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Immuno-fluorescence staining patterns of leukocyte subsets in the skin of taurine and indicine cattle

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Abstract

The immuno-staining patterns of skin leukocytes were investigated in three breeds of cattle: Holstein-Friesian, Brahman and Santa Gertrudis of similar age before and after tick infestation. The antibodies specific for CD45 and CD45RO reacted with cells in the skin of all Holstein-Friesian cattle but did not react with cells in the skin of any Brahman cattle. The same antibodies reacted with cells from the skin of four (CD45) and seven (CD45RO) of twelve Santa Gertrudis cattle. The antibodies specific for T cells and γδ subset of T cells recognized cells from all three breeds of cattle. The antibody specific for MHC class II molecules labelled cells of mostly irregular shape, presumably dermal dendritic cells and/or macrophages and Langerhans cells. The antibody specific for granulocytes (mAb CH138) reacted with cells only in sections cut from skin with lesions. The antibody specific for $CD25^{+}$ cells labelled regularly shaped cells that showed a wide range of intensities of staining.

Keywords: leukocyte, immuno-fluorescence, cattle, breed, skin

Introduction

The identification of the cells of the immune system *in situ* has become an important tool for both research and diagnosis in recent years (Gutierrez et al., 1999). Although many antibodies specific for various subsets of bovine leukocytes have been produced they are most commonly used in flow cytometry, very few of them have been used to probe tissue sections (Howard and Naessens, 1993; Niku et al., 2006). Consequently, for most of the antibodies that are available, limited or no information is available on the immuno-staining patterns and localisation of the cells recognized in tissue sections. Furthermore, most research on immuno-staining so far was

carried out on cells from cattle of unspecified breed (Keresztes et al., 1996; Niku et al., 2006). Things are further complicated by the fact that for many antibodies successful immuno-staining is only accomplished after the optimization of antigen retrieval, fixation, incubation times and dilutions of antibodies etc (Niku et al., 2006; Polak and Van Noorden, 2003). Furthermore, some cell membrane antigens (such as T-cell sub-set antigens) might not survive routine fixation and wax embedding and can be successfully demonstrated only on frozen sections (Beesley, 1993). Finally, although antibodies that recognize T and B cells in a wide range of mammalian species have been reported, many are species specific (Jones et al., 1993; Niku et al., 2006).

As part of a project investigating the local immune response in cattle infested with ticks (Constantinoiu et al., 2010) we evaluated a panel of antibodies for the identification of immune cells in the skin sections of three breeds of cattle, including representatives of *Bos taurus taurus* (Holstein-Friesian), *Bos taurus indicus* (Brahman) and a stabilised composite breed (Santa Gertrudis, 5/8 *B. t. taurus* and 3/8 *B. t. indicus*). Recent analyses of SNP variation have demonstrated the wide divergence between indicine (*B. t. indicus*) and taurine (*B. t. taurus*) cattle with respect to genetic variability and suggests that antibodies developed for one subspecies may not be suitable for use in another species (Decker et al., 2009; Gibbs et al., 2009). The immuno-staining patterns of cells in the skin of these breeds of cattle produced by a panel of 12 antibodies are reported here.

Materials and methods

Tissue samples

Tissue samples were collected from the perineum of three Holstein-Friesian cattle (100% *B. t. taurus*), three Brahman cattle (100% *B. t. indicus*) and twelve Santa Gertrudis cattle (5/8 *B. t.*

taurus and 3/8 *B. t. indicus*) of similar age (12-24 months) before and after infestation with *Rhipicephalus microplus*. For each mAb and breed of cattle at least 5 sections derived from skin samples collected before and after tick infestation were immuno-stained and analysed. However, the mAbs specific for CD45 and CD45RO were probed on sections cut from tissue samples collected before tick infestation only. The trial was conducted with the approval of the University of Queensland Animal Ethics Committee for Production and Companion Animals (Approval number: SVS/864/06/CRC and SVS/872/07/CRC).

The cattle were restrained in a crush and given an epidural injection of 5 mL of Lignocaine 20 mg/mL (Troy Laboratories Pty. Limited, Sydney, Australia) to desensitise the tail head and the escutcheon area. Skin biopsies were collected with 8 mm punches (Paramount Surgimed Ltd., New Delhi, India) and within 10 min of collection were placed in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan.) that was frozen in isopentane (Labscan Asia Co., Ltd., Bangkok, Thailand) cooled with liquid nitrogen.

Monoclonal antibodies evaluated

Twelve different monoclonal antibodies for specific bovine immune cell types were evaluated in this study. Their source, stated specificity and associated references are outlined in Table 1.

Immuno-fluorescence

Various combinations of antibodies were used by double immuno-fluorescence labelling to investigate the immuno-staining patterns of leukocytes and their location in the skin of the cattle from different breeds. Cryosections, 6 μm thick, were mounted on Polysine™ glass slides (Menzel-GmbH & Co KG, Braunschweig, Germany) and dried overnight at room temperature (RT) with a fan. Next the sections were fixed in cold ethanol (4 ºC) for 8 min. Because the method of embedding and fixation can alter the epitopes of interest or make them inaccessible

(Willingham, 1999) and determine whether an antibody labels the target cells or not, the intensity and pattern of staining, and the intensity of the background, four methods of fixation were tried (cold acetone for 10 min, cold methanol for 10 min, cold ethanol for 8 min or dried fixed). Following fixation the background staining was blocked with Image-iT FX signal enhancer (Invitrogen, Carlsbad, California, USA) followed by 10% [v/v] goat serum in 1% [w/v] bovine serum albumin (BSA, Sigma, St Louis, USA), in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.4 mM KH_2PO_4). The cryosections were further incubated overnight at 4 ºC in a humidified chamber with monoclonal antibodies (100 µL per section) for specific leukocyte receptors (Table 1) diluted in 1% [w/v] BSA/PBS. IgG1, IgG2a and IgM negative control mouse monoclonal antibodies (DakoCytomation, Carpinteria, California, USA) in similar concentrations to the receptor specific antibodies were used as negative controls. The cryosections were washed in PBS and incubated with goat anti-mouse isotype-specific antibodies (100 µL per section) conjugated with fluorescein isothiocyanate (FITC) or Texas Red (Invitrogen, Carlsbad, California, USA) (1/400 [v/v] in 1% [w/v] BSA/PBS for 40 min at RT. After washing with PBS the nuclei were stained with DAPI dilactate (100 µL per section) (Invitrogen, Carlsbad, California, USA) and the slides were mounted with mounting medium (KPL, Gaithersburg, Maryland, USA). The slides were examined and photographed using an epifluorescent microscope, Olympus BX 51 (Olympus, Tokyo, Japan), equipped with a digital camera (Model DP 70, Olympus, Tokyo, Japan). The intensity of cell staining was assessed visually and the differences between breeds with regard to the intensity of cell staining were mentioned only when they were obvious. The images to be published were imported into Microsoft Office Picture Manager and the contrast/brightness adjusted similarly for all images.

Results

Sensitivity of the epitopes to the fixatives

Out of the four methods of fixation tried ethanol proved to have a less harsh effect on the epitopes recognized by most of the antibodies (for most antibodies the reaction was more intense and the background reduced after ethanol fixation, the reaction of antibodies CACT80C, IL-A116 and MM61A with their epitopes was abolished by the methanol fixation etc) tested and was chosen as the routine fixative for all cryosections. However, there were antibodies (MCA837G, IL-A116, HM57) that stained better on acetone fixed sections. This method also preserved the structure of the tissues better.

Immuno-staining patterns of the leukocytes

The antibodies specific for CD45 and CD45RO antigens (Fig. 1a and b) reacted with cells in the skin samples of all three Holstein-Friesian cattle but with none in the skin of the three Brahman cattle. The antibody directed at CD45 antigens labelled cells from the skin of four out of the twelve Santa Gertrudis cattle while the antibody directed at CD45RO antigens stained cells of seven out of the twelve Santa Gertrudis cattle. All Santa Gertrudis cattle whose cells were positive for CD45 antigens were also positive for CD45RO antigens. However, three cattle whose cells were positive for CD45RO antigens were negative for CD45 antigens. These results were confirmed by flow cytometry using blood samples from the same animals in this study (Piper et al., 2008, unpublished data). The cells from the skin of the Holstein-Friesian and Santa Gertrudis cattle displayed a strong, similar staining with both antibodies.

The antibodies specific for T cells (CD3 complex) (Fig. 2a and 2b) and γδ subset (WC1) of T cells (Fig. 3a, 3b and 3c) reacted with cells from all three breeds of cattle. However, in sections cut from the skin samples collected before tick infestation the cells recognized by the antibody

specific to T cells (CD3 complex) and the γδ subset (WC1) of T cells stained marginally more intensely in Brahman than in Holstein-Friesian cattle.

The antibody IL-A12 (specific for $CD4^+$ cells) (Fig. 4a and 4b) and the antibodies CACT80C and MCA837G (specific for $CD8^+$ cells) (Fig. 5a and 5b) stained cells of regular shape and no obvious differences were observed among the three breeds of cattle. For all breeds of cattle in this trial the antibody specific for MHC class II molecules (mAb IL-A21) (Fig. 2a and 2b) reacted with cells that had different shapes (most of them irregular shape) located in dermis, presumably dermal dendritic cells (DDC) and/or macrophages and cells from the epidermis, the Langerhans cells (LC) (Larregina and Falo, 2005). Generally, the reaction of this antibody with cells from the dermis was stronger than with cells from the epidermis. No differences between the three breeds were observed for this antibody.

The antibody CH138 (Fig. 3a, 3b and 3c) stained cells only in the sections cut from the samples collected after tick infestation. This antibody reacted with cells from dermis in sections from areas of skin with injuries caused by ticks (tick mouthparts fixed in the skin). The stained cells had a tendency to migrate towards skin injury or accumulate in intra-epidermal vesicles. In contrast this antibody did not recognize any cells in the tissue samples collected from areas of intact skin (before tick infestation) of all cattle from the three breeds but it labelled cells from the lumen of the blood vessels of the skin samples collected from these animals. No differences between the three breeds were observed for this antibody.

