ABCD_AU543 recognizes collagen type II in cell-produced extracellular matrix by immunofluorescence

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Abstract

Animal-origin-free (AOF) antibodies are key to advancing ethical and reproducible *in vitro* research. In this study, we evaluated the recombinant AOF antibody ABCD_AU543 for the detection of collagen type II by immunofluorescence in cell-produced extracellular matrix (ECM). Collagen type II–rich ECM was generated using a CRISPRa-edited human adipose stem cell line overexpressing ACAN, COL2A1, and ZNF865. ABCD_AU543 specifically detected collagen type II using an animal-derived staining protocol, with staining patterns comparable to those previously reported. However, an entirely AOF staining protocol failed to detect collagen type II, despite successful cytoskeletal control staining. These results confirm the specificity of ABCD_AU543 for collagen type II and indicate that further optimization is required for fully animal-origin-free ECM immunostaining.

Introduction

Antibodies are among the most widely used reagents in basic and preclinical research. Thus, they are also crucial in the validation of *in vitro* models that are developed in demand for human-relevant *in vitro* systems, which offer more reproducible, ethical and physiologically accurate models of disease (Bédard *et al*., 2020). The reality is, that many *in vitro* models still depend on animal-derived components—such as fetal bovine serum (FBS) for cell culture, bovine serum albumin (BSA) in blocking solutions, and conventional polyclonal or hybridoma-derived antibodies (Duarte *et al*., 2023). This reliance on animal-origin reagents stands in contrast to the aim to reduce animal sufferance.

Recombinant antibodies have emerged as an important solution to these limitations. Compared to traditional animal-derived antibodies, recombinant antibodies offer enhanced reproducibility, long-term availability, and completely animal-free production workflows. In addition, they can be precisely engineered to improve affinity, specificity, stability, or molecular format—providing functional benefits that are difficult or impossible to achieve with conventional methods (Chandrasekaran, 2024).

Given the limitations of animal-derived research tools and the urgent need for robust, ethical, and translational systems, the development and validation of reliable animal-origin free (AOF) antibodies and protocols is an essential step toward fully animal-free experimental pipelines.

Human Collagen type II is a key structural protein in cartilage, providing tensile strength and forming the backbone of the ECM required to maintain tissue integrity, resilience, and load-bearing function (Wu *et al*., 2021). Collagen II consists of homotrimers of collagen type II alpha 1 chains. In this study, we tested a recombinant AOF antibody (ABCD_AU543) recognizing collagen type II alpha 1 chain (COL2A1, UniProt #P02458) for immunofluorescence studies.

For this purpose, we used cells developed by Levis *et al*., consisting of a stable adipose stem cell (ASC) line edited using CRISPR activation (CRISPRa) to upregulate the expression of ACAN, COL2A1, and ZNF865, resulting in the production of an ECM enriched in collagen type II (Levis *et al*., 2025). Two protocols were tested: one with an animal-derived staining protocol as well as an AOF protocol using AOF blocking solution. This work establishes the foundation for an entirely animal-free approach to collagen type II immunostaining and assesses efficiency in terms of specificity binding.

Materials & Methods

Antibodies: ABCD_AU543 and ABCD_AA344 (ABCD nomenclature, http://web.expasy.org/abcd/) were produced by the Geneva Antibody Facility (http://unige.ch/medecine/antibodies/) as mini-antibodies with the antigen-binding scFv portion fused to mouse IgG2a Fc. The scFv sequence of ABCD_AU543 (AU543, anti-collagen II) corresponds to the variable regions of the anti-collagen type II monoclonal antibody produced by the hybridoma M2139. This antibody was shown to bind to the J1 epitope of collagen type II (Karlsson *et al*., 1995). The scFv sequence of ABCD_AA344 (AA344, anti-tubulin) corresponds to the sequences of the variable regions of the clone S11B (Nizak *et al*., 2003). ScFv

variables sequences were joined by the peptide linker $(GGGGS)_4$. Antibodies were produced in HEK293T suspension cells growing in HEK TF medium (Xell #861-0001, Sartorius) supplemented with 0.1% Pluronic F68 (Sigma #P1300). Cells were transiently transfected using FectoPro® transfection reagent (Polyplus #101000014) with the vector coding for the mini-antibodies and supernatants were collected after 3 days.

