identify and manage these risks early, making it possible to take preemptive steps to reduce adverse effects.

Furthermore, T cell activation and proliferation measurements can assist in assessing immune health and tracking treatment progress. In non-cancer applications, activation levels are advantageously used to monitor immune

responses in infections, autoimmune diseases, and other conditions. Abnormal activation levels may indicate disease progression, treatment response, or the need for therapeutic adjustments. In cancer, activation markers can serve as indicators of treatment success over time, with sustained T cell activation suggesting continued tumor surveillance.

This measurement is also valuable in personalizing immunotherapy. T cell activation profiles vary widely among individuals, influenced by both genetic and environmental factors. Tailoring treatment to each patient's unique immune response can make therapy safer and potentially more effective. By identifying patients with low activation potential or resistance to activation, clinicians can consider alternative therapies or CAR-T modifications to enhance activation.

Overall, the ability to measure (low fractions of) proliferating T cells is crucial in clinical settings to evaluate treatment effectiveness, improve safety, and/or personalize therapy. It provides insights into immune response, helps manage potential side effects, and optimizes treatment regimens, all of which contribute to better outcomes in cancer and immune-mediated diseases.

LEGEND TO THE FIGURES

Figure 1: Schematic presentation of an exemplary protocol according to the invention.

Figure 2: Combination of APTES or branched PEI with glutaraldehyde (GA) for concentration of the cells. A) Representative widefield fluorescence microscopy images of DAPI stained peripheral blood lymphocytes (PBLs) using a PEI or APTES coating for adhering and concentrating of the cells. Poly-L-lysine (PLL) and poly(allylamine) (PAA), were included as

comparative examples. Scale bar: 50 µm. Cells were seeded at a density of 80 cells per 100 μm^2 followed by extensive washing. B) Cell density measured based on DAPI.

Figure 3: Identification of activated T cells using ProliSpot. A) Representative widefield fluorescence microscopy images of PBLs treated for 3 days with CD3 | CD28 activation beads and incubated for 2h with the nucleoside analog EdU. The Azide-488 detection probe was coupled via click chemistry to EdU. PBLs were also stained for CD3 (Alexa-647) and the nuclei were stained with DAPI. Scale bar: 50 µm. B) 20,000 cells for each condition are segmented based on DAPI. Fluorescence intensity of EdU (Azide-488) is plotted for each cell. C) Percentage of EdU positive cells, calculated with the threshold indicated in panel B. Average shown for 3 experiments combined each 20,000 cells ± SEM.

Figure 4: High sensitivity of the EdU based protocol for measuring T cell proliferation. (A) Exemplary ProliSpot result. T cells were activated with anti-CD3 anti-CD28 beads (1 bead/25 cells). Arrowheads indicate dividing T cells with incorporated EdU (spot forming units). Scale bar: 100 µm. (B) T cells were transfected with mRNA coding for a specific T cell receptor and incubated with an MHC antigen from the cancer-specific protein NY-ESO-1 in the presence of EdU. (C-D) Determination of cytokines IFN-gamma and IL-2 by ELISA. Note the good correlation with the EdU incorporation.

Figure 5: ProliSpot in PEI/GA coated 96 wells format. Peripheral blood lymphocytes (PBLs) were stimulated for 24h with CD3/CD28 beads and loaded for 2h with EdU. Cells were diluted 1:1 with untreated cells and transferred to PEI/GA coated 96-wells glass bottom plate. Wells were mosaic imaged with wide field fluorescence microscopy (10x air objective). Nuclei are segmented using DAPI channel in the stitched image. From each nucleus the EdU-488 fluorescence intensity was measured.

Figure 6: Detection of the fraction of T cells recognizing the SARS-CoV-2 Spike proteins in blood samples of three healthy and CMV-negative donors by ProliSpot. PBLs of three healthy donors were stimulated for 24 h with a peptide library from the Spike protein. EDU was added during the last 2 h of the culturing, the cells were adhered to a PEI-coated glass surface, and the labelling was performed. Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified. For all three donors, T cells specific for SARS-CoV-2 spike protein were detected with high sensitivity.

Figure 7: ProliSpot using brightfield microscopy and conversion of the chromogen DAB. Compared to fluorescent labeling, chromogen labeling has the advantage that the identification of the cells does not require a fluorescence microscope but can be performed on a brightfield microscope. Anti-CD3 anti-CD28 bead-stimulated PBLs were conjugated to a PEI/GA coating and labeled with azide-PEG-biotin. The azide-PEG-biotin consists of an azide moiety that reacts with the alkyne moiety of EDU, a PEG linker, and a biotin. Next, the biotin was coupled to streptavidin conjugated to horseradish peroxidase (HRP). The sample was then incubated with the DAB chromogen and H_2O_2, resulting in the HRP mediated conversion of DAB into the brown precipitate polybenzimidazole. Note the dark nuclei in a fraction of the T cells. Arrows: beads used for activation of the T cells.

Figure 8: Human peripheral blood mononuclear cells (PBMCs) were stimulated with or without anti-CD3 and anti-CD28 beads for 4 days, incubated with EdU for 8, 6, 4, 2, 1 or 0 hours and stained with an e780 cell viability dye. Afterwards, EdU-containing cells were labeled with Eterneon2 GREEN 488 dye. The PBMCs were then analyzed by flow cytometry to determine: (A-B) The percentage of dead cells for each incubation period. EdU has only minor effects on T cell viability. Note that longer incubation

times of activated T cells result in increased T cell viability as the T cells proliferate. (C-D) The percentage of EdU containing cells for each incubation period. Note that unactivated T cells still showed 0.01-0.05% EdU positive T cells as flow cytometry is prone to false positives.

Figure 9: Strong correlation between FluoroSpot and ProliSpot. Detection of the fraction of T cells recognizing the SARS-CoV-2 Spike proteins in blood samples of five healthy donors by ProliSpot. PBLs of five healthy donors were stimulated for 24 h with a peptide library from the Spike protein. (A) Determination of Interferon gamma (IFN-γ) producing cells using a sandwich-based FluoroSpot assay with antibodies from Mabtech (cat. nrs. 3420-3-1000 and 3420-12-1000). (B) Determination of proliferating cells by ProliSpot. EDU was added during the last 2 h of cell culturing, the cells were adhered to a PEI-coated glass surface, and the labelling was performed. Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified. (C) Correlation of the percentages of IFN-γ and proliferating T cells from panels A and B by linear regression analysis. The percentages of IFN-γ correlated with the percentages of proliferating T cells.

Figure 10: Order of glutaraldehyde (GA) addition for cell concentration. 50,000, 100,000 or 200,000 human peripheral blood lymphocytes (PBLs) were seeded in a well of a 96-well plate coated with PEI followed by extensive washing. The PEI was either preactivated with GA (pretreatment) or GA was added simultaneously with the cells (together). Cell density was measured based on DAPI.

Figure 11: GA and centrifugation for effective concentration of the cells. 50,000, 100,000 or 200,000 human peripheral blood lymphocytes (PBLs) were seeded in a well of a 96-well plate coated with PEI followed by extensive washing. In the centrifugation conditions, the plate was

centrifuged for 5 min at 300g. Glutaraldehyde (GA) or paraformaldehyde (PFA) was added simultaneously with the cells. Cell density was measured based on DAPI.

Figure 12: Potency testing of CAR-T drug product using ProliSpot approach (ProliCART). CD19-directed CAR-T cells derived from three patients with B-Cell hematological malignancies were stimulated with control antigen or with the CD19 antigen for 24 hr at 37°C. EdU was added during the last 2 h of cell culturing. The cells were concentrated by covalent immobilization to a GA-activated PEI-coated glass surface, and the labelling of incorporated nucleoside analog was performed. Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified.

Figure 13: ProliSpot on complete blood samples. Shown is a ProliSpot experiment on 400 µl complete blood samples of a healthy blood donor. The complete blood sample was incubated with a peptide library from the Spike protein or control peptides for 24 hr at 37°C. EdU was added during the last 2 h of cell culturing. The red blood cells were removed by Ficoll density gradient centrifugation. The cells were adhered to a PEI-coated glass surface, and the EdU labelling was performed. Shown is the average with standard deviation from three repeats. Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified.

Figure 14: Comparison of different types of branched PEI for concentration of the cells. 500,000 human peripheral blood lymphocytes (PBLs) were seeded in an 8-well chamber slide coated with two different PEI forms (Sigma, P3143, Mn ~60,000; and Sigma, 408727, Mn 25,000) followed by centrifugation for 5 min at 300g and extensive washing. Glutaraldehyde (GA) was added simultaneously with the cells to activate the PEI coating and allow for covalent immobilization of the cells. Cell density was measured based on DAPI.

EXPERIMENTAL SECTION

Example 1: Covalent adhesion of cells to (poly) amine-coated surfaces using GA crosslinking.

This example demonstrates that glutaraldehyde (GA)-mediated covalent immobilization of cells to a branched PEI or APTES coating allows for concentrating and subsequent detection of the EdU-loaded cells. Two other polyamine compounds, poly-L-lysine (PLL), widely used compound for immobilization of suspension cells, and poly(allylamine) (PAA), were included as comparative examples.

Glass surfaces were coated with different polyamine compounds according to the following protocol.

APTES: a glass surface was plasma cleaned for 10 minutes and placed immediately for 10 seconds in 2 w/v% APTES (Sigma-440140) in acetone, washed once in acetone and stored under vacuum till use. Just prior to use, 1 % glutaraldehyde (GA) in PBS was added to the glass surface and incubated for 30 minutes at RT and washed five times with bidest H_2O.

PEI, PLL, PAA: solid glass was immersed in a coating solution of either 0.2 w/v % branched PEI (Sigma; p3143), 0.001% PLL (Sigma-Aldrich, p4707) or 0.02% PAA (Sigma-Aldrich, 479136), all in bidest H_2O for 24 hours. Solutions were removed and air dried. Just prior to use, 1 % glutaraldehyde (GA) in PBS was added to the glass surface and incubated for 30 minutes at RT and washed five times with bidest H_2O.

Peripheral blood leukocytes (PBLs) were activated, seeded and immobilized at a density of about 80 cells per 100 μm^2, fixed with formaldehyde, and subjected to the bio-orthogonal labeling reaction as follows: First, PBLs were

thawed, washed twice with PBS, pelleted at 300 ×g for 5 minutes and resuspended at 1.25×10^6 cells per ml in RPMI medium supplemented with 10% decomplemented human serum, 2 mM L-glutamine, 1 % antibiotic-antimycotic, IL-2 (Miltenyi Biotec,130-097-742) and Dynabeads CD3 | CD28 (Gibco, 11131D). 100,000 cells/well were cultured in a 24 wells plate for 24 hours at 37°C and 5% CO_2. The last 2 hours of stimulation, 5-EdU (Baseclick GmbH, BCK-EdUPro-FC488) was added to the cells at a 10 µM concentration. Next, the cells were pelleted at 300 ×g for 5 minutes and resuspended at 4×10^5 cells per ml in PBS. 200 µl of cell suspension was added to surface treated 0.13-0.16 mm diameter cover slips (Epredia, BC02600760a140mnz0) and incubated for 2 hours at 4 °C to let cells adhere to the coated surface. Chambers were washed twice with cold PBS and cells were fixed for 15 minutes with 4% formaldehyde in PBS at 4 °C.