Antibody specific for CD25 (mAb IL-A111) cells labelled regular cells that showed a wide range of intensities of staining, from very weak to very strong (probably depending on the number of CD25 molecules on the surface of the cells related to the level of activation of the cells) (Fig. 4a and 4b). The same range of staining intensities was observed in the three breeds of cattle.

For all cattle breeds in the trial a relatively weak reaction was observed with antibody HM57 (CD79ά specific) that labelled two types of cells, some that have a circular shape, presumably B cells, and others whose shape resembled that of dendritic cells. Very few B cells could be observed in the skin of all breeds of cattle (Fig. 5a and 5b).

The antibody designated CC37 stained two types of cells, with regular and irregular shape (Fig. 6). The reactivity of this antibody was checked only with skin samples from Santa Gertrudis cattle so no comparison among the three breeds was carried out.

Generally, in all breeds of cattle the antibodies specific for CD3 cells, γδ T cells, CD4 cells, CD25 cells (interleukin 2 receptor α-chain (IL-2Rα)), CD8 cells and CD21 cells (mAb CC37) labelled cells that were located mainly in the dermis, most of them in the superficial dermis (within 0.5-0.6 mm from the epidermis). Occasionally a few of these cells were observed in the epidermis (mostly γδ T cells and CD25 cells). Except for mAb CH138 that did not stain any cells in intact skin sections no other differences were observed in the staining patterns of the cells before and after tick infestation.

No reaction was seen on cryosections that were incubated with IgG1, IgG2a and IgM negative control mouse monoclonal antibodies (data not shown).

Discussion

Although the largest proportion of material is formalin fixed, paraffin embedded this combination is not the best choice for preserving antigenicity of tissues (Beesley, 1993; Seitzer et al., 2002). Some cell membrane antigens, including those present on some T- and B-cell phenotypes do not survive paraffin embedding and the chemical processing that follows (Beesley, 1993; Polak and Van Noorden, 2003). This is the reason why O.C.T. embedded, frozen

tissues are preferred for immune-phenotyping these cells and generally more specific cell types can be detected with frozen sections than with paraffin sections (Beesley, 1993; Ward et al., 2006).

Monoclonal antibodies CACTB51A (specific for CD45) and mAb Il-A116 specific for CD45RO have been used in the past for labelling cattle leukocytes by both flow cytometry (Bembridge et al., 1995; Pelan-Mattocks et al., 2001) and immuno-histochemistry (Niku et al., 2006) but no differences in the reactivity of these antibodies in relation to the genetic composition of cattle have been reported. These differences might affect the interpretation of research involving *B. t. indicus* cattle and their hybrids as well as the results of research comparing the immune response mounted against various pathogens by *B. t. taurus* and *B. t. indicus* cattle. Furthermore, CD45 expression on bovine leukocytes using the same mAb as in the present study (CACTB51A) has been proposed as a tool for differentiation of lymphocytes and monocytes in leukograms (Pelan-Mattocks et al., 2001). Our data suggest that the antibody is not suitable for that application in *B. t. indicus* breeds and would at best give inaccurate results.

These antibodies have been reported by the producer (VMRD, Inc., Pullman, USA) to react with leukocytes from water buffalo (*Bubalus bubalis*) and Cape buffalo (*Syncerus caffer*) (information sheet for mAb CACTB51A) or sheep (information sheet for mAb IL-A116), which are more distantly related to *B. t. taurus* than *B. t. indicus*. However, it has been shown that there is allelic polymorphism in the gene encoding CD45 among cattle (Ballingal et al., 2001). Ballingal et al (2001) also demonstrated considerable polymorphism among European *B. t. taurus*, African *B. t. taurus* and Asian *B. t. indicus* with distinct genotype families common to each group. These genotype families were associated with specific cellular staining patterns in flow cytometric analyses of PBL. African and European taurine cattle stained uniformly with

mAb IL-A116 (as used in the current study) and also with mAb IL-A150 (another antibody that recognizes bovine CD45RO). In contrast the indicine Boran and Sahiwal cattle stained in a highly variable manner. The results of the present study are highly consistent with the findings of Ballingal et al. (2001), suggesting that further investigation of the immune phenotype of animals with divergent CD45 genotypes would be warranted. Furthermore, the small number of Brahman and Holstein-Friesian cattle in this experiment warrants trials including larger number of animals from all breeds as well as a larger panel of antibodies specific for CD45/CD45RO antigens.

The higher intensity of staining of T cells and γδ subset of T cells in skin of Brahman cattle suggests higher abundance of these antigens on the surface of cells from Brahman cattle than on the surface of cells from Holstein-Friesian cattle. However, this observation requires further studies to be confirmed as the fluorescence of individual cells from the two breeds was not quantified. CD3 proteins are associated with T cell receptor (TCR) and T cells that express fewer TCRs might be less responsive to antigen activation (Viola and Lanzavecchia, 1996). Activated T cells, including γδ T cells, might express MHC class II molecules (Bujdoso et al., 1993) but in our experiments little overlap if any between T cells (CD3 receptor) and cells bearing MHC class II molecules (IL-A21) was observed in the skin cells of all breeds (Fig. 2a and 2b). This is in contrast to previous research carried out by flow cytometry with antibody IL-A21 (specific for MHC class II molecules), which identified class II proteins on the majority of activated $CD4^+$ and $CD8^+$ T cells cultured for 4-5 days in the presence of IL-2 and either phytohemagglutinin or pokeweed mitogen (Taylor et al., 1993). Currently we have no explanation for this but in the present trial activated cells (CD45RO or CD25 positive) were identified in large numbers in the skin of cattle from all breeds. Furthermore, the lower analytical sensitivity of immuno-fluorescence in comparison to flow cytometry might not be the cause of the differences between

these two trials as in the present trial the mAb IL-A21 at the dilution used reacted very intensely with many cells in the skin of all breeds of cattle (Fig. 2a and b).

Dual fluorescence experiments showed that not all cells bearing CD4 antigens expressed CD3 antigens while all $CD8^+$ cells expressed CD3 antigens. The $CD4^+$ cells that did not express CD3 complex might have been monocytes or macrophages that can also bear CD4 antigens (Janeway, 2005). Furthermore, some γδ T cells expressed CD8 antigens but none of them expressed CD4 antigens.

The antibody CH138 (Fig. 3a, 3b and 3c) is believed to react with granulocytes but the exact identity of the cells recognized by this antibody is unknown (information sheet for mAb CH138, VMRD, Inc., Pullman, USA). If it had not been for the lesions induced by the ticks no reaction with this antibody would have been observed in the layers of the skin of cattle from this trial. Under certain circumstances granulocytes might be induced to express MHC class II antigens on their surface (Culshaw et al., 2008; Gosselin et al., 1993). However, in the present trial the cells that reacted with mAb CH138 did not bear MHC class II antigens.

CD25 (interleukin 2 receptor α-chain) is expressed on activated cells, including T cells, B cells and monocytes as well as on T regulatory (T reg) cells (Barclay et al., 1997; Belkaid, 2007). The different intensities of staining of cells by mAb mAb IL-A111might reflect different densities of interleukin 2 receptor α-chain on the surface of the cells that relates to the level of cell activation.

In addition to the CD79α specific antibody (mAb HM57, DakoCytomation) two other antibodies specific for B cells (BAQ155A, VMRD and IL-A30, ILRI) were used to probe the skin sections but neither of them labelled any cells at all. However, under the same conditions all three antibodies showed a strong reactivity with cells of germinal centres on cryosections cut from the

spleen of a *B. t. taurus* calf (data not shown), indicating that the lack of detectable B cells in skin was not an artefact.

The antibody designated CC37 has previously been shown to react with B cells and follicular dendritic cells (Naessens and Howard, 1991). Considering that few B cells seem to reside in the skin of these cattle it is likely that this antibody reacted mainly with dendritic cells. Some of the cells recognized by this antibody expressed MHC class II molecules recognized by the antibody IL-A21 while others did not (Fig. 7).

Apart from the differences in the staining patterns among breeds described in the present paper, differences in the numbers of cells in the skin of the three breeds were reported. The numbers of $CD4^+$, $CD8^+$, $CD25^+$ and γδ T cells were significantly higher in the skin of *B. t. indicus* cattle than in the skin of *B. t. taurus* while the numbers of $CD3^+$ cells tended to be higher in the skin of *B. t. indicus* cattle but not significantly higher (Constantinoiu et al., 2010). Furthermore, the numbers of $CD4^+$, $CD8^+$, $CD25^+$, $CD3^+$ and γδ T cells were significantly higher in the skin of *B. t. indicus* than in the skin of Santa-Gertrudis cattle (Constantinoiu et al., unpublished data).

Conclusions

This paper describes immuno-staining patterns of cells labelled by twelve monoclonal antibodies (Table 1) in the skin of cattle and the associations of the epitopes recognized by these antibodies on different cell subpopulations. The findings of this paper add to current knowledge of leukocyte markers for immuno-staining of cattle cells, especially of those from *B. t. indicus* breeds and they will be very helpful for future research investigating the immune response in the skin of cattle. The differences observed in the reactivity of the antibodies tested with cell populations from three breeds of cattle are also presented. The results of this paper show that the

276 epitopes recognized by some antibodies (CACTB51A and Il-A116) might not be present on the
277 cell populations from all breeds of cattle or might be expressed in different levels (MM1A and
278 IL-A29). This suggests that caution should be exercised when using some antibodies to compare
279 the immune response in different breeds of cattle or when extrapolating the results obtained
280 within one breed to other breeds. Furthermore the cells recognized by some antibodies might be
281 present in the skin only under specific circumstances as it happened with mAb CH138 that
282 labelled cells only in the skin with lesions.
283

284 **Conflict of interest**

285 None of the authors of this paper has a financial or personal relationship with other people or
286 organisations that could inappropriately influence or bias the content of the paper
287

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293

294 **References**

295 Ballingal, K.T., Waibochi, L., Holmes, E.C., Woelk, C.H., MacHugh, N.D., Lutje, V.,
296 McKeever, D.J., 2001. The CD45 locus in cattle: allelic polymorphism and evidence for
297 exceptional positive natural selection. Immunogenetics 52, 276-283.