Antigen: The ability of AU543 to recognize collagen type II was assessed against cell-produced ECM sheets. ACAN/Col2a1/ZNF865 CRISPRa-edited human ASCs cell line (ACAN-COL2-ZNF hASC) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 5% human platelet lysate (HPL; UltraGRO-PURE GI, AventaCell) and 1% antibiotic–antimycotic (A/A; Gibco) until reaching approximately 90% confluency. A detailed description of the CRISPRa-modified hASC cell line can be found elsewhere (Levis et al., 2025).

Protocol: For immunostaining, cells were fixed for 20 min at room temperature with 4% paraformaldehyde (PFA; Santa Cruz Biotechnology). Cells were washed for 5 min with 1% BSA (Sigma-Aldrich) or an AOF blocking solution (AOF-BS, 1x, Cell Signaling). Permeabilization was performed for 15 min at room temperature, either with a blocking solution of 0.03% Triton X-100 (Sigma-Aldrich), 0.9 % BSA and 10 % FBS in Phosphate Buffered Saline (PBS, Gibco) for the animal-derived protocol, or 0.03% Triton X-100 (Sigma-Aldrich) in AOF-BS for the animal-free protocol. Primary antibody staining was performed overnight at 4 °C in blocking solution. ABCD_AU543 anti-collagen type II was tested at 1 and 5 µg mL^{-1}. After washing with 1% BSA or 1% AOF-BS, respectively, cells were incubated for 1 h at room temperature in the dark with the secondary antibody Alexa Fluor™ 488 goat anti-mouse IgG (H+L) secondary antibody (1:500; Invitrogen).
ABCD_AA344 (anti–β-tubulin, 1 µg mL^{-1};) served as a positive control for the staining protocol, while the negative control consisted of cells stained with the secondary antibody only.
Nuclei were stained with DAPI (1.5 µg mL^{-1}; Sigma-Aldrich) for 5 min at room temperature, followed by a PBS wash. Fluorescence images were acquired using a Leica DMI3000B microscope equipped with DAPI (Excitation: 340-380 nm / Beam splitter: LP 400 nm / Emission: 435nm, 500ms exposure, 1.5 contrast), and FITC (Excitation: 460-500 nm / Beam Splitter: LP 505 nm / Emission: 512-542 nm, highest intensity, 2s exposure, 1.5 contrast) filter sets (Leica). Data were processed using ImageJ software (ImageJ 1.54r, National Institutes of Health USA). Brightness and contrast were adjusted linearly for ABCD_AU543 stained samples to optimize visualization of the individual channel (FTIR: 0–60). This adjustment was applied uniformly across images and did not affect the underlying raw data.

Results & Discussion

Antibody AU543 was evaluated for its ability to detect cell-secreted collagen type II in the extracellular matrix (ECM) by immunofluorescence. Two staining protocols were compared: one using animal-origin-free (AOF) blocking reagents and one based on bovine serum albumin (BSA) and fetal bovine serum (FBS) (animal-derived protocol). Using the animal-derived protocol, AU543 specifically detected collagen type II at a concentration of 1 µg mL^{-1}, with increased signal intensity observed at 5 µg mL^{-1} (Fig. 1). The resulting staining patterns were comparable to those reported in the original publication employing animal-derived primary and secondary antibodies (Levis *et al.*, 2025).
In contrast, no collagen type II signal was detected using the AOF protocol at any antibody concentration. However, staining of the β-tubulin control was successful with both protocols, indicating that the AOF protocol itself was functional (Fig. 2).