Cells were permeabilized for 20 minutes in 1:10 saponin (Sigma, 47036-50G) buffer in PBS. The cells were then incubated for 40 minutes with the BCK-EdUPro-FC488 click assay cocktail which contained Eterneon2 GREEN 488 dye (BaseClick; BCK-EdUPro-FC488 kit). After incubation cells were washed once with saponin buffer.

Cells were labeled with 1 µg/ml DAPI (Sigma, 32670) in PBS and identified by automatic segmentation based on the DAPI signal. Covalent immobilization of the cells to the PEI or APTES coating substantially increased sensitivity, as the absence of glutaraldehyde resulted in a loss of ~75% of the cells (Figure 2). This loss could be largely prevented by glutaraldehyde fixation for PEI and APTES. In contrast, GA fixation could not prevent cell loss from surfaces coated with either PLL or PAA.

Figure 14 demonstrates that different types of branched PEI can be used for concentrating the cells. 500,000 human PBLs were seeded in an 8-well chamber slide (Ibidi; 80821) coated with either PEI with Mn ~60,000

(Sigma, P3143) or Mn ~25,000 (Sigma, 408727) followed by centrifugation for 5 min at 300g and extensive washing (5x bidest). 1% GA was added simultaneously with the cells and cell density was measured based on DAPI, as described above.

Example 2: Identification of activated T cells using EdU.

This example demonstrates that bead-activated T cells can be detected using ProliSpot, and that this can be multiplexed with antibody labelling (in this case for T cell marker CD3, using a spectrally-shifted fluorescent secondary antibody).

PBLs were activated with beads conjugated to antibodies raised against CD3 and CD28 (Gibco, 11131D) for 24 hours at 37°C and 5% CO_2. During the last 2 hours of stimulation, EdU was added to the cells at a 10 µM concentration. The cells were concentrated (to a density of about 250,000 cells/cm^2) by covalent immobilization toto an activated polyamine coated surface (PEI/GA) by centrifugation for 5 min at 300g and incubation for 2 hours at 4 °C. Following washing steps with cold PBS, the cells were fixed for 15 minutes with 4% formaldehyde in PBS at 4 °C. Afterwards, cells were washed extensively with PBS and incorporated nucleoside analog was stained (labeled) with Eterneon^2 GREEN 488 dye azide (BaseClick; BCK-EdUPro-. FC488; see example 1).

For CD3 labeling, the cells were blocked and permeabilized in 0.1 M PBS with 20 mM glycine + 3% BSA and 0.1% saponin pH 7.4 for 60 minutes at RT. Cells were incubated with rabbit polyclonal IgG against CD3 (Abcam, ab5690) at 1:200 diluted in the same buffer. Subsequently cells were washed three times in PBS and incubated with donkey anti-rabbit IgG (H+L) Alexa Fluor 647 (Invitrogen, A31571) at 1:800 dilution. Nuclei were stained with DAPI in PBS.

EdU labeling was highly efficient, and EdU-positive cells showed a more than 10-fold increase in fluorescence compared to the non-stimulated control (Figure 3, Figure 4A). Moreover, all dividing cells were positively labeled for CD3, confirming that they were T cells.

Figure 4B demonstrates that activation of T cells expressing specific T cell receptors can also be measured accurately with ProliSpot and that the production of cytokines IFN-gamma and IL-2 detected by conventional ELISA correlates well with EDU positive cells. T cells were transfected with mRNA coding for a specific T cell receptor and incubated with an MHC antigen from the cancer-specific protein NY-ESO-1 and EdU.

Example 3: ProliSpot in 96-well plate format.

Bead activated PBLs (see Example 2) were diluted 10-fold with unstimulated cells and concentrated by covalent immobilization to a PEI-coated glass bottom 96-well plate activated with GA. Click labelling was performed as described for Example 1 above. Cells were imaged in PBS with DAPI. Using FIJI, the cells were quantified using segmentation of the DAPI stained nuclei. Figure 5 demonstrates that ProliSpot can be performed in a 96-well plate format.

For measuring the percentage of EdU positive cells, the wells were tile imaged. Stitched images were used for counting the number of DAPI positive spots and EDU-488 positive spots set on a threshold based on the background signal in unstained cells. The EDU positive (i.e. dividing/proliferating) T cells could be detected with a sensitivity of less than 0.01% (e.g. <5 cells / 50,000 cells).

Example 4: Sensitive detection of SARS-CoV-2 positive cells in blood samples.

This example demonstrates that ProliSpot can be used to determine the fraction of T cells recognizing the SARS-CoV-2 Spike proteins in blood samples of healthy donors. Since the large majority of the adult population is vaccinated against SARS-CoV-2, resulting in 0.01 – 0.1% activated T cells within blood samples, this is a suitable system for further validation of the ProliSpot technique.

PBLs of three random healthy CMV-negative donors were stimulated for 24 h with a peptide library from the Spike protein (PepTivator SARS-CoV-2 130-127-951 (Milteny) or PepTivator CMV pp65 130-093-438 (Milteny) in a 96-wells plate (SensoPlate, 96w, F, glassbottom, Greiner, 655892). EdU was added during the last 2 h of the culturing, the cells were concentrated by covalent linkage to a PEI-coated surface activated with GA, and the bioorthogonal labelling was performed. Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified. For all three donors, we could detect T cells specific for SARS-CoV-2 spike protein with high sensitivity (Figure 6).

In addition, it was found that the ProliSpot assay can also be used to determine the fraction of T cells recognizing the SARS-CoV-2 Spike proteins in complete blood samples of healthy donors. 400 µl blood samples of a random healthy donor were stimulated for 24 h with a peptide library from the Spike protein (PepTivator SARS-CoV-2 130-127-951 (Milteny)) or without antigen in a 96-wells plate. EdU was added during the last 2 h of the culturing.

The red blood cells were removed by Ficoll density gradient centrifugation as follows. The blood samples were mixed with 1600 µl PBS + 5mM EDTA. The suspension was transferred slowly by pipetting on top of 1 ml Ficoll

(Lymphoprep). The cells were collected at room temperature, 800 g for 22 min (without brakes). The peripheral blood lymphocytes (PBLs; white ring of cells) were collected into a fresh 15 ml tube and diluted with PBS + 5mM EDTA to a final volume of 15 ml. Cells were collected by centrifugation at room temperature for 10 min at 300g. Cells were washed three times with PBS + 5mM EDTA + 0.1% BSA and resuspended in RPMI with 10% FBS, 1% antibiotic-antimycotic and 50 IU/ml IL-2 to a final concentration of 2.5 x 10^6 cells/ml.

250,000 cells were added per well of a flat bottom 96-wells plate with PEI-coating and preactivation with GA, and the bio-orthogonal labelling was performed (see example 1). Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified. We could detect T cells specific for SARS-CoV-2 spike protein with high sensitivity (Figure 13).

Example 5: ProliSpot based on azide-coupled enzyme detection probe.

This example describes the ProliSpot concept based on the detection of peroxidase-mediated conversion of DAB instead of fluorescence. The advantage is that any cheap brightfield microscope can be used. Bead-stimulated PBLs were concentrated by immobilization to a PEI/GA coating and labeled with azide-PEG-biotin. Attaching horseradish peroxidase (HRP) to the EdU was performed as described for Figure 2-3, except that the Eterneon2 Green 488 detection probe was replaced by 10 mM Azide-PEG3-biotin. After the click reaction, the cells were incubated for 30 minutes in PBS with 20 mM glycine + 3% BSA. Endogenous HRP was blocked by adding 1% H_2O_2 in PBS for 10 minutes. EDU was further labeled by attaching streptavidin-HRP 1:1000 in PBS with 20 mM glycine + 3%

BSA. Cells were incubated in 2 mg/ml DAB and 0.03 % H_2O_2 in PBS until a DAB precipitate was visible (~10 minutes).
Compared to fluorescent labeling, chromogen labeling has the advantage that the identification of the cells does not require a fluorescence microscope but can be performed on a brightfield microscope, as EdU positive cells show a dark nucleus (Figure 7).

Example 6: No effect of nucleoside labeling on T cell viability.

Figure 8 demonstrates that (1) labeling T cells with nucleoside analog (EdU) does not affect T cell viability, (2) incubation for 1 hr with nucleoside analog suffices for labeling, and (3) flow cytometry gives a relatively high background (false positives).

PBLs were stimulated with or without anti-CD3 and anti-CD28 beads (Gibco, 11131D) for 4 days, incubated with EdU for 8, 6, 4, 2, 1 or 0 hours and stained with an e780 cell viability dye (ThermoFisher, 65-0865-14). Afterwards, EdU containing cells were labeled with Eterneon2 GREEN 488 dye. The PBLs were then analyzed by flow cytometry.

EdU only has minor effects on T cell viability as cell death did not increase for longer incubation periods. In fact, longer incubation times of activated T cells seemingly resulted in increased T cell viability as the T cells proliferate.
Incubation for 1 hr with EdU suffices for labeling, because the percentage of EdU-positive cells only marginally increased for prolonged labeling times. Flow cytometry gives a relatively high background, because unactivated T cells still showed 0.01-0.05% EdU positive T cells and therefore flow cytometry is prone to false positives.

Example 7: Performance of ProliSpot.

This example describes a side-by-side comparison of ProliSpot with a commercial FluoroSpot kit for IFN-γ for five additional healthy donors. EDUspot was performed as described in Example 4.

For FluoroSpot, 2.5×10^6 cells/ml in RPMI medium supplemented with 10% decomplemented human serum, 2 mM L-glutamine, 1% antibiotic-antimycotic, IL-2 (Miltenyi Biotec,130-097-742) and 0.12 nM SARS-CoV-2 spike protein peptide library (Milteny, 130-127-951) in 100 µl was added to a multiscreen HTS IP filter plate (Millipore, S5EJ104I07) coated with α-IFN-γ Mab-1-D1K (MabTech, 3420-3-1000; 5 µg/ml in PBS) and cultured for 24 hours at 37°C and 5% CO2. Subsequently, the back covers from the multiscreen HPS IP filter plates were removed and filters were washed at both sides 5 times with PBS + 0.05% Tween.

The back covers were placed back and the samples were labeled with the IFN-γ detection antibody Mab-7-B6-1, BAM (MabTech, 3420-12-1000) diluted 1:400 in PBS + 0.1% BSA for 2 hours at room temperature. The back covers were removed and filters were washed from both sides 5 times with PBS + 0.05% Tween. The back covers were placed back and α-BAM mAb-490 (MabTech, 3640-2-1000) was added 1:600 in PBS + 0.1% BSA and incubated for 1 hour in the dark. The back covers from the multiscreen HPS IP filter plates were removed and the filters were washed both sides 2 times with PBS + 0.05% Tween and twice with PBS. The back covers are placed back and filters were incubated for 15 minutes with Fluorescence enhancer II (MabTech, 3641-F10). The wells were emptied and covers were removed and dried for 24 hours before detection with a FluoroSpot reader (Autoimmun Diagnostika, Strassberg, Germany).

As shown in Figure 9, the percentages of SARS-CoV-2 spike protein recognizing T cells detected by ProliSpot (i.e. proliferating T cells) correlate very well with the percentages of IFN-γ producing T cells as determined using FluoroSpot.

Example 8: Activation with GA.

This example demonstrates that a polyamine coating can be either preactivated with glutaraldehyde (GA), or GA can be added to the polyamine-coated solid support simultaneously with the cells.

96-well glass bottom plates were coated with PEI according to Example 1.