298 Barclay, A., Brown, M., Alex Law, S., McNight, A., Tomlinison, M., Anton van der Merwe, P.,
299 1997. The Leukocyte Antigen Factsbook, 2nd Edition, Academic Press, San Diego, 613 pp.
300 Beesley, J.E., 1993. Immunocyto-chemistry: a practical approach. IRL Press at Oxford
301 University Press, Oxford, 248 pp.
302 Belkaid, Y., 2007. Regulatory T cells and infection: a dangerous necessity. Nature reviews.
303 Immunology 7, 875-888.
304 Bembridge, G.P., MacHugh, N.D., McKeever, D., Awino, E., Sopp, P., Collins, R.A., Gelder,
305 K.I., Howard, C.J., 1995. CD45RO expression on bovine T cells: relation to biological function.
306 Immunology 86, 537-544.
307 Bensaid, A., Hadam, M., 1991. Individual antigens of cattle. Bovine CD4 (BoCD4). Veterinary
308 immunology and immunopathology 27, 51-54.
309 Bujdoso, R., Lund, B.T., Evans, C.W., McConnell, I., 1993. Different rates of interleukin 2
310 receptor expression by ovine gamma/delta and alpha/beta T cells. Veterinary Immunology and
311 Immunopathology 39, 109-114.
312 Collins, R.A., Werling, D., Duggan, S.E., Bland, A.P., Parsons, K.R., Howard, C.J., 1998.
313 Gammadelta T cells present antigen to CD4+ alphabeta T cells. Journal of Leukocyte Biology
314 63, 707-714.
315 Constantinoiu, C.C., Jackson, L.A., Jorgensen, W.K., Lew-Tabor, A.E., Piper, E.K., Mayer,
316 D.G., Venus, B., Jonsson, N.N., 2010. Local immune response against larvae of Rhipicephalus
317 (Boophilus) microplus in Bos taurus indicus and Bos taurus taurus cattle. International Journal
318 for Parasitology 40, 865-875.
319 Culshaw, S., Millington, O.R., Brewer, J.M., McInnes, I.B., 2008. Murine neutrophils present
320 Class II restricted antigen. Immunology letters 118, 49-54.

Davis, W.C., MacHugh, N.D., Park, Y.H., Hamilton, M.J., Wyatt, C.R., 1993. Identification of a monoclonal antibody reactive with the bovine orthologue of CD3 (BoCD3). Veterinary Immunology and Immunopathology 39, 85-91.

Decker, J.E., Pires, J.C., Conant, G.C., McKay, S.D., Heaton, M.P., Chen, K., Cooper, A., Vilkki, J., Seabury, C.M., Caetano, A.R., Johnson, G.S., Brenneman, R.A., Hanotte, O., Eggert, L.S., Wiener, P., Kim, J.J., Kim, K.S., Sonstegard, T.S., Van Tassell, C.P., Neibergs, H.L., McEwan, J.C., Brauning, R., Coutinho, L.L., Babar, M.E., Wilson, G.A., McClure, M.C., Rolf, M.M., Kim, J., Schnabel, R.D., Taylor, J.F., 2009. Resolving the evolution of extant and extinct ruminants with high-throughput phylogenomics. Proceedings of the National Academy of Sciences of the United States of America 106, 18644-18649.

Gibbs, R.A., Taylor, J.F., Van Tassell, C.P., Barendse, W., Eversole, K.A., Gill, C.A., Green, R.D., Hamernik, D.L., Kappes, S.M., Lien, S., Matukumalli, L.K., McEwan, J.C., Nazareth, L.V., Schnabel, R.D., Weinstock, G.M., Wheeler, D.A., Ajmone-Marsan, P., Boettcher, P.J., Caetano, A.R., Garcia, J.F., Hanotte, O., Mariani, P., Skow, L.C., Sonstegard, T.S., Williams, J.L., Diallo, B., Hailemariam, L., Martinez, M.L., Morris, C.A., Silva, L.O., Spelman, R.J., Mulatu, W., Zhao, K., Abbey, C.A., Agaba, M., Araujo, F.R., Bunch, R.J., Burton, J., Gorni, C., Olivier, H., Harrison, B.E., Luff, B., Machado, M.A., Mwakaya, J., Plastow, G., Sim, W., Smith, T., Thomas, M.B., Valentini, A., Williams, P., Womack, J., Woolliams, J.A., Liu, Y., Qin, X., Worley, K.C., Gao, C., Jiang, H., Moore, S.S., Ren, Y., Song, X.Z., Bustamante, C.D., Hernandez, R.D., Muzny, D.M., Patil, S., San Lucas, A., Fu, Q., Kent, M.P., Vega, R., Matukumalli, A., McWilliam, S., Sclep, G., Bryc, K., Choi, J., Gao, H., Grefenstette, J.J., Murdoch, B., Stella, A., Villa-Angulo, R., Wright, M., Aerts, J., Jann, O., Negrini, R., Goddard, M.E., Hayes, B.J., Bradley, D.G., Barbosa da Silva, M., Lau, L.P., Liu, G.E., Lynn, D.J.,

344 Panzitta, F., Dodds, K.G., 2009. Genome-wide survey of SNP variation uncovers the genetic
345 structure of cattle breeds. Science 324, 528-532.
346 Gosselin, E.J., Wardwell, K., Rigby, W.F., Guyre, P.M., 1993. Induction of MHC class II on
347 human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor,
348 IFN-gamma, and IL-3. Journal of Immunology 151, 1482-1490.
349 Gutierrez, M., Forster, F.I., McConnell, S.A., Cassidy, J.P., Pollock, J.M., Bryson, D.G., 1999.
350 The detection of CD2+, CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in
351 fixed and paraffin embedded bovine tissue using a range of antigen recovery and signal
352 amplification techniques. Veterinary Immunology and Immunopathology 71, 321-334.
353 Howard, C.J., Naessens, J., 1993. Summary of workshop findings for cattle (tables 1 and 2).
354 Veterinary Immunology and Immunopathology 39, 25-47.
355 Janeway, C., 2005. Immunobiology: the immune system in health and disease, 6th ed. Garland
356 Science, New York, 823 pp.
357 Jones, M., Cordell, J.L., Beyers, A.D., Tse, A.G., Mason, D.Y., 1993. Detection of T and B cells
358 in many animal species using cross-reactive anti-peptide antibodies. Journal of Immunology 150,
359 5429-5435.
360 Keresztes, G., Takacs, L., Vilmos, P., Kurucz, E., Ando, I., 1996. Monoclonal antibodies
361 detecting components of the bovine immune system in formaldehyde-fixed paraffin-embedded
362 tissue specimens. Veterinary Immunology and Immunopathology 52, 383-392.
363 Larregina, A.T., Falo, L.D., Jr., 2005. Changing paradigms in cutaneous immunology: adapting
364 with dendritic cells. The Journal of investigative dermatology 124, 1-12.
365 Liebana, E., Marsh, S., Gough, J., Nunez, A., Vordermeier, H.M., Whelan, A., Spencer, Y.,
366 Clifton-Hardley, R., Hewinson, G., Johnson, L., 2007. Distribution and activation of T-

lymphocyte subsets in tuberculous bovine lymph-node granulomas. Veterinary Pathology 44, 366-372.

Morrison, W.I., Davis, W.C., 1991. Individual antigens of cattle. Differentiation antigens expressed predominantly on CD4- CD8- T lymphocytes (WC1, WC2). Veterinary Immunology and Immunopathology 27, 71-76.

Naessens, J., Howard, C.J., 1991. Individual antigens of cattle. Monoclonal antibodies reacting with bovine B cells (BoWC3, BoWC4 and BoWC5). Veterinary Immunology and Immunopathology 27, 77-85.

Naessens, J., Nthale, J.M., Muiya, P., 1996. Biochemical analysis of preliminary clusters in the non-lineage panel. Veterinary Immunology and Immunopathology 52, 347-356.

Niku, M., Ekman, A., Pessa-Morikawa, T., Iivanainen, A., 2006. Identification of major cell types in paraffin sections of bovine tissues. BMC Veterinary Research 2, 5.

Pelan-Mattocks, L.S., Pesch, B.A., Kehrli, M.E., Jr., 2001. Flow cytometric analysis of intracellular complexity and CD45 expression for use in rapid differentiation of leukocytes in bovine blood samples. American Journal of Veterinary Research 62, 1740-1744.

Polak, J.M., Van Noorden, S., 2003. Introduction to immunocytochemistry, 3rd ed. BIOS Scientific Publishers, Oxford, 176 pp.

Seitzer, U., Endl, E., Gerdes, J., Hollmann, C., 2002. Measuring immune responses in situ: Immunofluorescent and immunoenzymatic techniques. Methods in microbiology 32, 751-765.

Taylor, B.C., Choi, K.Y., Scibienski, R.J., Moore, P.F., Stott, J.L., 1993. Differential expression of bovine MHC class II antigens identified by monoclonal antibodies. Journal of Leukocyte Biology 53, 479-489.

Viola, A., Lanzavecchia, A., 1996. T cell activation determined by T cell receptor number and tunable thresholds. Science 273, 104-106.

Ward, J.M., Erexson, C.R., Faucette, L.J., Foley, J.F., Dijkstra, C., Cattoretti, G., 2006. Immunohistochemical markers for the rodent immune system. Toxicologic pathology 34, 616-630.

Willingham, M.C., 1999. Conditional epitopes: Is your antibody always specific? Journal of Histochemistry & Cytochemistry 47, 1233-1235.

