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Supporting Information

Paper-Based Antibody Detection Devices Using Bioluminescent BRET-Switching Sensor Proteins

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Experimental Procedures

Materials and instruments. Ultrapure water (18.2 MΩ cm) from a PURELAB flex water purification system was used throughout all experiments. LUMinescent AntiBody Sensing proteins (LUMABS) against HIV1-p17 antibody (HIV-LUMABS), dengue virus type 1 antibody (DEN-LUMABS) and hemagglutinin (HA-LUMABS) were prepared following the previously reported procedure[1] and stored in freeze dried form at -80˚C. NanoGlo Luciferase assay substrate (N1110, Furimazine, with enclosed Promega buffer) was purchased from Promega Corporation (Tokyo, Japan). anti-Dengue Virus Type Ⅰ (Antibody, clone 15F3-1) (anti-DEN1) was purchased from MERCK MILLIPORE (Tokyo, Japan). Hemagglutinin (HA) Tag Monoclonal Antibody (2-2.2.14) (anti-HA) was purchased from Thermo Fisher SCIENTIFIC (Kanagawa, Japan). HIV1-p17 (clone 32/1.24.89) antibody (anti-HIV1) was purchased from Zeptometrix (New York, America). PBS ×10 (phosphate buffered saline, pH 7.4) was purchased from NIPPON GENE (Tokyo, Japan). Porcine whole blood with 0.3 wt % citric acid and porcine serum were purchased from Tokyo Shibaura Zouki CO (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

The filter paper used was a Whatman No.1 filter paper (GE Healthcare, Tokyo, Japan). Wax patterning was performed as previously described.[2] In short, hydrophobic wax patterns on A4 sized filter papers were designed in Adobe Illustrator CC software and printed with a ColorQube 8570 wax printer (Xerox, Norwalk, CT, USA), followed by a heating process where printed wax was melted into the paper by placing it on a hot plate (Nissin 99 NHS-450ND) at 150˚C for 180 s. Vivid™ plasma separation membranes grade GR (Pall Corporation, Tokyo, Japan) were cut into 7.9 mm circles by a hand craft punch (McGill, d = 7.9 mm). In order to encase devices comprising multiple layers, hot lamination with a QHE325 laminator (Meiko Shokai, Tokyo, Japan) using 100 μm thick (including top and bottom layer) hot lamination sheets (Jointex, Tokyo, Japan) was performed. Inlet holes on the top lamination sheet layer were cut by a Silhouette Cameo electronic knife blade cutting device (Silhouette, Lehi, UT, USA). For capturing bioluminescence signals from multi-layer 3D-μPADs, a DMC-FZ50 digital camera (Panasonic, Osaka, Japan) was used.

Paper layer modification. In general, to prevent the non-specific adsorption of target antibodies and LUMABS, all filter papers (wax-patterned or non-patterned) were first soaked for 15 min in 0.5 wt% bovine serum albumin (BSA) solution in PBS for blocking,

subsequently immersed for 15 min into water to wash out excess amounts of BSA and allowed to completely dry for 30 min at 40˚C, before any further modification. Figure S1a shows the procedure of paper modification for performing simple spot tests. The filter paper was wax-patterned into spots of 4.0 mm diameter. LUMABS was applied as solution in PBS buffer containing 1 mg/mL of BSA. Figure S1b shows the modification of the upper paper layer for 3D-µPADs carrying the light emitting NanoLuc substrate furimazine. 15 µL of 50 times diluted furimazine in ethanol were deposited, followed by complete drying and cutting into round shapes of 7.9 mm diameter. Figure S1c shows the schematic of the second paper layer (lowermost paper layer of the 3D-µPAD) patterned into multiple signal readout areas (Fig. S1d). In cases of running triplicate assays for a single target antibody from a single sample application, all of the signal readout areas were impregnated with identical LUMABS solutions (in PBS buffer with 1mg/ml BSA) (Fig. S1d-1). When running assays for a single target antibody with different amounts of sensing proteins, different volumes of identical LUMABS solutions were deposited in different signal readout areas (Fig. S1d-2). Finally, for the simultaneous detection of multiple targets in parallel, multiple LUMABS targeting different antibodies (anti-HIV, anti-DEN and anti-HA) were deposited in different signal readout areas (Fig. S1d-3).