For the "preactivation" condition, the polyamine-coating was incubated with 1% GA for 30 min. Peripheral blood leukocytes (PBLs) were activated, seeded and covalently immobilized at 50,000, 100,000 or 200,000 cells per well. For the "simultaneous" condition, 1% GA was added together with the cells.

The plates were centrifuged for 5 min at 300g for concentrating the cells. Cells were then subjected to the bio-orthogonal labeling reaction as in Example 1. Cells were labeled with 1 µg/ml DAPI (Sigma, 32670) in PBS and identified by automatic segmentation based on the DAPI signal. As is shown in Figure 10, the order of activation with GA did not affect the extent of cell concentration.

Example 9: Role of GA and effect of centrifugation.

This example demonstrates that centrifugation in conjunction with GA-mediated covalent immobilization of cells to a PEI or APTES coating allows for concentrating and subsequent detection of the nucleoside-labeled cells. Paraformaldehyde (PFA), another widely used compound for cell fixation, was included as comparative example.

96-well glass bottom plates were coated with PEI according to Example 1. Peripheral blood leukocytes (PBLs) were activated, seeded, centrifuged (5 min 300g) and immobilized at 50,000, 100,000 or 200,000 cells per well, fixed with 1% GA or 4% PFA for 10 min, and subjected to the bio-orthogonal labeling reaction as in Example 1. Cells were labeled with 1 µg/ml DAPI (Sigma, 32670) in PBS and identified by automatic

segmentation based on the DAPI signal. Centrifugation and GA-mediated covalent immobilization of the cells onto the PEI coating substantially increased sensitivity (Figure 11). In contrast, PFA fixation could not prevent cell loss.

Example 10: Measuring potency of CAR-T cells (ProliCART).

200,000 CD19-directed CAR-T cells that were derived from three patients with B-cell hematological malignancies were cultured in a flat bottom 96-wells plate in RPMI with 10% FBS, 1% antibiotic-antimycotic and 50 IU/ml interleukin-2. The cells were stimulated with uncoated beads (control antigen) or beads coated with CD19 for 24 hr at 37°C. EdU was added during the last 2 h of cell culturing. The cells were transferred to a 96 wells V bottom plate and washed 2x with PBS. The cells were concentrated by centrifugation (5 min 300g) and covalent immobilization to a PEI-coated 96-well plate (SensoPlate, 96w, F, glass bottom, Greiner, 655892) preactivated with GA, and the bioorthogonal labelling was performed (see example 1). Cells were segmented based on the DAPI signal, and the EdU-positive cells were automatically identified. For all three patients, stimulation with the CAR-T cell receptor antigen CD19 resulted in increased EdU incorporation, reflecting antigen-induced proliferation (Figure 12).

REFERENCES

1. Davis, N. Does Covid immunity wane and will vaccine booster jabs be needed? *The Guardian* 25 Aug (2021).

2. Jalkanen, P. *et al.* COVID-19 mRNA vaccine induced antibody responses against three SARS-CoV-2 variants. *Nat. Commun.* **12**, 3991 (2021).

3. Garcia-Beltran, W. F. *et al.* mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant. *Cell* **185**, 457–466 (2022).

4. Barros-Martins, J. *et al.* Immune responses against SARS-CoV-2 variants after heterologous and homologous ChAdOx1 nCoV-19/BNT162b2 vaccination. *Nat. Med.* **27**, 1525–1529 (2021).

5. Angyal, A. *et al.* T-cell and antibody responses to first BNT162b2 vaccine dose in previously infected and SARS-CoV-2-naive UK health-care workers: a multicentre prospective cohort study. *The Lancet. Microbe* **3**, e21–e31 (2022).

6. Lyons, A. B. & Parish, C. R. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* **171**, 131–137 (1994).

7. Gratzner, H. G. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* **218**, 474–475 (1982).

8. Karlsson, A. C., *et al.* Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *J Immunol Methods*. **283**, 141–153 (2003).

9. Magbanua, M. J. M., *et al.* Expanded Genomic Profiling of Circulating Tumor Cells in Metastatic Breast Cancer Patients to Assess Biomarker Status and Biology Over Time (CALGB 40502 and CALGB 40503, Alliance). *Clin Cancer Res.* **24**, 1486–1499 (2018).

10. Stempels, F. C. *et al.* A sensitive and less cytotoxic assay for identification of proliferating T cells based on bioorthogonally-functionalized uridine analogue. *J. Immunol. Methods* **502**, 113228 (2022).

11. Sun, Y., *et al.* Multicolor flow cytometry analysis of the proliferations of T-lymphocyte subsets in vitro by EdU incorporation. *Cytometry A.* **81**, 901–909 (2012).

12. Kuddannaya, S., Chuah, Y. J., Lee, M. H., Menon, N. V., Kang, Y., Zhang, Y. Surface chemical modification of poly(dimethylsiloxane) for the enhanced adhesion and proliferation of mesenchymal stem cells. *ACS Appl Mater Interfaces.* **5**, 9777–9784 (2013).

Claims

1. An *in vitro* method for detecting proliferating cells, comprising the steps of:

(i) culturing cells in the presence of a nucleoside analog comprising a first reactive unsaturated group to allow for incorporation of the nucleoside analog in the DNA of the cells;

(ii) concentrating the nucleoside-labeled cells by covalent immobilization to a solid surface comprising a coating of (3-aminopropyl)triethoxysilane (APTES) or branched polyethyleneimine (PEI) that is activated by glutaraldehyde (GA);

(iii) optionally fixing the concentrated and covalently immobilized cells with formaldehyde;

(iv) contacting the covalently immobilized cells with a detection probe comprising a second reactive unsaturated group, such that a [3+2] or [4+2] cycloaddition occurs between the first and second reactive unsaturated groups; and

(v) determining the amount of detection probe attached to the DNA of the immobilized cells to measure cellular proliferation.

2. The method according to claim 1, wherein either:

a) the first reactive unsaturated group comprises an azide group and the second reactive unsaturated group comprises an alkynyl group, or

b) the first reactive unsaturated group comprises an alkynyl group and the second reactive unsaturated group comprises an azide group.

3. The method according to claim 2, wherein the nucleoside analog is selected from the group consisting of 5-ethynyl-2'deoxyuracil (EdU), 5-ethynyl-2'-deoxycytidine (5-EdC), 5-azido-2'-deoxyuracil (5-AdU) 5-Azidomethyl-2'-deoxyuridine (5-AmdU) and 5-vinyl-2'-deoxyuridine (5-VdU),

as well as their triphosphate and phosphoramidite forms, preferably wherein the nucleoside analog is EdU.

4. The method according to any one of the preceding claims, wherein the nucleoside analog is EdU and wherein the detection probe is an azide-coupled detection probe, preferably an azide-coupled fluorophore or an azide-coupled enzyme, more preferably azide-coupled HRP.

5. The method according to any one of the preceding claims, wherein step (ii) comprises concentrating nucleoside-labeled cells to a density of at least 40 cells per 100 μm^2, preferably at least 60 cells per 100 μm^2, more preferably at least 80 cells per 100 μm^2.

6. The method according to any one of the preceding claims, wherein step (ii) comprises concentrating nucleoside-labeled cells at a density of at least 20,000 cells per well, preferably at least 40,000, more preferably at least 60,000 cells, most preferably at least 80,000 cells per well of a 96-well plate.

7. The method according to any one of the preceding claims, comprising the culturing and labeling of T cells, cancer cells or stem cells, preferably T cells.

8. The method according to claim 7, comprising culturing and labeling of T cells in the presence of an antigen of interest, preferably a tumor antigen or a pathogenic antigen, such as a cytomegalovirus (CMV), influenza, or SARS-CoV-2 antigen.

9. The method according to any one of the preceding claims, wherein step (i) comprises culturing and labeling cells expressing a chimeric antigen receptor (CAR) in the presence of a CAR antigen, preferably wherein the CAR-expressing cells are CAR-T cells, more preferably wherein the CAR-T cells are labeled in the presence of an antigen selected from the group consisting of CD19, CD20, CD22, BCMA (B-cell maturation antigen), CD30,

CD7, CD123, CLL1 (human C-type lectin-like molecule-1), Mesothelin, HER2 (Human Epidermal Growth Factor Receptor 2), EGFRvIII (Epidermal Growth Factor Receptor variant III), Glypican-3 (GPC3), PSMA (Prostate-Specific Membrane Antigen), and any combination thereof.

10. The method according to any one of the preceding claims, wherein the solid surface is a transparent solid surface, preferably the surface of a glass or plastic support, more preferably a microscope glass slide or a flat-bottom multi-well plate.

11. The method according to any one of the preceding claims, wherein step (iv) further comprises contacting the cells with one or more additional detection reagent(s), preferably one or more spectrally-shifted reagent(s), such as a DNA-specific probe and/or a fluorochrome-conjugated antibody.

12. The method according to any one of the preceding claims, wherein step (v) comprises the use of an optics-based readout system.

13. A kit-of-parts for *in vitro* detection of proliferating cells, comprising

- a first container comprising (3-aminopropyl)triethoxysilane (APTES) or a branched polyethyleneimine (PEI), preferably branched PEI;
- a second container comprising a nucleoside analog comprising a first reactive unsaturated group, preferably 5-ethynyl-2′-deoxyuridine (EdU);
- a third container comprising a detection probe comprising a second reactive unsaturated group which can undergo a [3+2] or [4+2] cycloaddition reaction with the first reactive group;
- a fourth container comprising glutaraldehyde (GA); and
- at least one antigen of interest,
- optionally an additional container containing an antioxidant, preferably a salt of L-ascorbate.

14. The kit-of-parts according to claim 13, comprising a tumor antigen or a pathogenic antigen, or antigenic fragment thereof.

15. The kit-of-parts according to claim 13, comprising a viral antigen, preferably an antigen selected from the group of CMV antigens, SARS-CoV-2 antigens and influenza antigens.

16. The kit-of-parts according to claim 13, comprising an antigen recognized by a chimeric antigen receptor (CAR), preferably an antigen selected from the group consisting of CD19, CD20, CD22, CD7, BCMA (B-cell maturation antigen), CD30, CD123, CLL1 (human C-type lectin-like molecule-1), Mesothelin, HER2 (Human Epidermal Growth Factor Receptor 2), EGFRvIII (Epidermal Growth Factor Receptor variant III), Glypican-3 (GPC3), PSMA (Prostate-Specific Membrane Antigen), and any combination thereof.

17. Kit-of-parts according to any one of claims 13-16, wherein either:
a) the nucleoside analog comprises an azide group and the detection probe comprises an alkynyl group, or
b) the nucleoside analog comprises an alkynyl group and the detection probe comprises an azide group.

18. Kit of parts according to claim 17, wherein the nucleoside analog is 5-ethynyl-2'deoxyuracil (EdU) or 5-ethynyl-2'-deoxycytidine (5-EdC).

19. Kit of parts according to any one of claims 13-18, wherein the detection probe is an azide-coupled detection probe, preferably an azide-coupled fluorophore or an azide-coupled enzyme, more preferably azide-coupled HRP.

20. Kit of parts according to any one of claims 13-19, further comprising a carrier for receiving cells, preferably wherein the carrier comprises a coated glass or plastic surface, more preferably wherein the carrier is a multiwell plate, most preferably a flat-bottom, optically transparent multi-well plate.

21. The use of a method according to any one of claims 1-12 or a kit according to any one of claims 13-20 in the detection of rare circulating (MRD) cancer cells, for stem cell research, for determining T cell responses in allergies, or for monitoring the efficacy of a vaccine.