Table 1 Monoclonal antibodies used to characterize the bovine skin immune cells

Monoclonal antibody designation	Source	Antigen specificity	Isotype	Cellular expression	Dilution used	Reference
CACTB51A	VMRD	CD45	IgG2a	Leukocytes	1/800	(Keresztes et al., 1996; Niku et al., 2006)
Il-A116	VMRD	CD45RO	IgG3	Activated cells	1/400	(Bembridge et al., 1995)
MM1A	VMRD	CD3	IgG1	T cells	1/800	(Davis et al., 1993)
CH138	VMRD	unknown	IgM	Granulocytes	1/400	(Keresztes et al., 1996; Naessens et al., 1996)
CACT80C	VMRD	CD8	IgG1	T cytotoxic cells	1/50	(Gutierrez et al., 1999)
MCA837G	AbD Serotec	CD8	IgG2a	T cytotoxic cells	1/50	(Gutierrez et al., 1999; Liebana et al., 2007)
HM57	DakoCytomation	CD79ά	IgG1	B cells	1/100	(Jones et al., 1993)

IL-A29[a]	ILRI[b]	γδ form of the T cell receptor	IgG1	γδ T cells	1/25	(Morrison and Davis, 1991)
IL-A21[a]	ILRI[b]	MHC class II antigen	IgG2a	Macrophages, dendritic cells, B cells, activated T cells	1/200	(Taylor et al., 1993)
IL-A12[a]	ILRI[b]	CD4	IgG2a	T helper cells	1/25	(Bensaid and Hadam, 1991)
IL-A111[a]	ILRI[b]	CD25	IgG1	Activated cells (IL2-R bearing cells)	1/25	(Collins et al., 1998)
CC37	ILRI[b]	CD21	IgG1	B cells,	1/25	(Naessens and Howard, 1991)

	follicular	398
		399
	dendritic	400
		401
	cells	402
		403
		404

405
406 [a] Monoclonal antibodies from tissue culture supernatant
407 [b] International Livestock Research Institute, Nairobi, Kenya
408

409 **Figure legends**

410

411 Fig. 1 $CD45^+$ and CD45 RO^+ cells in the skin of a Holstein-Friesian cow. The green cells bear
412 CD45 antigens while the cells with different shades of orange bear both CD45 and CD45RO
413 antigens (Fig 1a overview and Fig. 1b detail). Cells nuclei are in blue. E: epidermis, D: dermis,
414 HF: hair follicle.

415

416 Fig. 2 MHC class II^+ (red) and $CD3^+$ (green) cells in the skin of a Santa Gertrudis cow (Fig 2a
417 overview and Fig. 2b detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle.

418

419 Fig. 3 γδ T cells (red) and granulocytes (green) in the skin of a Holstein-Friesian cow (Fig. 3a)
420 and a Brahman cow (Fig. 3b and c) (Fig 3a overview showing two places where ticks were fixed,
421 Fig. 3b detail and Fig. 3c epidermal vesicle). Cells nuclei are in blue. E: epidermis, D: dermis,
422 HF: hair follicle, TMP: tick mouth parts.

423

424 Fig. 4 $CD4^+$ cells (red) and $CD25^+$ cells (green) in the skin of a Holstein-Friesian cow. The cells
425 with different shades of yellow-orange are likely to be T regulatory cells ($CD4^+/CD25^+$) (Fig 4a
426 overview and Fig. 4b detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair follicle.

427

428 Fig. 5 $CD8^+$ cells (red) (MCA837G) and B cells (green) (HM57) in the skin of a Brahman cow
429 (Fig. 5a overview and Fig. 5b detail). Cells nuclei are in blue. E: epidermis, D: dermis, HF: hair
430 follicle.

431

Fig. 6 Immuno-staining pattern of cells recognized by mAb CC37 in skin of a Santa Gertrudis cow. E: epidermis, D: dermis, HF: hair follicle.

Fig. 7 Cells bearing MHCII molecules (red) and CD21 molecules (green, mAb CC37) in the skin of a Santa Gertrudis cow. E: epidermis, D: dermis, HF: hair follicle.