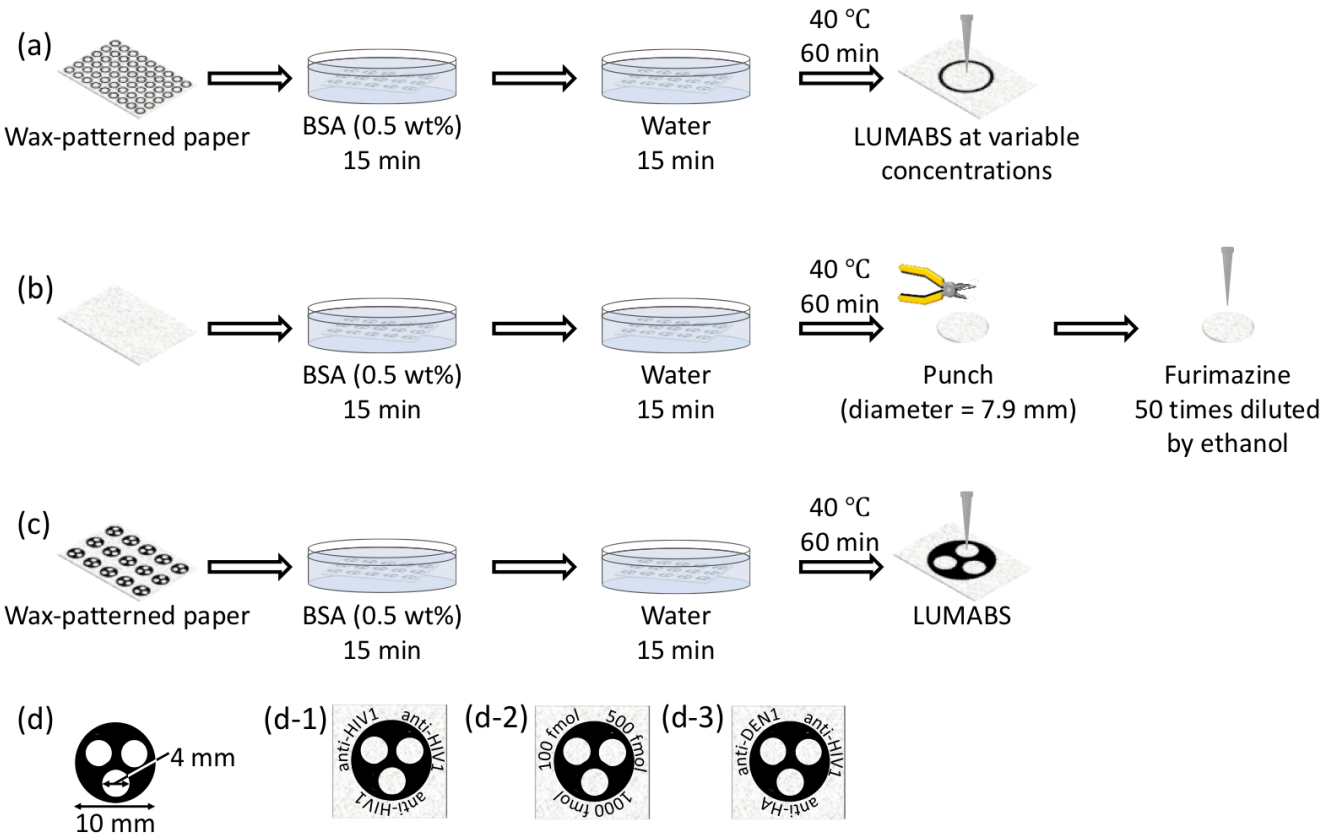

Figure S1. (a) Procedure of paper modification for performing spot tests; (b) modification of the upper paper layer (furimazine impregnated) for 3D-µPADs; (c) modification of the lower paper layer (LUMABS impregnated) for 3D-µPADs; (d) outline of wax pattern set in Adobe Illustrator CC; multiple detection areas impregnated with identical amounts of identical LUMABS (d-1), variable amounts of identical LUMABS (d-2), or different LUMABS (d-3).

Multi-layer device assembly. Devices were assembled in sets of 8 pieces. Onto a wax-patterned and LUMABS impregnated paper sheet (Fig. S1c) with two rows of 4 wax patterns, furimazine impregnated paper discs (Fig. S1b) were set to be centered on each of the underlying wax patterns, followed by placing a plasma separation membrane disc on top of each paper disc. This 3-layered structure (LUMABS impregnated paper, furimazine impregnated paper, blood separation membrane) was sandwiched between the two layers of the hot lamination film with the cut-out sample inlet holes of the top layer being aligned to the plasma separation membrane discs and passed through the hot laminator.

Selection of filter paper substrate. Two different filter paper substrates have been considered for use in 3D-µPADs. These are Whatman No.1 filter paper (GE Healthcare, Tokyo, Japan) and Advantec 5C filter paper (Toyo Roshi Co., Ltd, Tokyo, Japan). The differences between the 2 paper substrates are in their pore size (particle retention size) and their typical thickness. Whatman No.1 filter paper is the most widely applied paper substrate in the field of µPADs. The Whatman No. 1 substrate features relatively large pore size (particle retention size of 11 µm) and a low thickness (180 µm) compared to the Advantec 5C substrate (particle retention size of 1 µm and thickness of 220 µm). The filter paper pore size directly influences the wicking speed of the sample, with larger pores resulting in faster sample liquid transport. In order to achieve a short response time, Whatman No. 1 filter paper was chosen as the paper substrate for the fabrication of multi-layer devices. In addition, its lower thickness compared to the Advantec 5C substrate results in shorter vertical flow distances, further contributing to short response times. Finally, the thinner and more porous substrate

with lower cellulose surface area is advantageous in terms of reducing non-specific interaction between proteins and the paper surface.