22. The use of a method according to claim 9 or a kit according to claim 16 for monitoring the efficacy of CAR-T cell therapy or for determining the therapeutic potential and/or safety of a CAR-T cell drug product.

Fig. 1

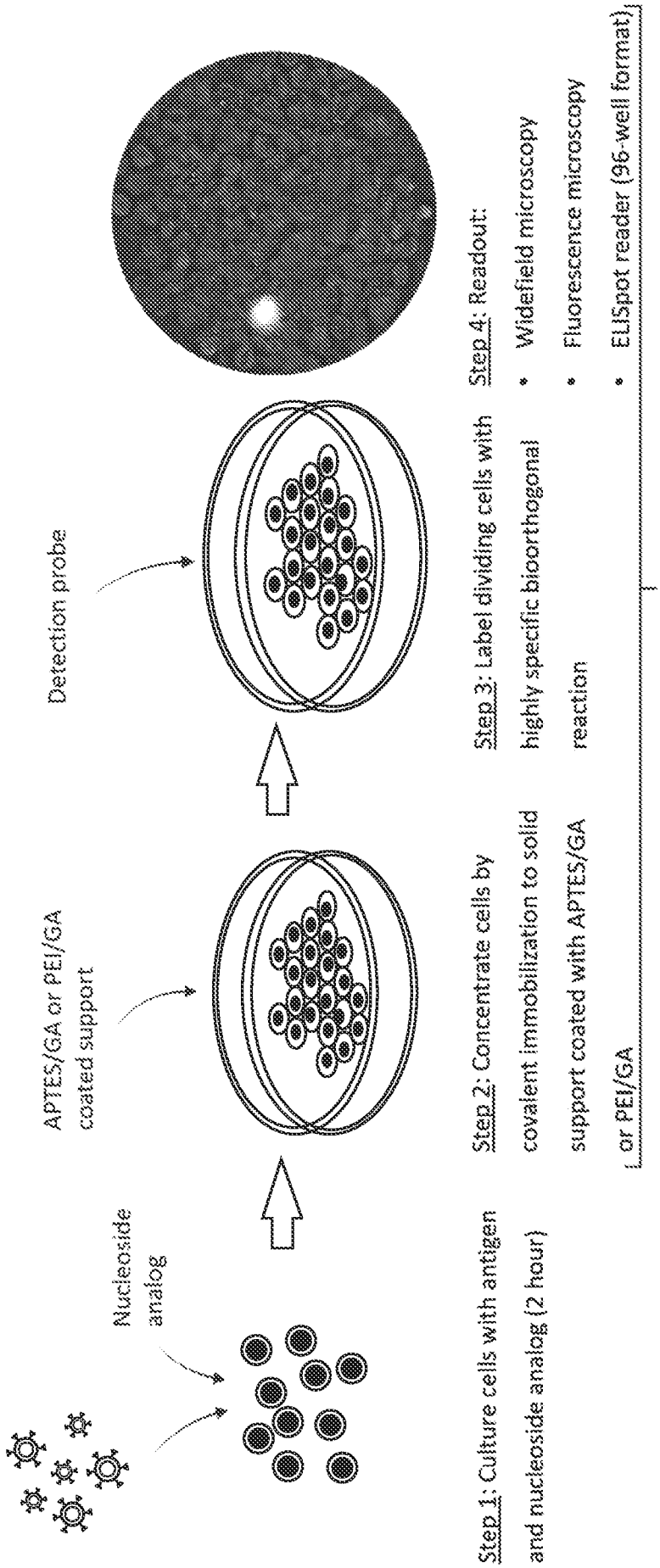
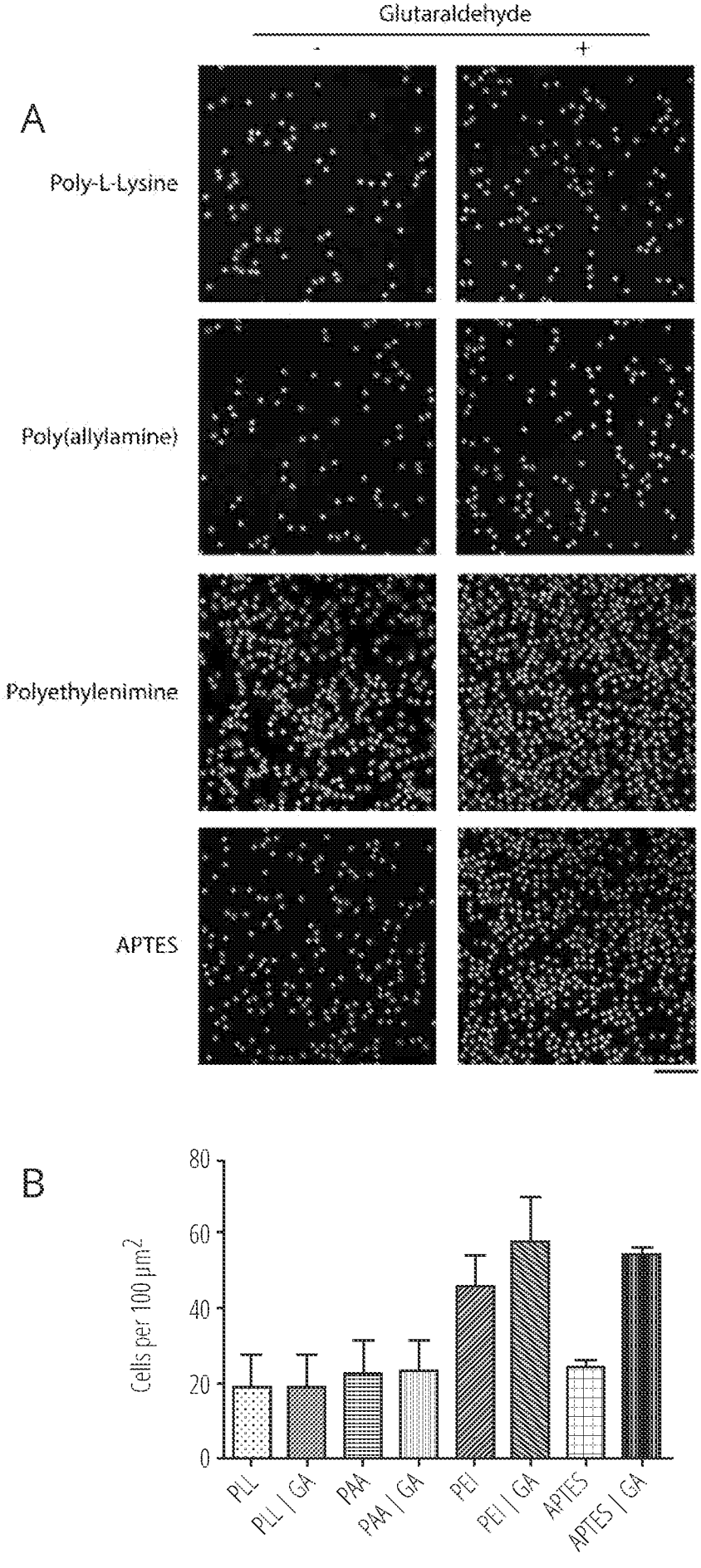

Nucleoside analog
APTES/GA or PEI/GA coated support
Detection probe
Step 1: Culture cells with antigen and nucleoside analog (2 hour)
Step 2: Concentrate cells by covalent immobilization to solid support coated with APTES/GA or PEI/GA
Step 3: Label dividing cells with highly specific bioorthogonal reaction
Step 4: Readout:
• Widefield microscopy
• Fluorescence microscopy
• ELISpot reader (96-well format)
3 hour

Fig. 2


Glutaraldehyde
-
+
A
Poly-L-Lysine
Poly(allylamine)
Polyethylenimine
APTES
B
Cells per 100 µm²
0
20
40
60
80
PLL
PLL | GA
PAA
PAA | GA
PEI
PEI | GA
APTES
APTES | GA

3/14
Fig. 3

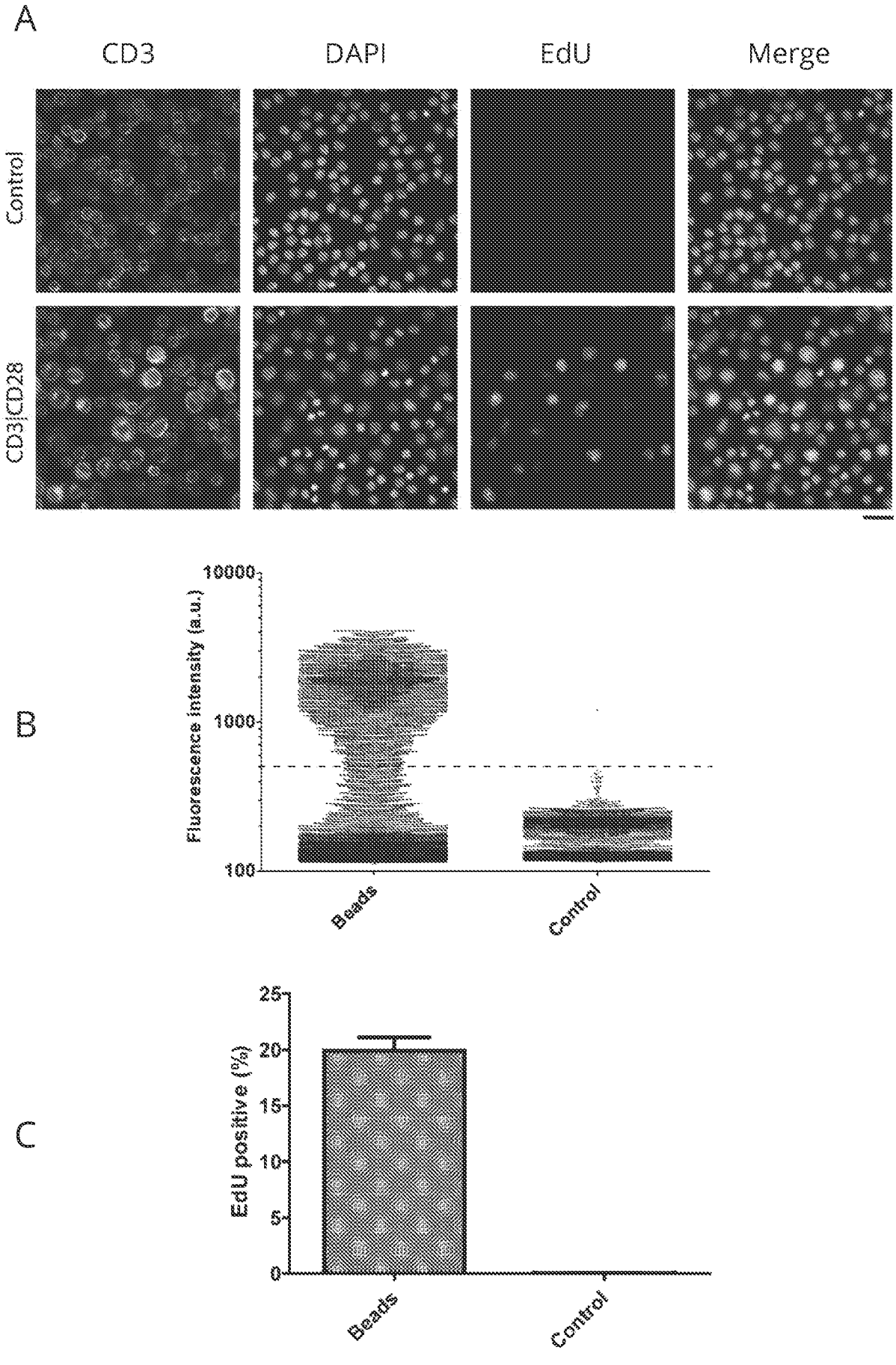

A
CD3
DAPI
EdU
Merge
Control
CD3|CD28
B
Fluorescence intensity (a.u.)
10000
1000
100
Beads
Control
C
EdU positive (%)
25
20
15
10
5
0
Beads
Control