Figure 1a

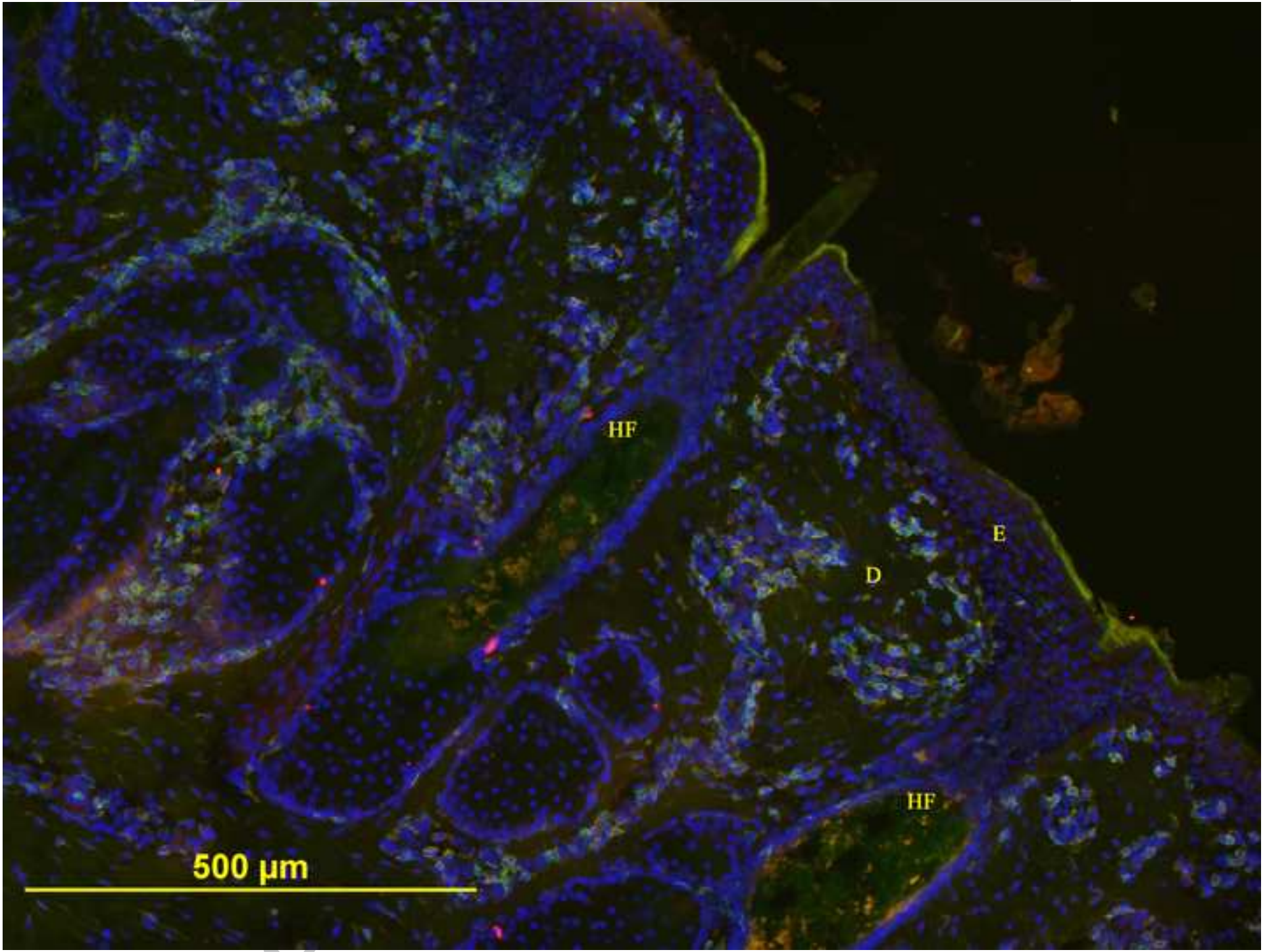

Figure 1b

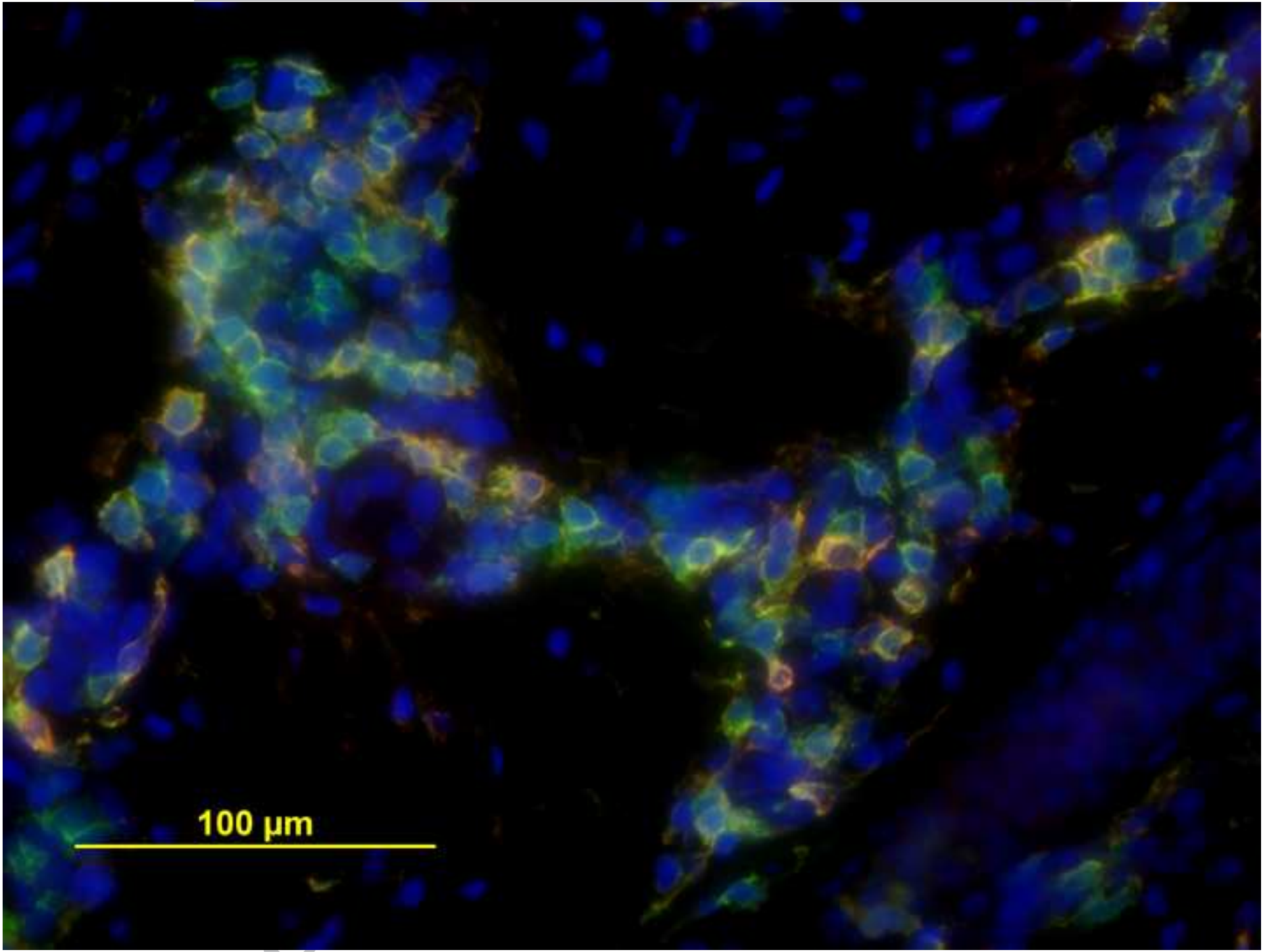

Figure 2a

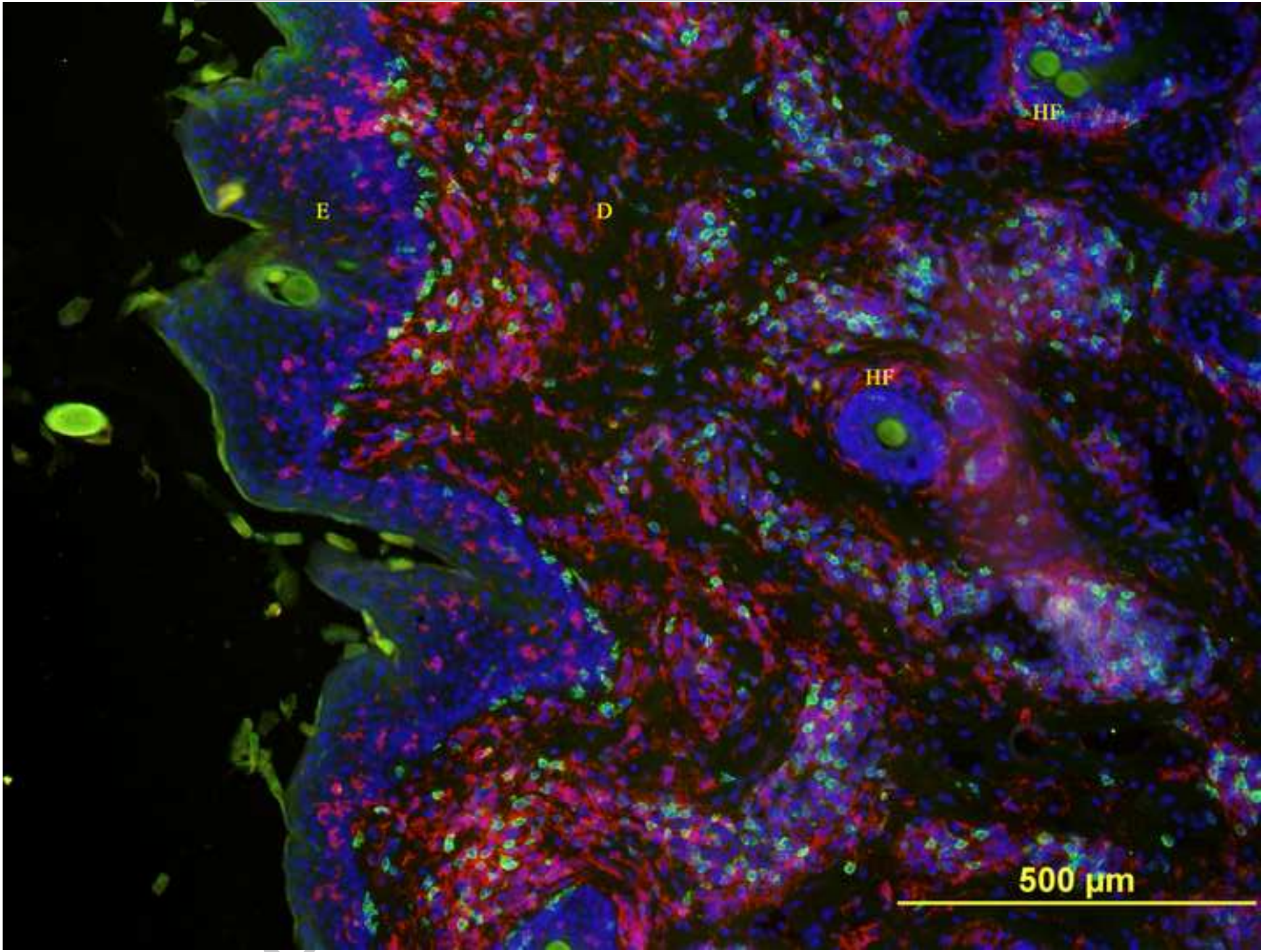