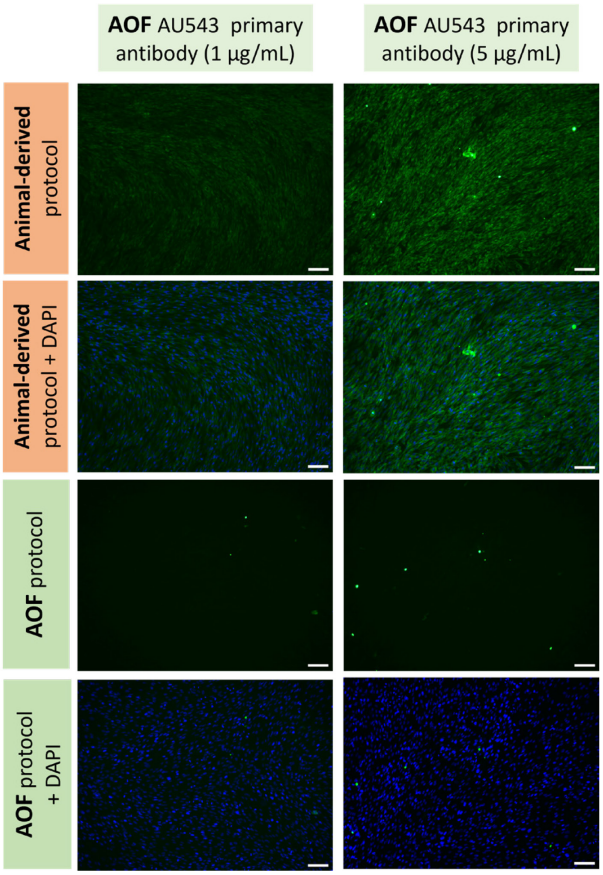

Fig. 1. Immunofluorescence detection of cell-secreted collagen type II (green) in the extracellular matrix using the AOF primary antibody AU543. AU543 was tested at 1 and 5 µg mL^{-1} using two staining protocols: an animal-derived blocking protocol (FBS/BSA) and an animal-origin-free (AOF) protocol based on AOF blocking solution. Each condition is shown with or without DAPI nuclear counterstaining (blue). Scale bar: 100 µm.

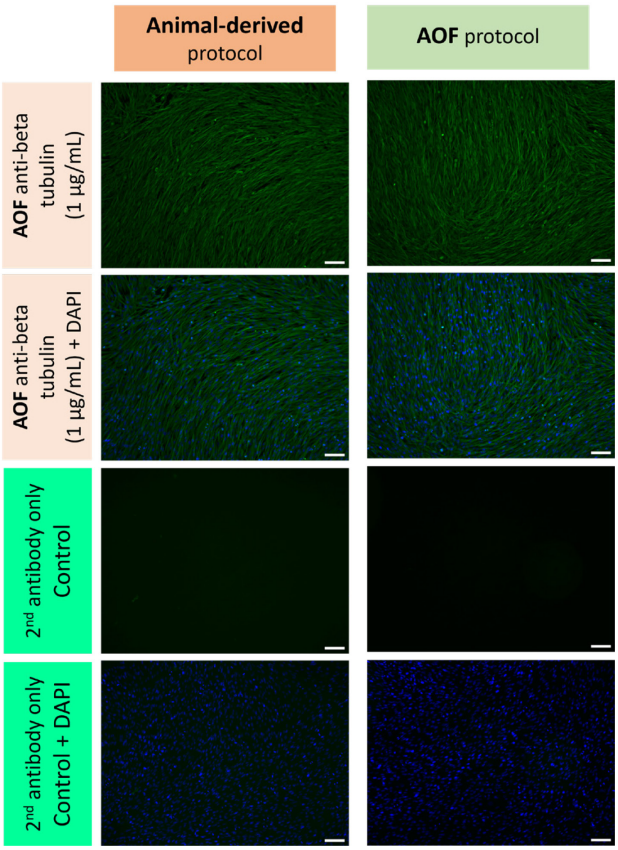

Fig. 2. Immunofluorescence staining of control conditions. Top: positive control showing cytoskeletal staining with an anti-β-tubulin antibody (1 µg mL^{-1}, green) using both staining protocols. Bottom: negative control performed in the absence of primary antibody (secondary antibody only), confirming the specificity of the secondary antibody. All conditions are shown with or without DAPI nuclear counterstaining (blue). Scale bar: 100 µm.

No signal was observed in the absence of the primary antibody, confirming the specificity of the secondary antibody (Fig. 2).

Together, these results demonstrate that AU543 specifically recognizes collagen type II in cell-produced ECM by immunofluorescence, while underscoring the need for further optimization to establish a fully animal-origin-free staining protocol for ECM proteins. Under the conditions tested, the animal-free blocking buffer from Cell Signaling Technology displayed high stringency, which may have limited signal retention. Other commercially available protein-free blocking solutions are available and could be evaluated in future studies to improve blocking efficiency and signal preservation, with the goal of achieving a fully animal-free staining protocol.

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Conflict of interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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