Fig. 4

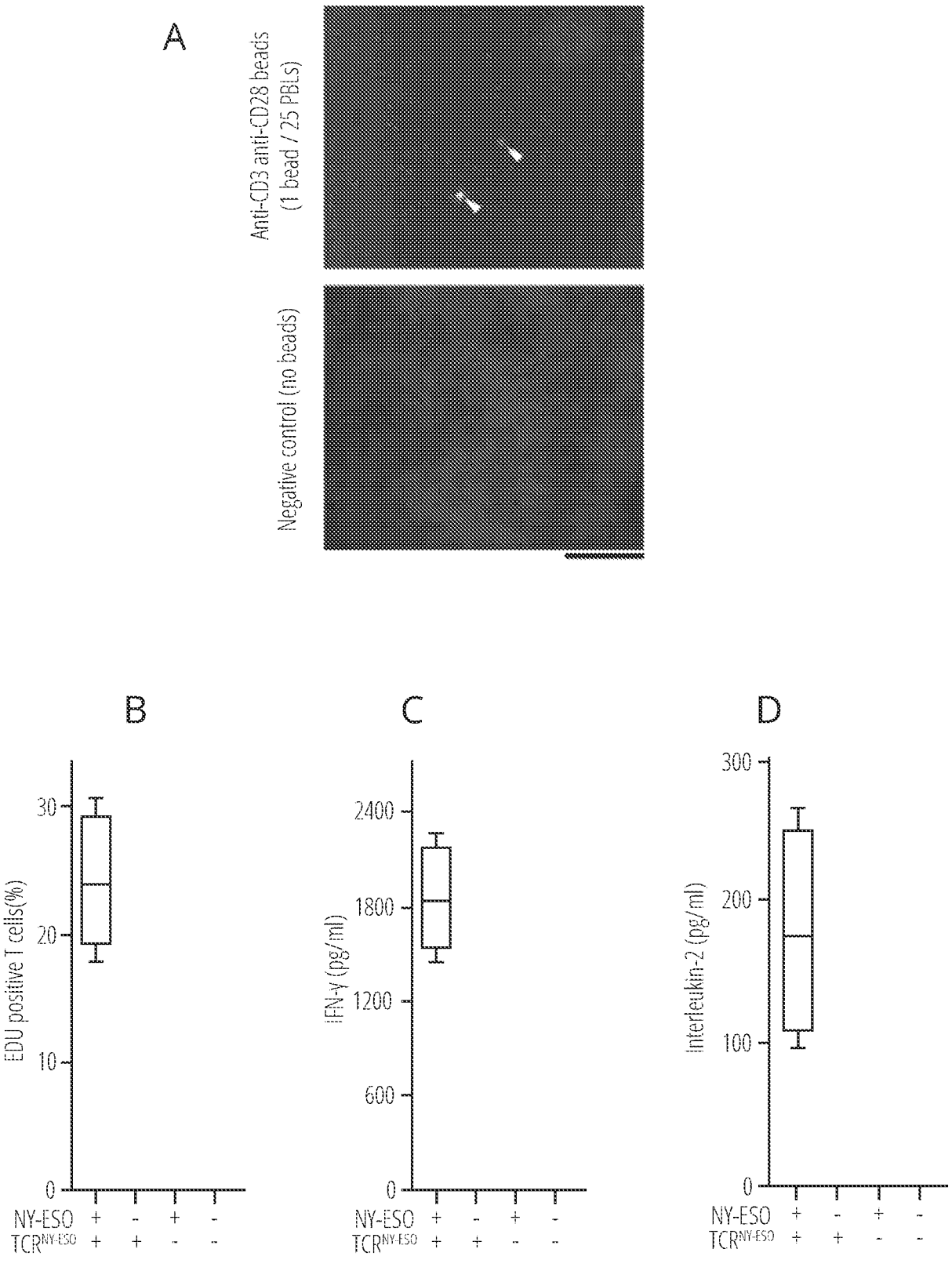

A
Anti-CD3 anti-CD28 beads
(1 bead / 25 PBLs)
Negative control (no beads)
B
EDU positive T cells(%)
30
20
10
0
NY-ESO + - + -
TCR^NY-ESO + + - -
C
IFN-γ (pg/ml)
2400
1800
1200
600
0
NY-ESO + - + -
TCR^NY-ESO + + - -
D
Interleukin-2 (pg/ml)
300
200
100
0
NY-ESO + - + -
TCR^NY-ESO + + - -

Fig. 5

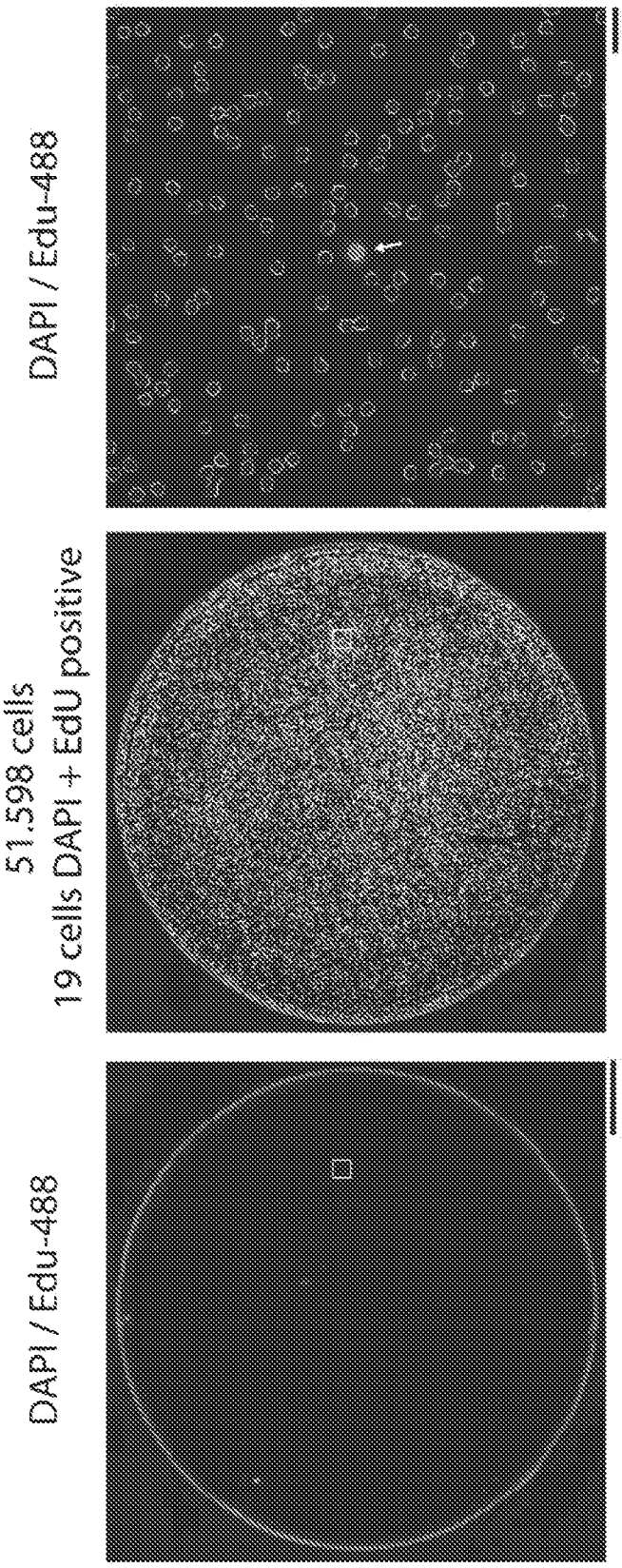
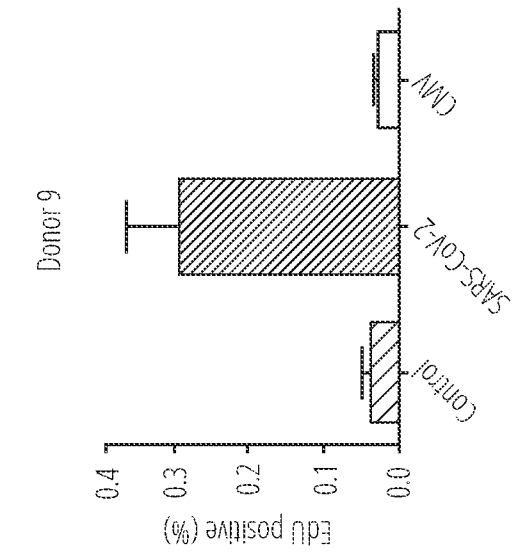
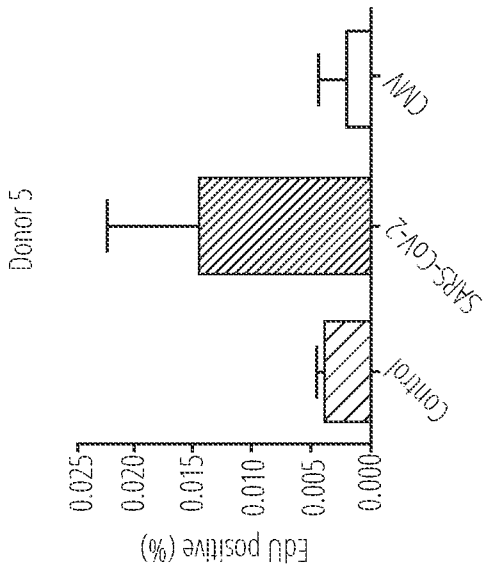

DAPI / Edu-488
51.598 cells
19 cells DAPI + EdU positive
DAPI / Edu-488

Fig. 6


Donor 9
Control
SARS-CoV-2
CMV
0.4
0.3
0.2
0.1
0.0
EdU positive (%)


Donor 5
Control
SARS-CoV-2
CMV
0.025
0.020
0.015
0.010
0.005
0.000
EdU positive (%)

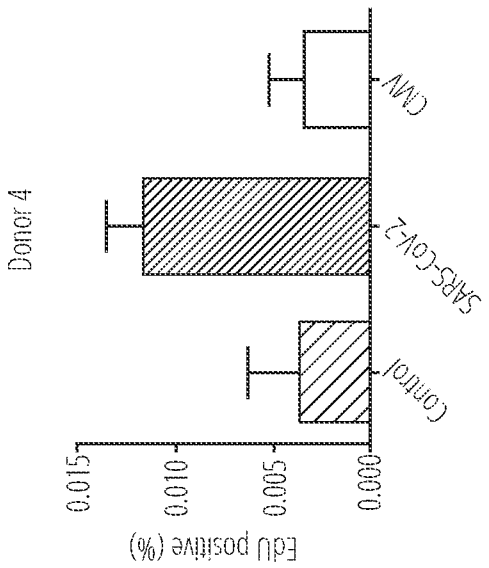

Donor 4
Control
SARS-CoV-2
CMV
0.015
0.010
0.005
0.000
EdU positive (%)