Figure 2b

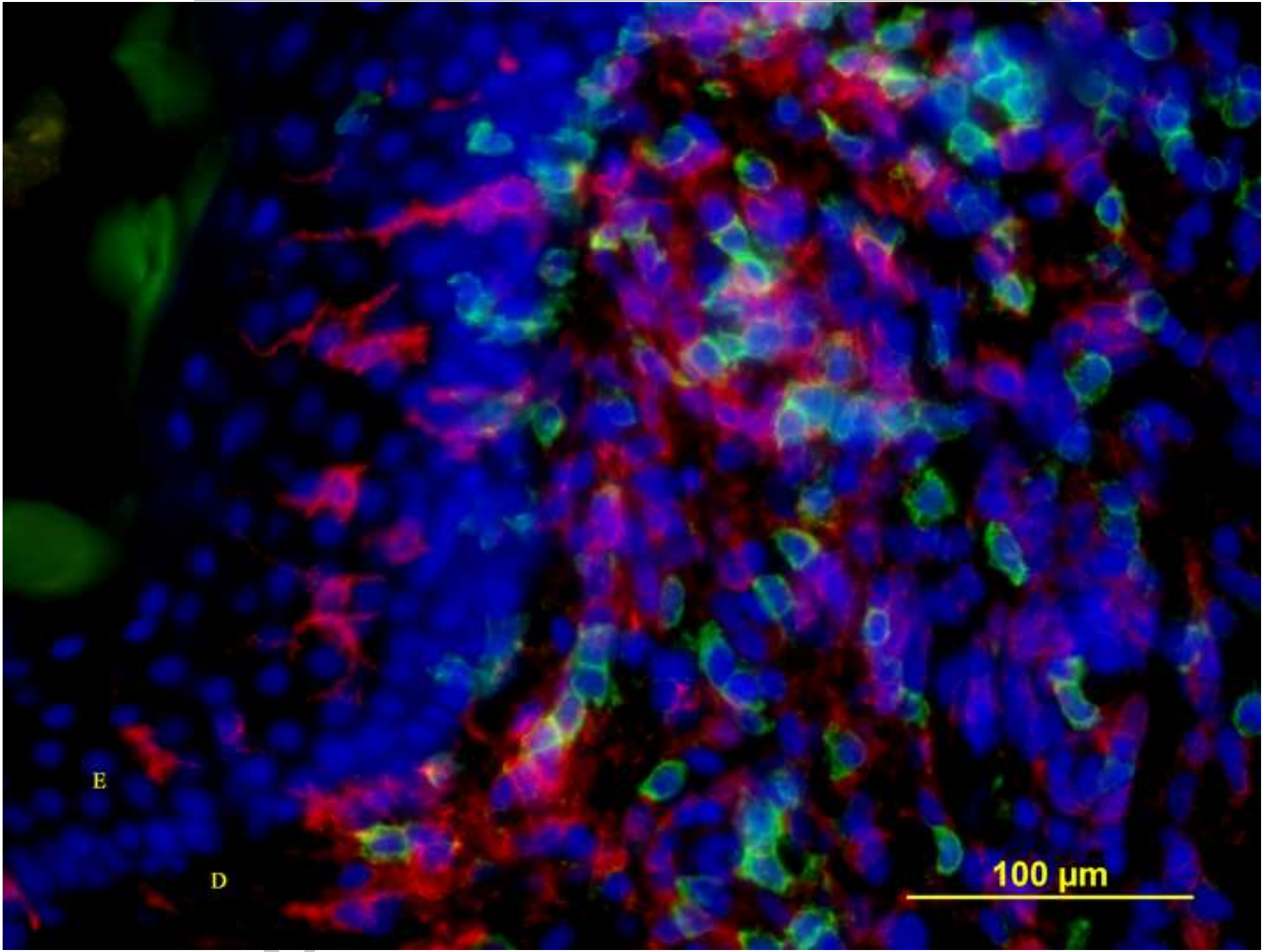

Figure 3a

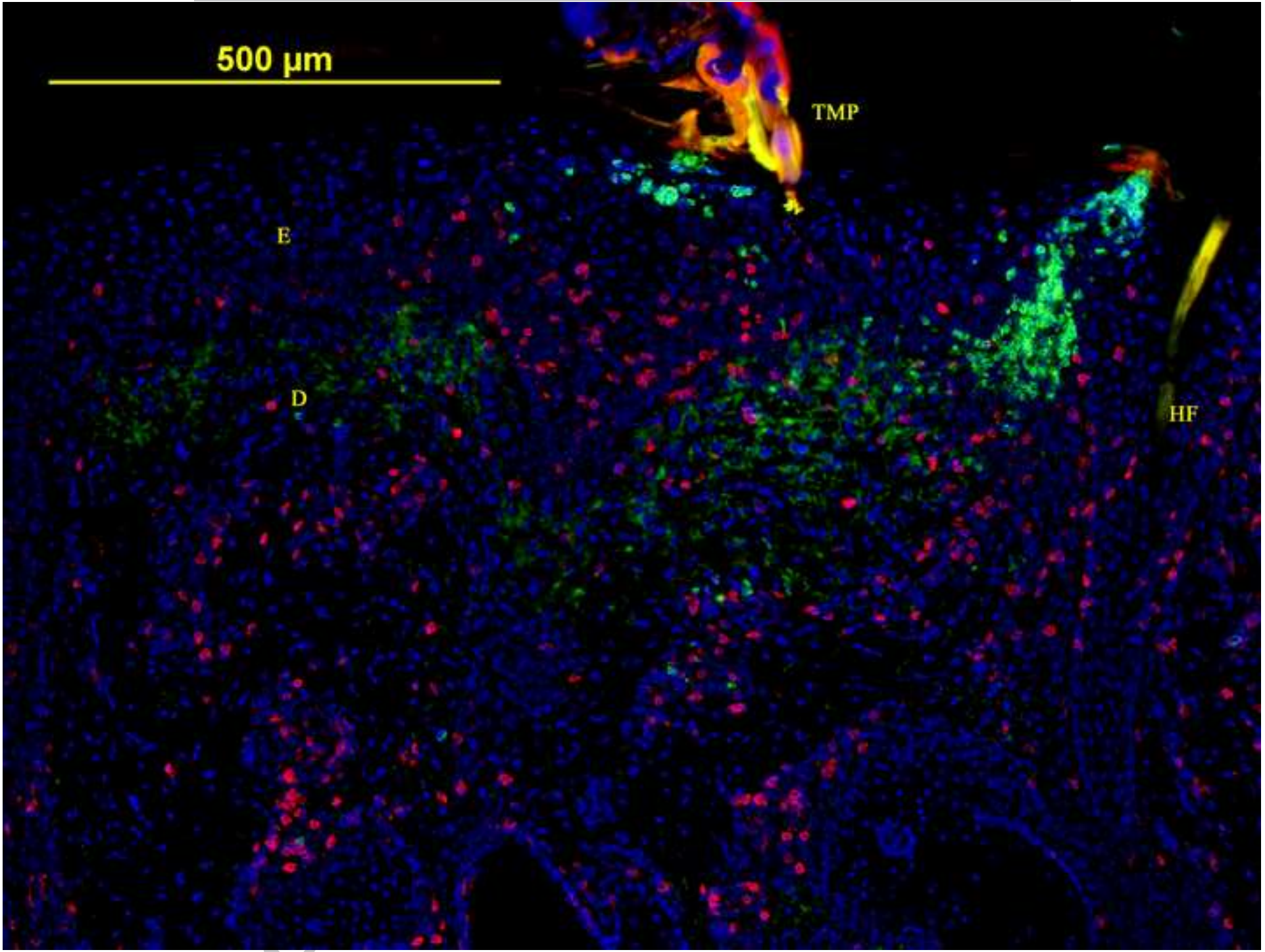

Figure 3b

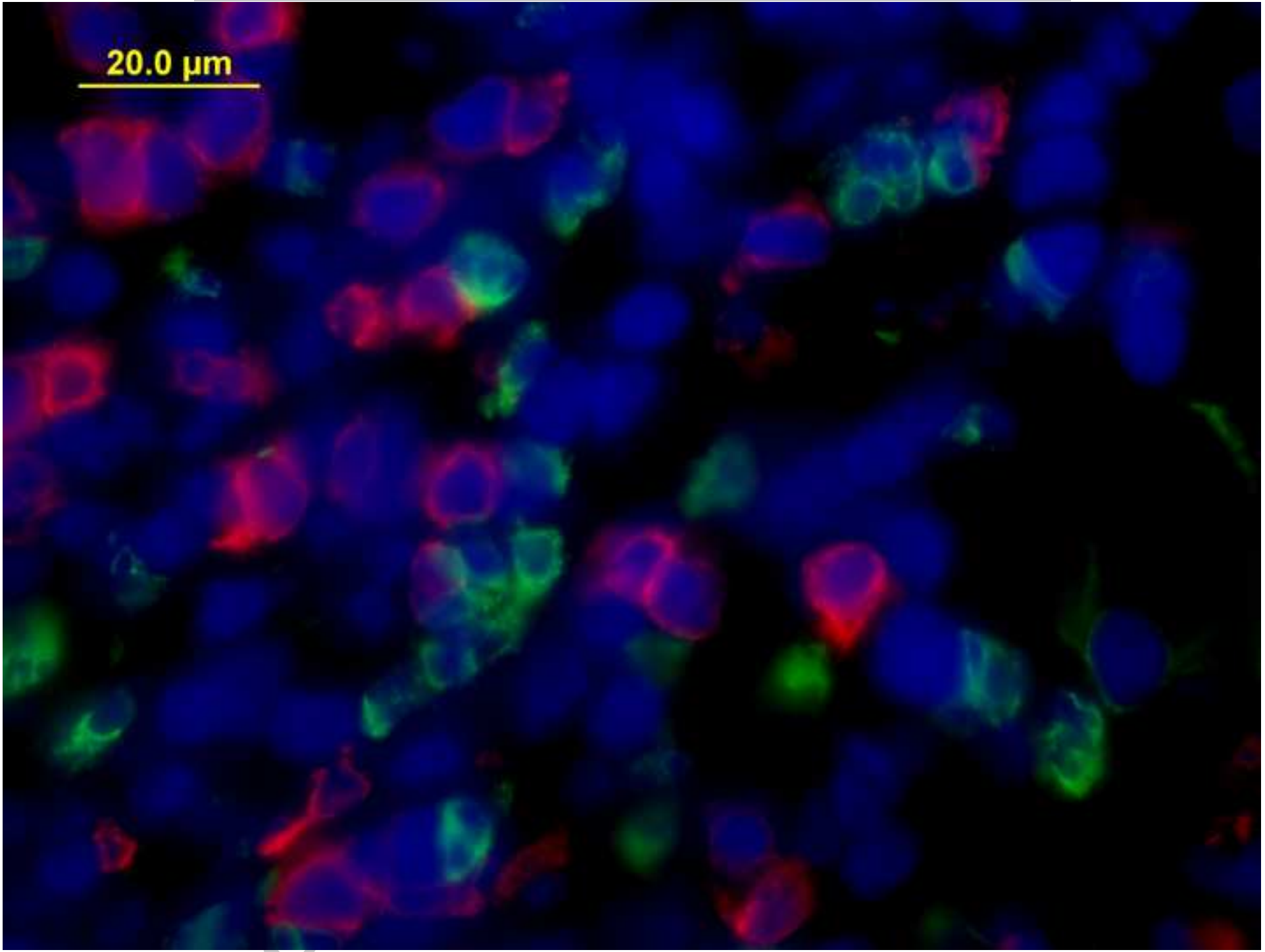