Fig. 7

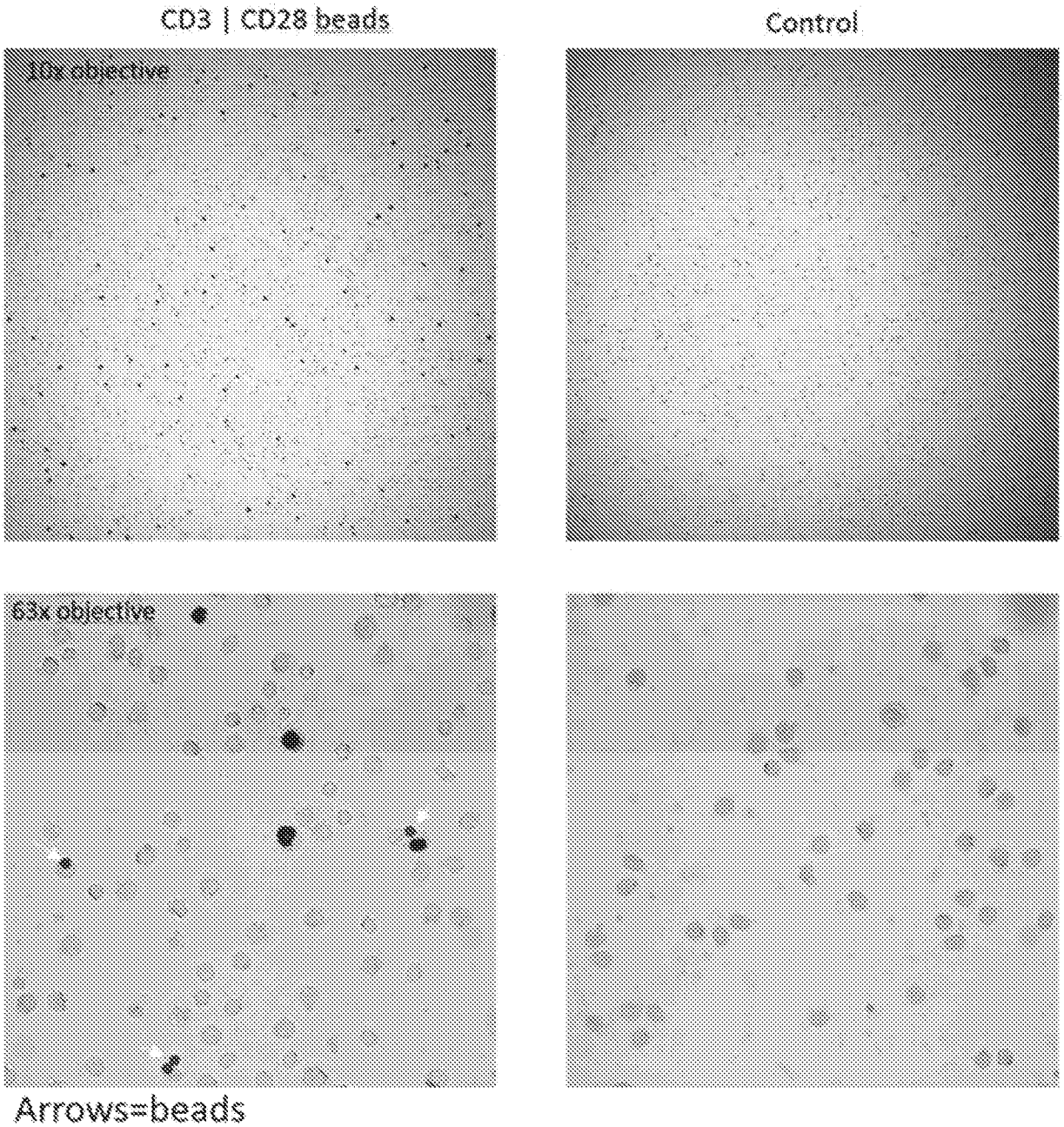

CD3 | CD28 beads
Control
10x objective
63x objective
Arrows=beads

Fig. 8

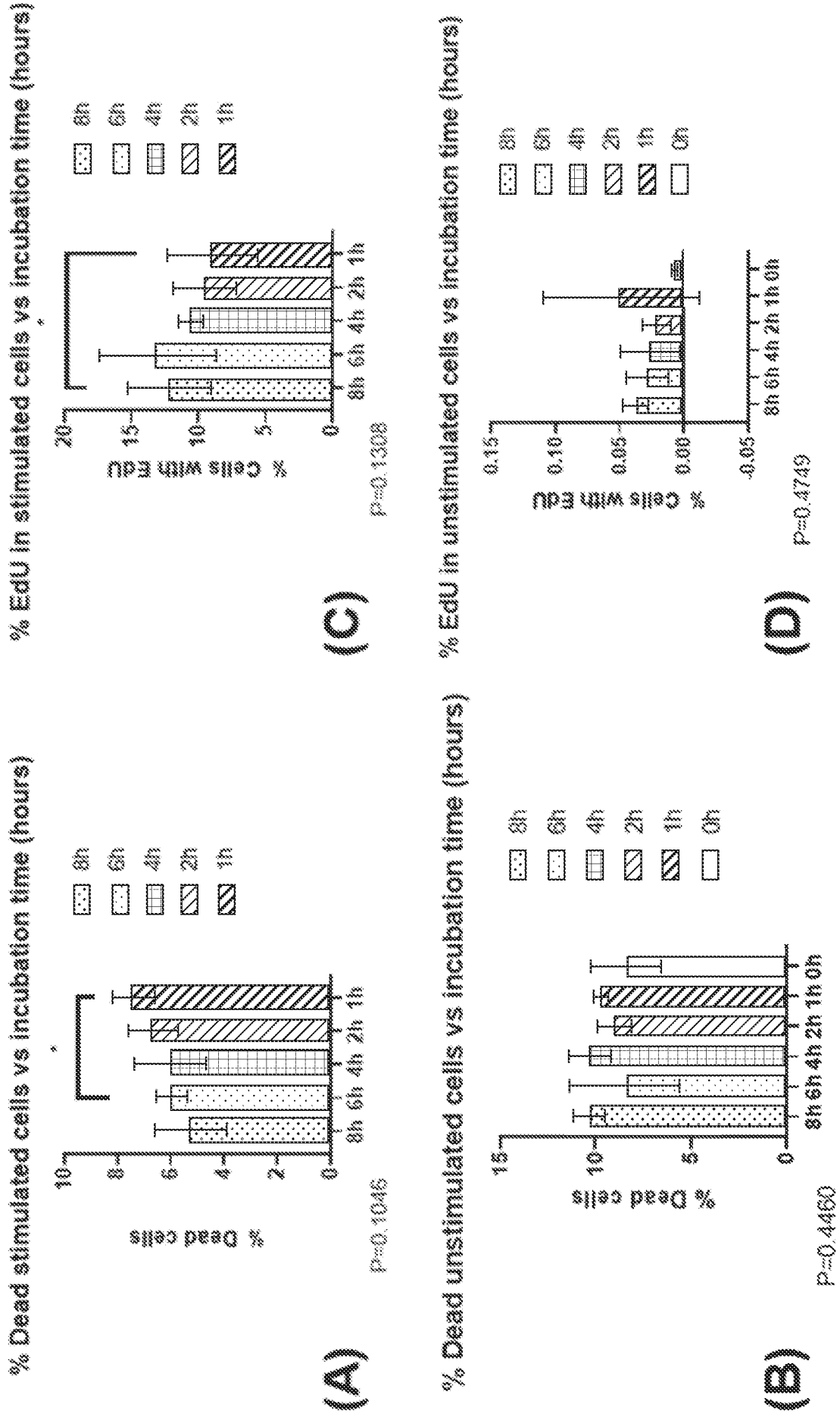

% Dead stimulated cells vs incubation time (hours)
8h 6h 4h 2h 1h
% Dead cells
10 8 6 4 2 0
8h 6h 4h 2h 1h
P=0.1045
(A)
% Dead unstimulated cells vs incubation time (hours)
8h 6h 4h 2h 1h 0h
% Dead cells
15 10 5 0
8h 6h 4h 2h 1h 0h
P=0.4460
(B)
% EdU in stimulated cells vs incubation time (hours)
8h 6h 4h 2h 1h
% cells with EdU
20 15 10 5 0
8h 6h 4h 2h 1h
P=0.1308
(C)
% EdU in unstimulated cells vs incubation time (hours)
8h 6h 4h 2h 1h 0h
% cells with EdU
0.15 0.10 0.05 0.00 -0.05
8h 6h 4h 2h 1h 0h
P=0.4749
(D)

Fig. 9

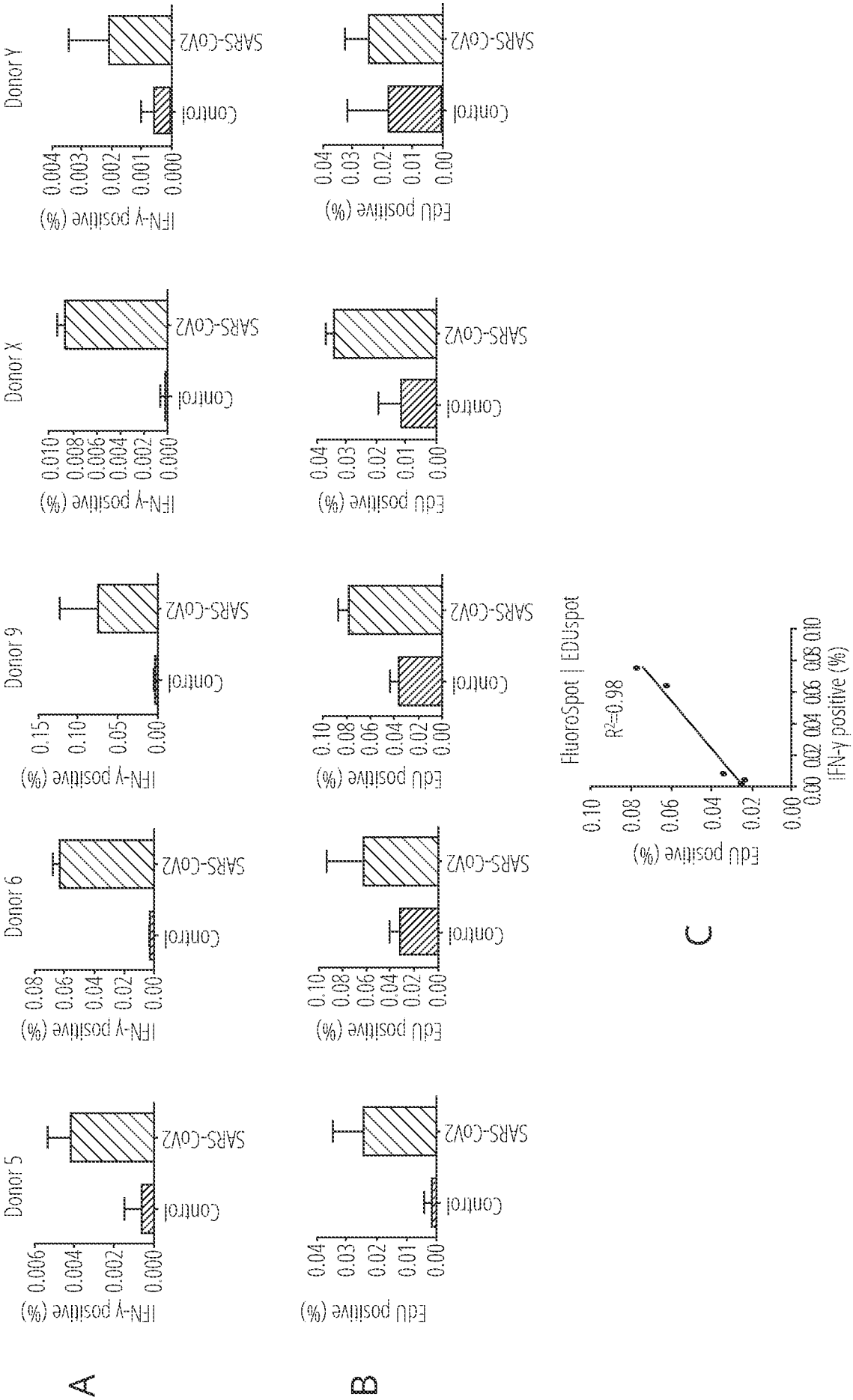

A
B
C
Donor 5
Donor 6
Donor 9
Donor X
Donor Y
IFN-γ positive (%)
EdU positive (%)
Control
SARS-CoV2
FluoroSpot | EDUspot
R²=0.98
IFN-γ positive (%)