Figure 3c

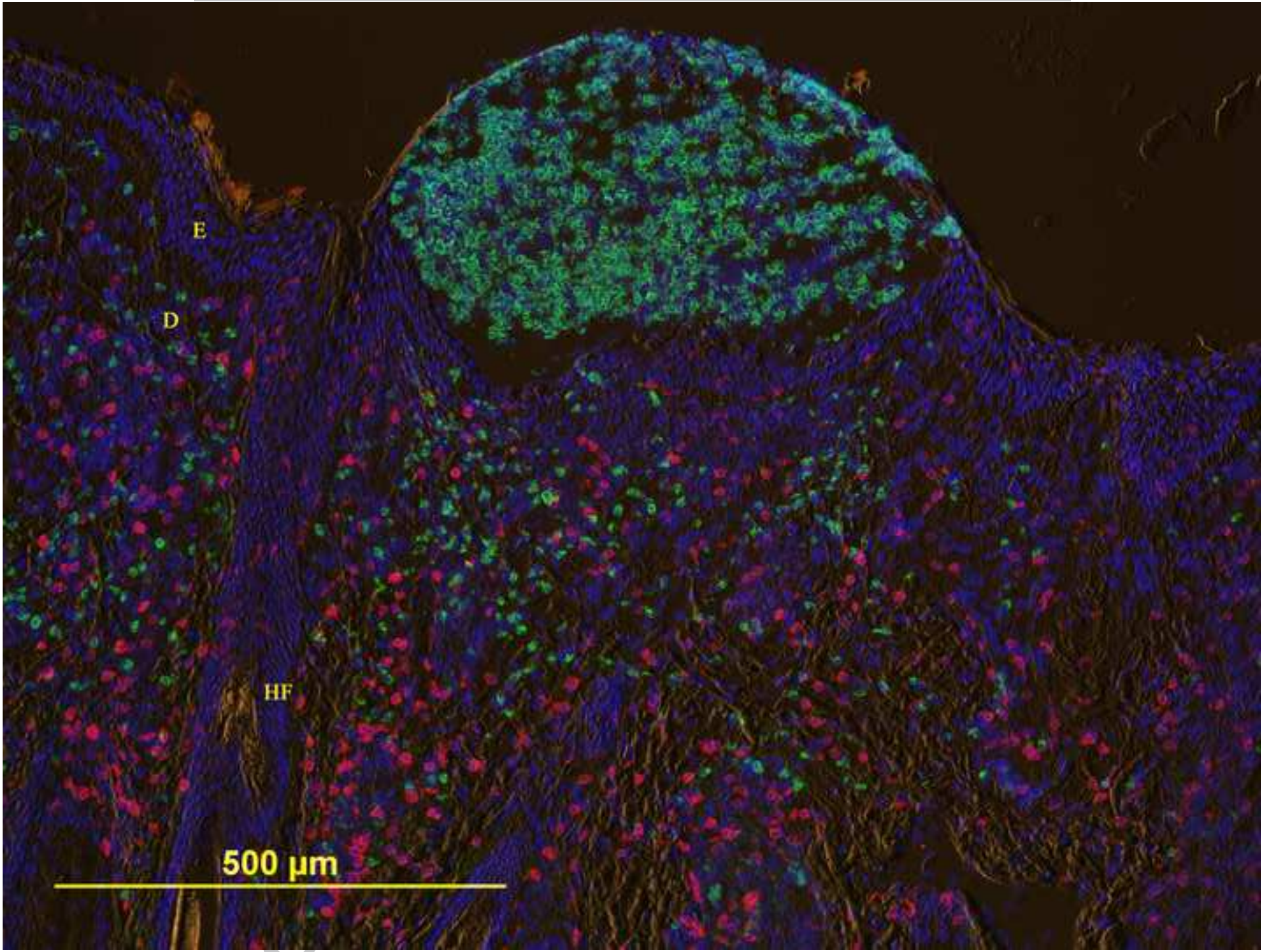

Figure 4a

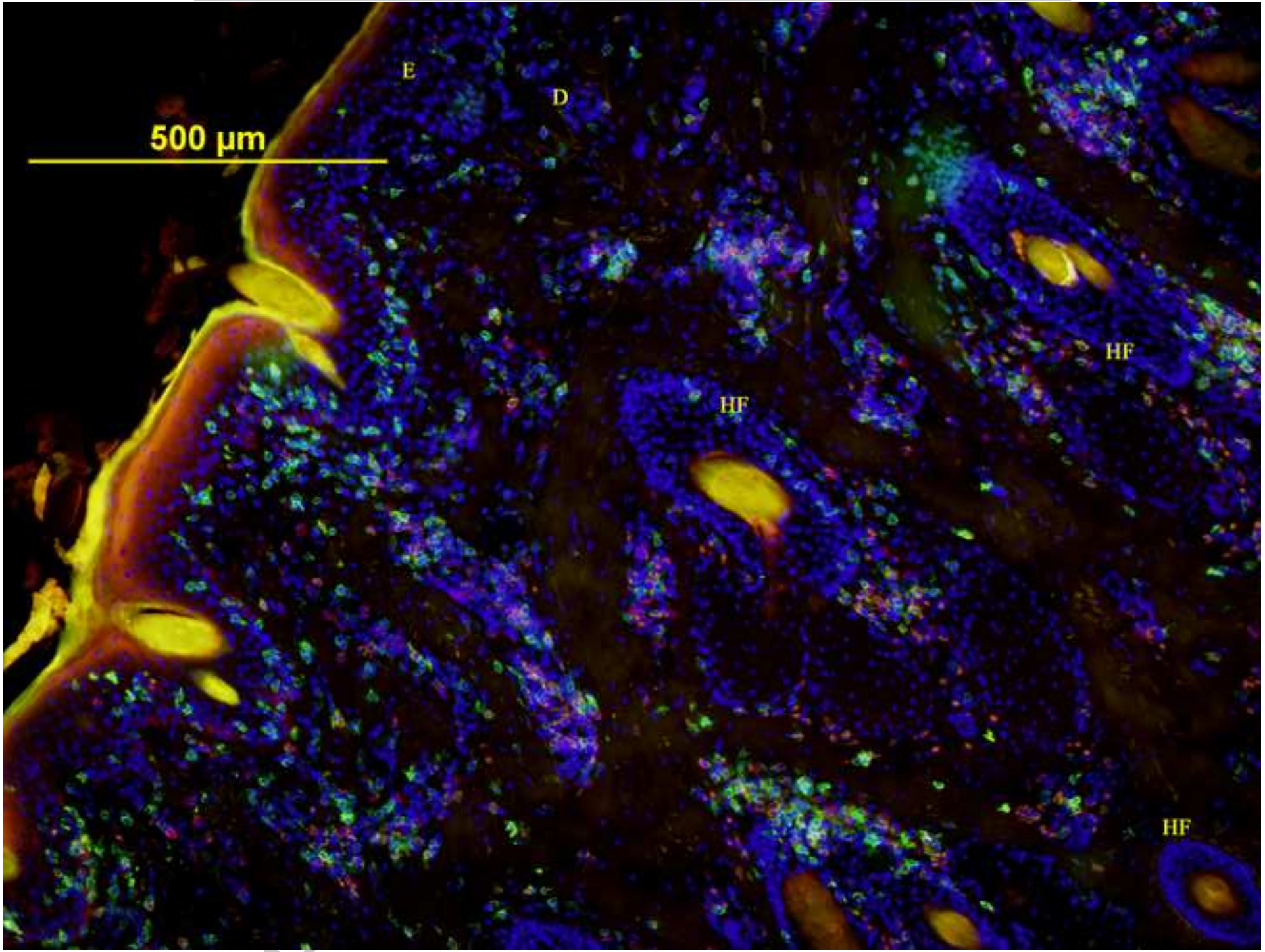

Figure 4b

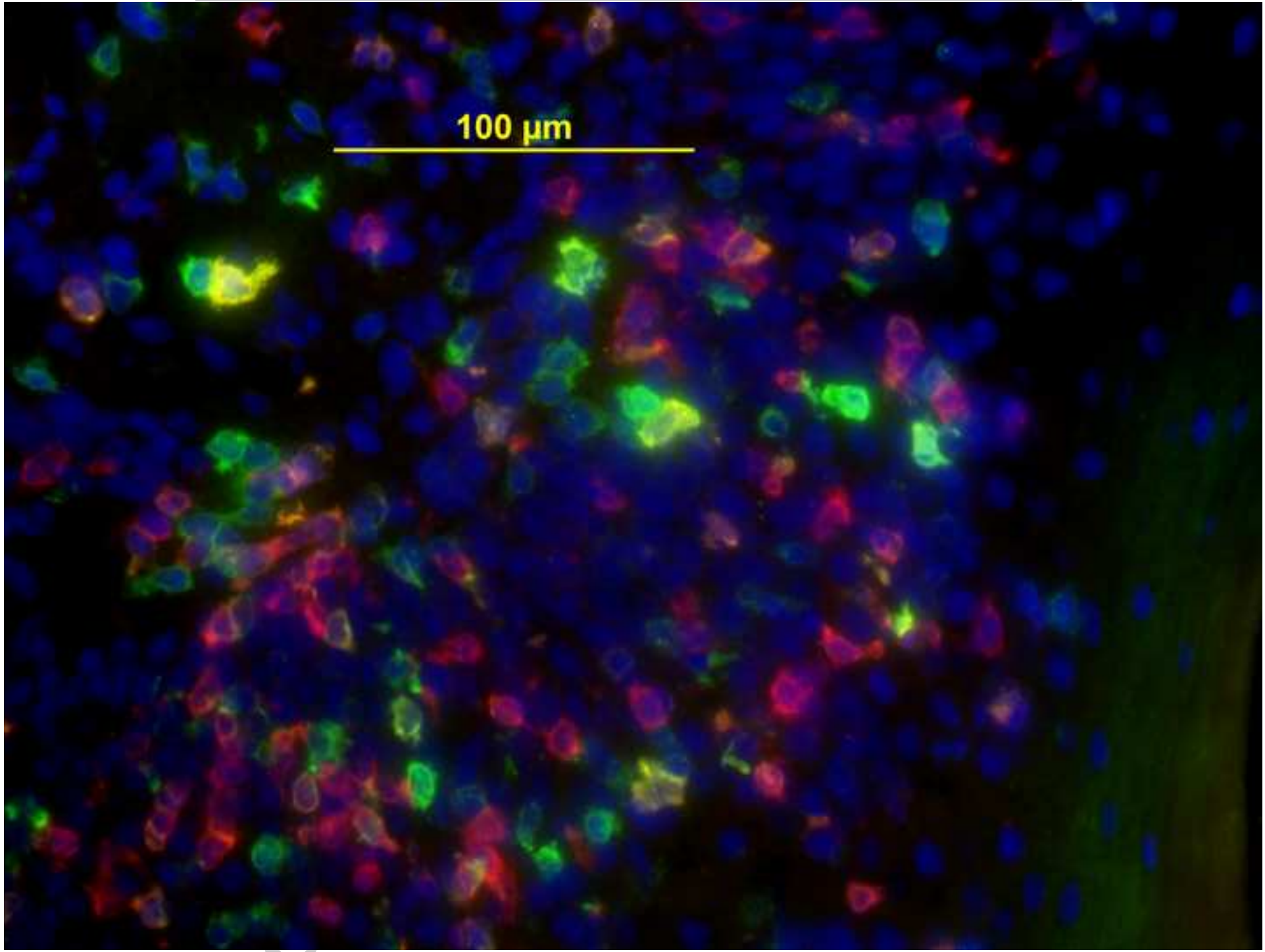

Figure 5a

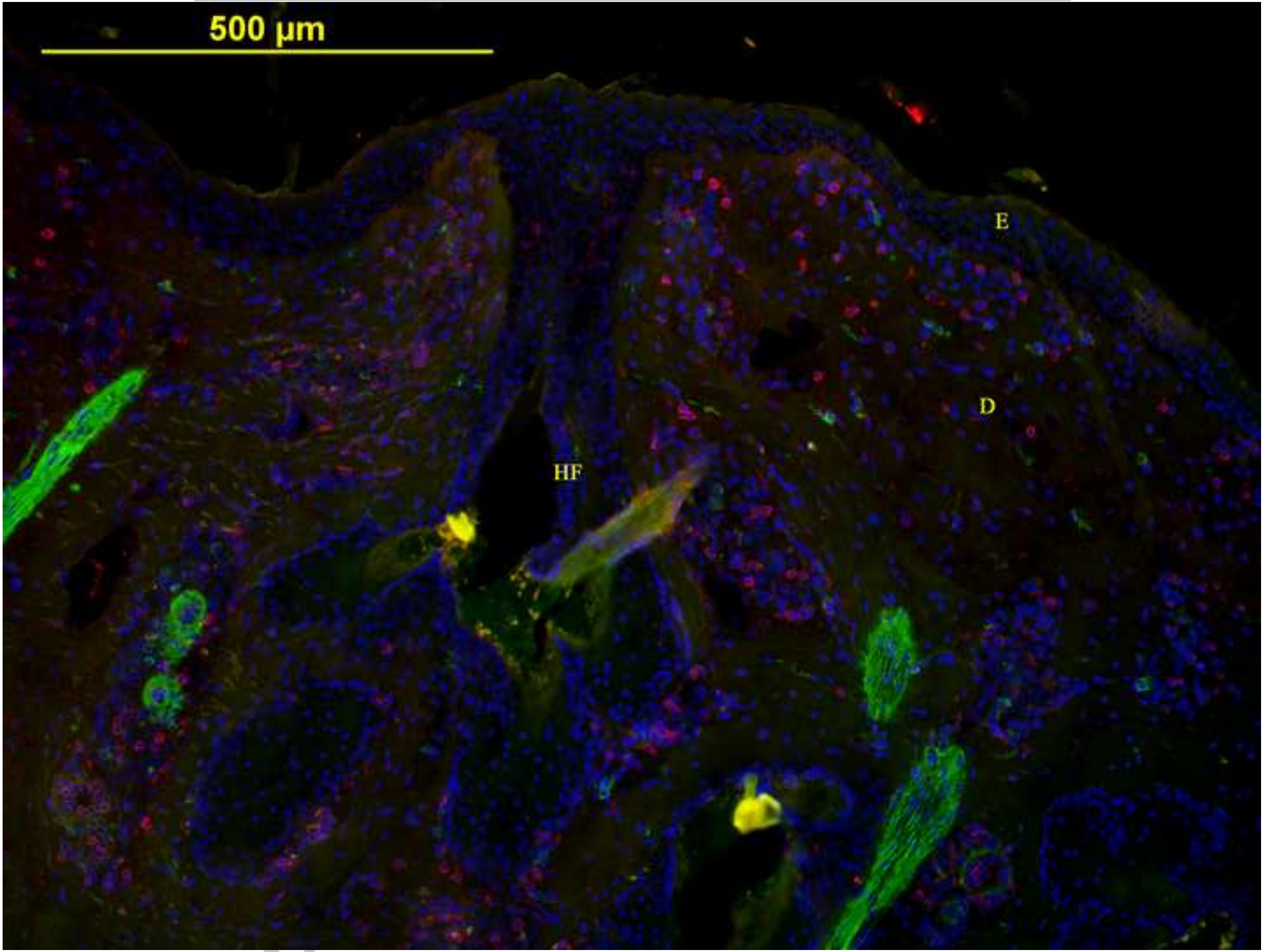

Figure 5b

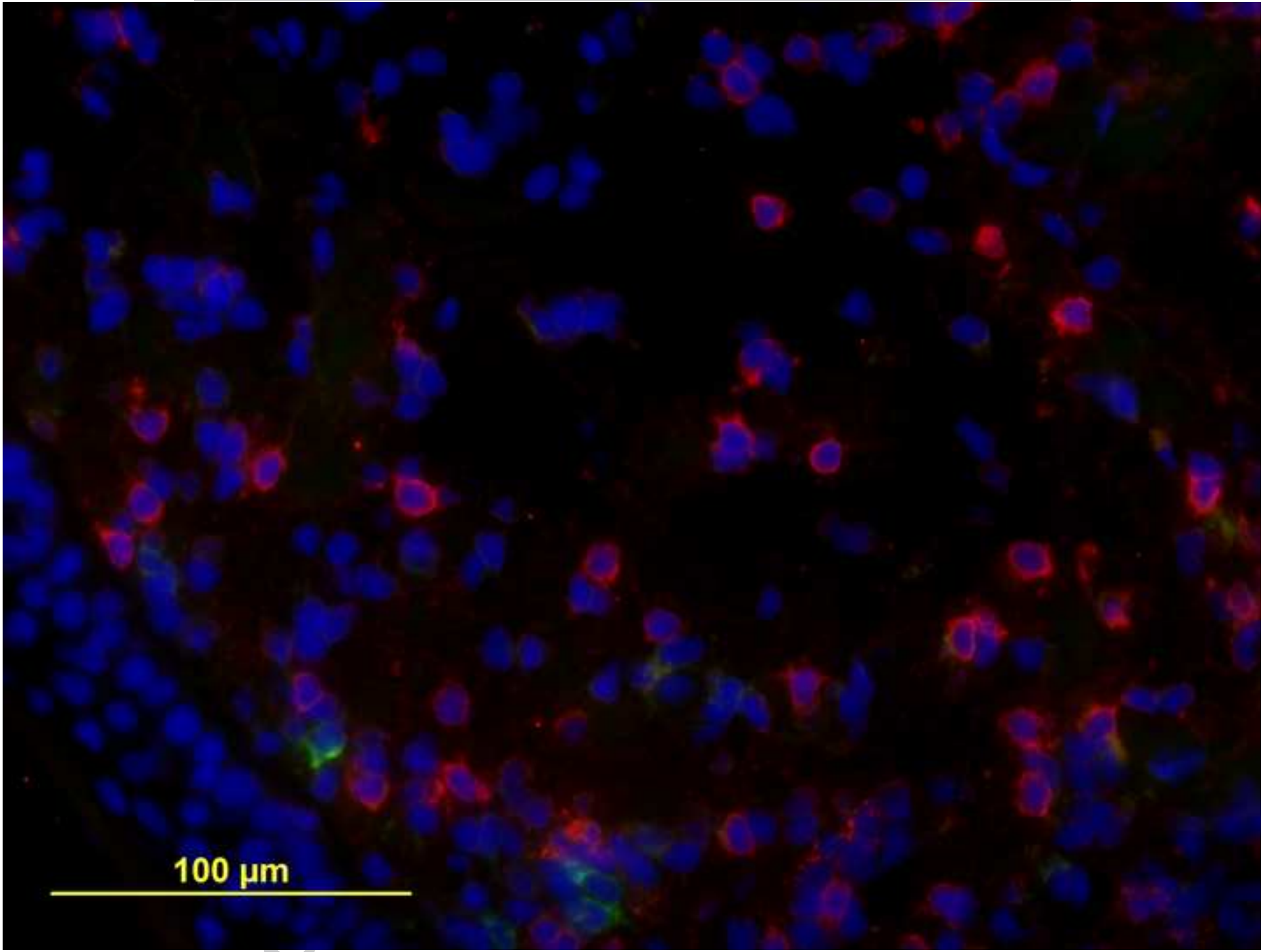

Figure 6

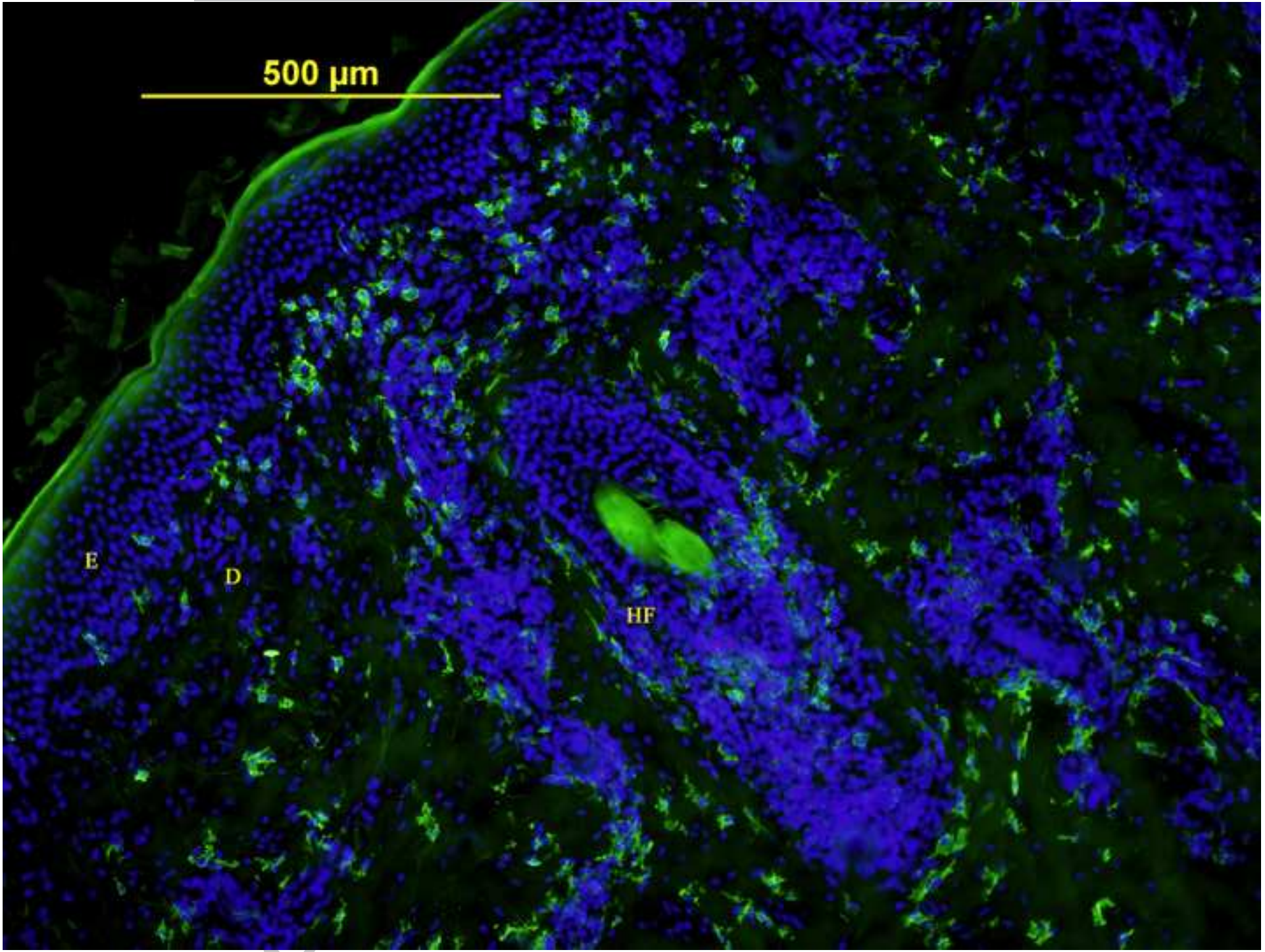

Figure 7