Fig. 10

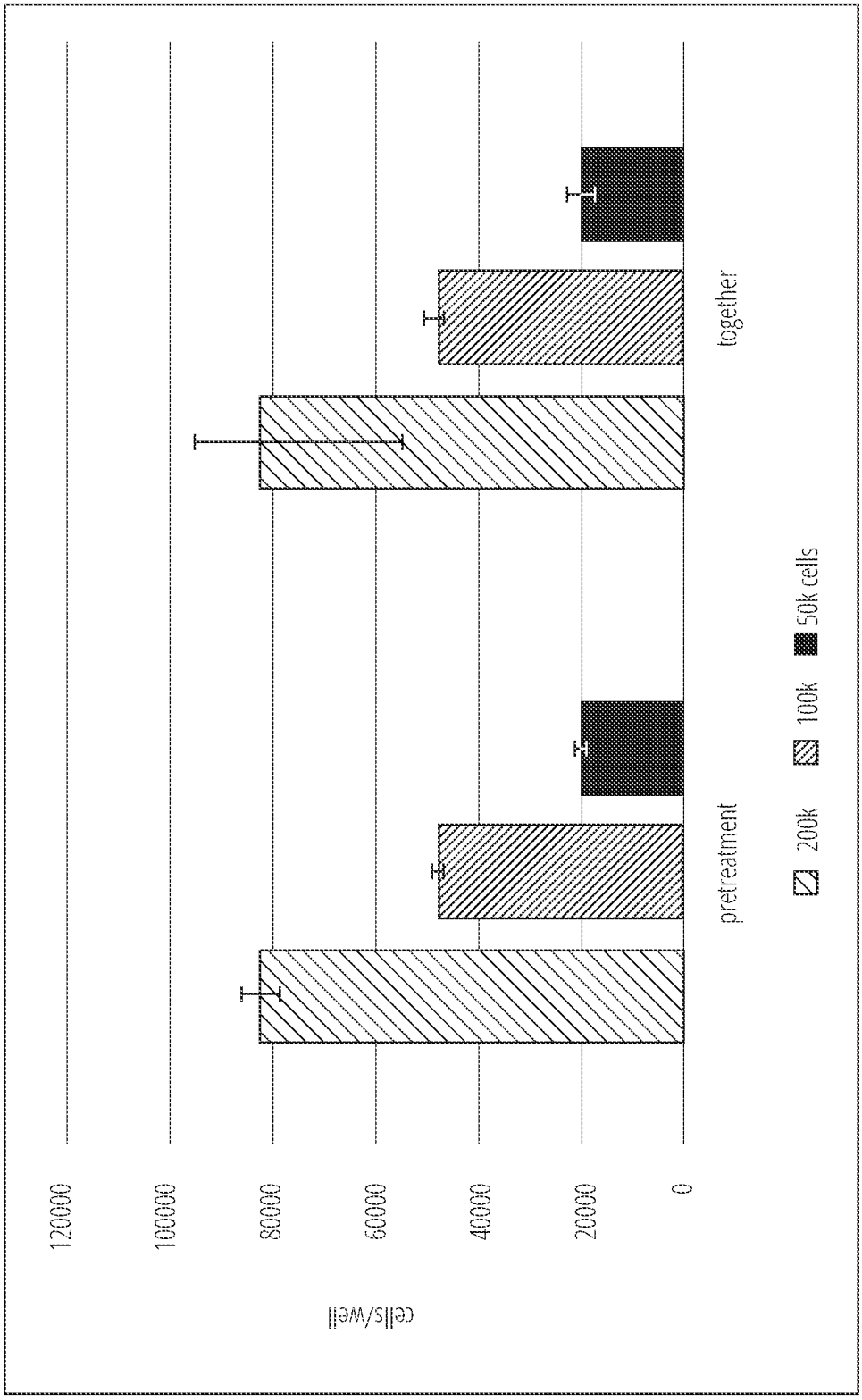

cells/well
120000
100000
80000
60000
40000
20000
0
pretreatment
together
200k
100k
50k cells

Fig. 11

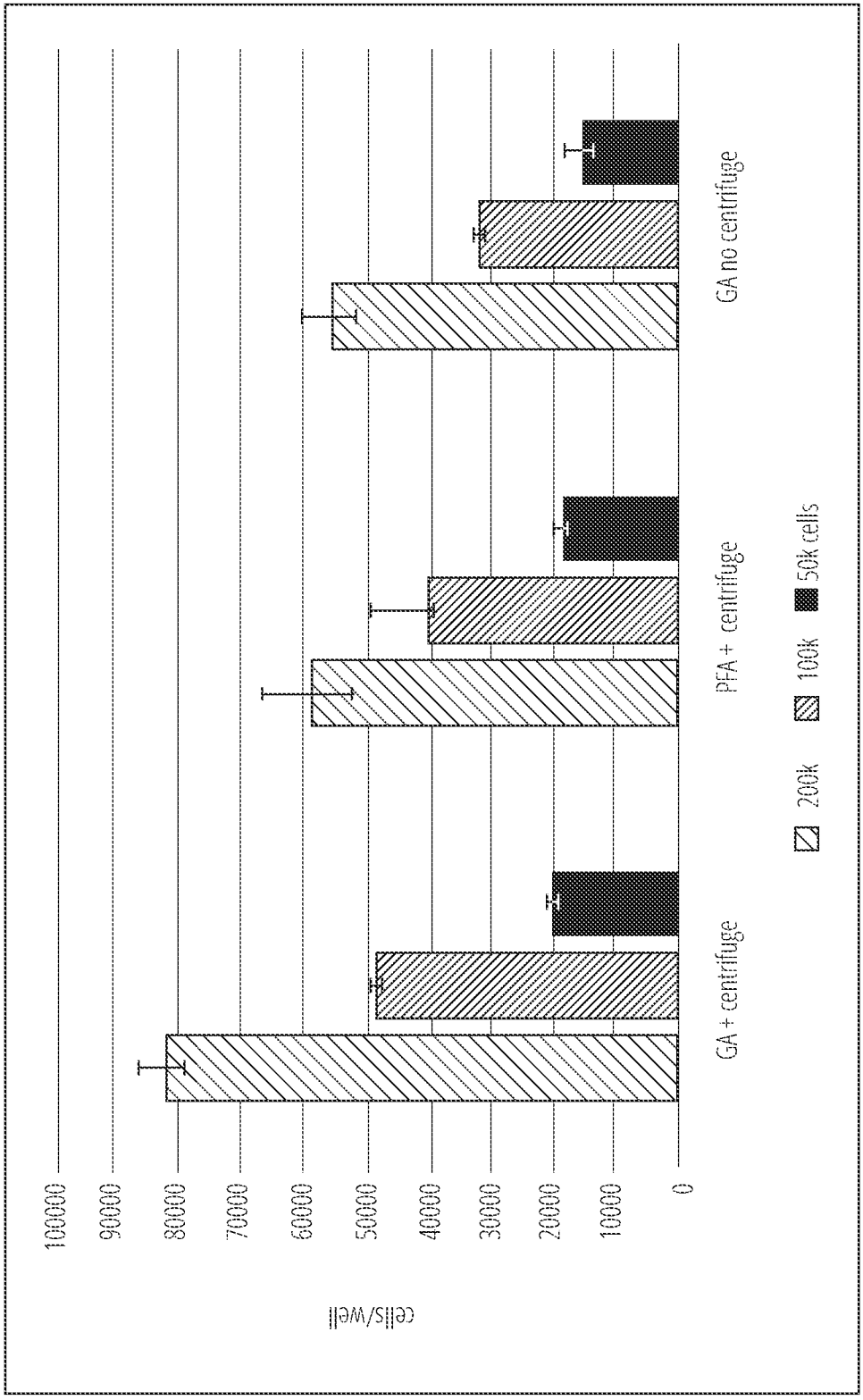

cells/well
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
GA + centrifuge
PFA + centrifuge
GA no centrifuge
200k
100k
50k cells

Fig. 12

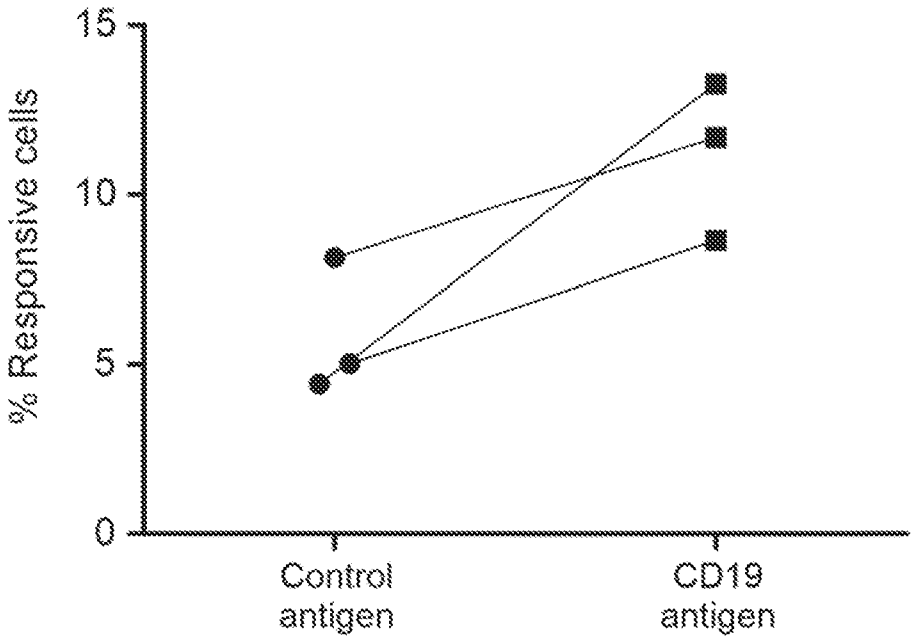


Fig. 13

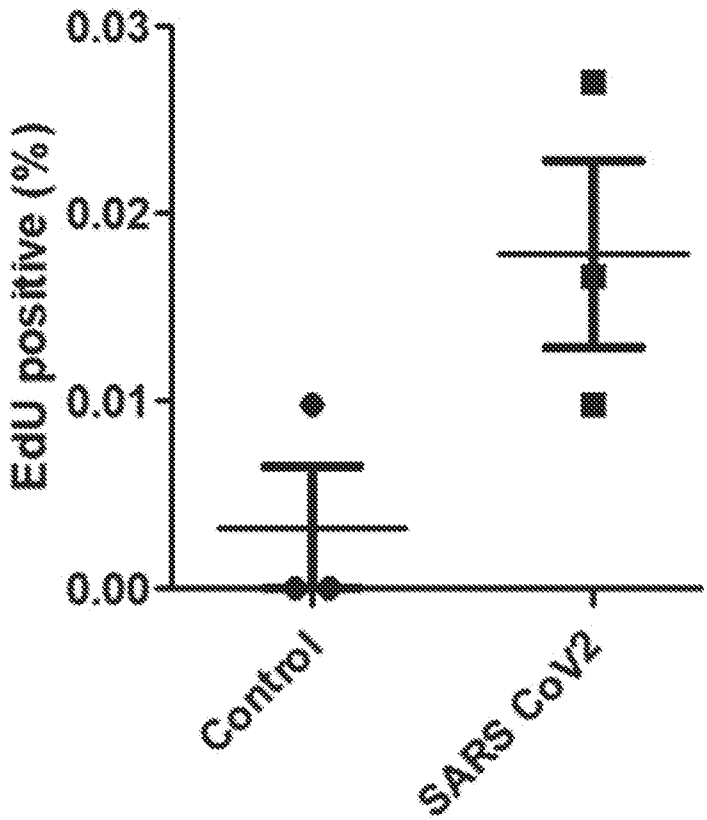
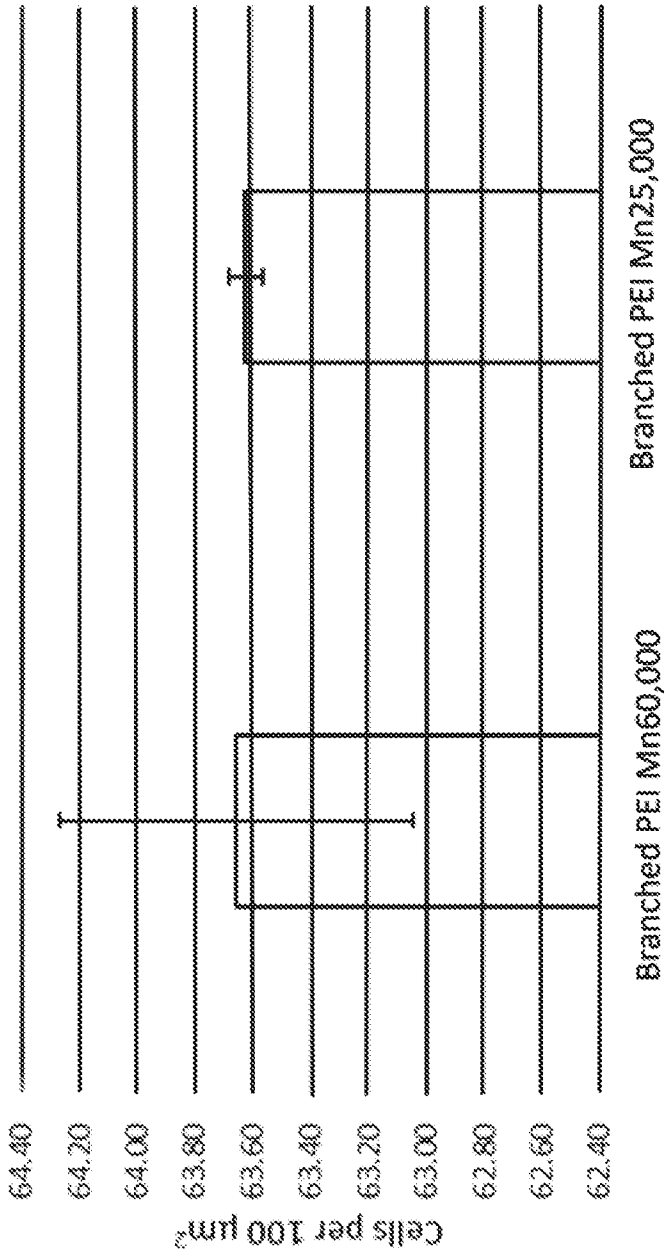


Fig. 14


Cells per 100 µm²
64.40
64.20
64.00
63.80
63.60
63.40
63.20
63.00
62.80
62.60
62.40
Branched PEI Mn60,000
Branched PEI Mn25,000

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2024/050641

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/00 C12Q1/6816
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PRICE ANDREW J ET AL: "A semi-interpenetrating network of polyacrylamide and recombinant basement membrane allows pluripotent cell culture in a soft, ligand-rich microenvironment", BIOMATERIALS, ELSEVIER, AMSTERDAM, NL, vol. 121, 9 December 2016 (2016-12-09), pages 179-192, XP029890661, ISSN: 0142-9612, DOI: 10.1016/J.BIOMATERIALS.2016.12.005 see abstract; section "2.1. Proliferation of composite substrate" on p. 180; section "2.8. EdU staining and analysis" on p. 182; p. 190, first column, first par.; fig. 1, 7	1-22

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2025	10/02/2025

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fischer, V

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANDRI K. RIAU ET AL: "Surface Modification of PMMA to Improve Adhesion to Corneal Substitutes in a Synthetic Core-Skirt Keratoprosthesis", APPLIED MATERIALS & INTERFACES, vol. 7, no. 39, 28 September 2015 (2015-09-28), pages 21690-21702, XP055459845, US ISSN: 1944-8244, DOI: 10.1021/acsami.5b07621 see abstract; section "Coating of PMMA surface with Alkoxysilane" on p. 21692; p. 21691, second column, second par.; section "Cell Proliferation Assay" on p. 21693; section "Casting of Collagen Hydrogel on modified PMMA Surface" on p. 21692; section "Cell Viability and Cell Proliferation on Modified PMMA Surface"; fig. 7	1-22
A	CN 114 272 436 A (CHEN DONG) 5 April 2022 (2022-04-05) see fig. 3; example 2, 3	1-22
A	WO 2007/012050 A2 (SURMODICS INC [US]; DONALDSON CO INC [US] ET AL.) 25 January 2007 (2007-01-25) see fig. 1; p. 6, l. 1-14; examples 1-4	1-22
A	GUITING LIN: "Labeling and tracking of mesenchymal stromal cells with EdU", CYTOTHERAPY , vol. 11, no. 7 3 May 2010 (2010-05-03), pages 864-873, XP093157604, GB ISSN: 1465-3249, DOI: 10.3109/14653240903180084 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2862589/pdf/nihms196752.pdf see abstract; p. 3, second par. relating to time course studies; fig. 1C-E	1-22
A	WO 2015/001085 A1 (GENMAB BV [NL]) 8 January 2015 (2015-01-08) see example 4	1-22

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Roche: "Cell Proliferation ELISA, BrdU (colorimetric)", , 10 December 2020 (2020-12-10), XP093158367, Retrieved from the Internet: URL:https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/224/344/11647229001.pdf Protocol on p. 7	1-22
A	SHIMADA A ET AL: "Improved methods for immunohistochemical detection of BrdU in hard tissue", JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 339, no. 1, 30 November 2008 (2008-11-30), pages 11-16, XP025504737, ISSN: 0022-1759, DOI: 10.1016/J.JIM.2008.07.013 [retrieved on 2008-08-15] see abstract; sections "2.2. Fixation, demineralization, and preparation of section" and "2.3. Pretreatment for immunodetection of BrdU", "2.4. Immunohistochemistry of BrdU" on p. 12; fig. 1	1-22
A	WO 2023/212290 A1 (SINGULAR GENOMICS SYSTEMS INC [US] ET AL.) 2 November 2023 (2023-11-02) see claims, especially 34, 35	1-22
A	SHREYAS KUDDANNAYA ET AL: "Surface Chemical Modification of Poly(dimethylsiloxane) for the Enhanced Adhesion and Proliferation of Mesenchymal Stem Cells", APPLIED MATERIALS & INTERFACES, vol. 5, no. 19, 9 October 2013 (2013-10-09), pages 9777-9784, XP055241189, US ISSN: 1944-8244, DOI: 10.1021/am402903e see abstract; fig. 1, 8	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2024/050641

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 114272436 A	05-04-2022	NONE	
WO 2007012050 A2	25-01-2007	CA 2615717 A1	25-01-2007
		EP 1937796 A2	02-07-2008
		JP 2009502242 A	29-01-2009
		US 2007082393 A1	12-04-2007
		WO 2007012050 A2	25-01-2007
WO 2015001085 A1	08-01-2015	AU 2014286116 A1	04-02-2016
		AU 2020202792 A1	21-05-2020
		AU 2022203851 A1	23-06-2022
		BR 112015032986 A2	22-08-2017
		BR 122023002590 B1	05-12-2023
		CA 2915575 A1	08-01-2015
		CN 105722529 A	29-06-2016
		CN 106255507 A	21-12-2016
		CN 113248615 A	13-08-2021
		CN 118063621 A	24-05-2024
		CY 1122840 T1	05-05-2021
		DK 3016681 T3	09-03-2020
		EA 201690167 A1	30-06-2016
		EP 3016681 A1	11-05-2016
		EP 3091998 A1	16-11-2016
		EP 3693385 A1	12-08-2020
		EP 4249515 A2	27-09-2023
		ES 2776706 T3	31-07-2020
		HK 1231369 A1	22-12-2017
		HR P20200382 T1	12-06-2020
		HU E048722 T2	28-08-2020
		IL 276530 A	30-09-2020
		IL 290613 A	01-04-2022
		IL 311371 A	01-04-2024
		JP 6546162 B2	17-07-2019
		JP 6619342 B2	11-12-2019
		JP 7104000 B2	20-07-2022
		JP 7208129 B2	18-01-2023
		JP 7389833 B2	30-11-2023
		JP 2016529882 A	29-09-2016
		JP 2017505121 A	16-02-2017
		JP 2020005635 A	16-01-2020
		JP 2020048564 A	02-04-2020
		JP 2022101539 A	06-07-2022
		JP 2022163009 A	25-10-2022
		JP 2024026114 A	28-02-2024
		KR 20160027177 A	09-03-2016
		KR 20220025210 A	03-03-2022
		KR 20230073341 A	25-05-2023
		LT 3016681 T	25-03-2020
		ME 03675 B	20-10-2020
		NZ 715896 A	25-02-2022
		PL 3016681 T3	15-06-2020
		PT 3016681 T	26-03-2020
		SG 10201800982Q A	28-03-2018
		SG 11201510739T A	28-01-2016
		SI 3016681 T1	30-04-2020
		SM T202000287 T1	08-07-2020
		UA 119646 C2	25-07-2019
		US 2016168247 A1	16-06-2016

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2024/050641

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		US 2016333095 A1	17-11-2016
		US 2020123255 A1	23-04-2020
		US 2020199229 A1	25-06-2020
		US 2023374131 A1	23-11-2023
		WO 2015001085 A1	08-01-2015
		WO 2015104346 A1	16-07-2015
		ZA 201800385 B	25-09-2019
WO 2023212290 A1	02-11-2023	NONE	