Optical signal acquisition. After passing of the respective incubation time, the bioluminescence emission signal was monitored in 3-minute intervals by taking a picture in a darkroom box made out of Styrofoam for eliminating ambient light interference. The settings of the digital camera were as follows: white balance fixed; exposure time 60 s; ISO 1600; f-value 2.8. Images were captured in camera RAW format, followed by conversion into JPEG format by the image processor of the Adobe Photoshop CC software (color temperature 3200 K, tint -150). Using the converted JPEG file, the hue value (on the 0-360 degree scale)[3] of the signal readout area(s) was measured with the image processing software ImageJ (National Institutes of Health). Figure S2 shows photographs of the hand-made box and the auxiliary equipment used to immobilize the paper devices at a constant distance from the camera lens and to monitor the signal from the bottom side of devices.

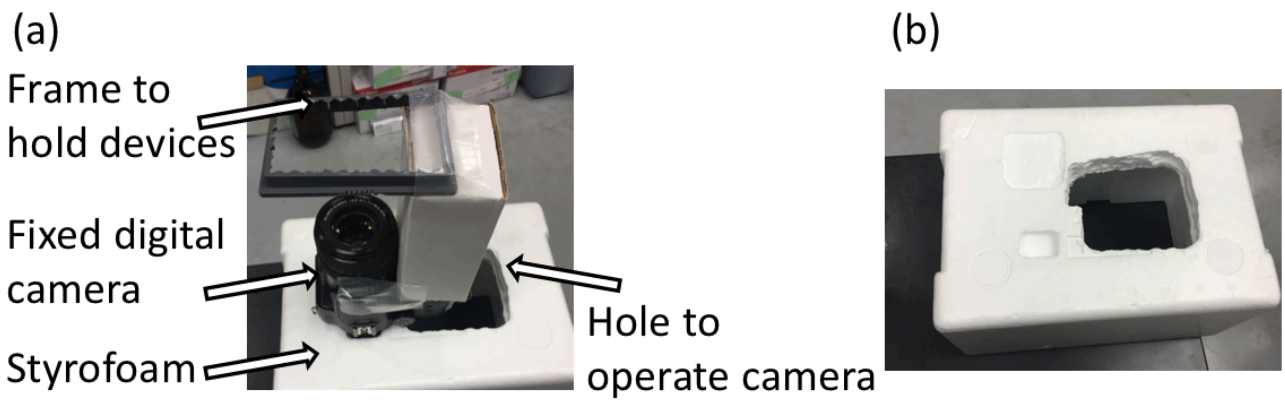

Figure S2. Setup used for bioluminescence signal capture from devices by a digital camera: (a) photograph of the system. The distance between the camera lens and the frame is 8.5 cm; (b) photograph of the hand-made box without camera.

Spot tests and microplate assay. For initial paper spot tests, 1 µL of 100 nM of anti-HIV-LUMABS (corresponding to an absolute amount of 100 fmol of sensor protein) was deposited into the test spots as described above and shown in Figure S1a. After drying completely, 0.3 µL furimazine (30 times diluted by Promega buffer), followed by 4 µL of sample containing HIV1-p17 antibody at different concentrations (in PBS with 1 mg/mL BSA) were added to the test spots. For comparison purposes, the assay was also performed in a white 96-well microplate. 20 µL of HIV1-p17 antibody sample solutions were supplemented with furimazine and LUMABS at a final concentration of 25 nM. In all cases (paper platform and microplate), photographs were taken in the darkroom box starting 15 min after deposition of all reagents.

Assays with multi-layer devices in aqueous buffer solutions or in porcine serum. If not mentioned otherwise, 20 µL of the corresponding sample solution (in PBS buffer with 1 mg/mL BSA or in porcine serum) with a specified antibody concentration was applied to the sample inlet area of device. Photographs were taken in the darkroom box starting 15 min after sample application. For experiments evaluating the influence of applied sample amounts, sample volumes were varied between 20 µL (minimum) and 60 µL (maximum).

Selectivity and cross-reactivity evaluation. The response of 3D-µPADs with signal detection areas modified with different LUMABS (Figure S1d-3) to aqueous solutions of a single antibody or to mixed antibody solutions was evaluated. To adjust for differences in absolute hue values between different sensor proteins, ∆hue values normalized to signals obtained with antibody-free solutions have been used.

Storage stability evaluation. Fully assembled 3D-µPADs modified with anti-HIV-LUMABS were placed into capped vials filled with argon gas containing silica gel desiccant and stored in a freezer at -20˚C. Bioluminescence signal evolvement in aqueous solutions was evaluated at room temperature after various storage periods. Concentrations of pre-deposited furimazine and anti-HIV-LUMABS were as mentioned above for multi-layer devices.

Antibody-spiked porcine whole blood assays with multi-layer devices. Porcine whole blood containing 0.3 wt % citric acid as an anticoagulant was spiked with various amounts of either a single antibody or a mixture of antibodies. Experiments were performed as in the case of working with aqueous buffers or serum, however with the applied sample volume generally being 30 µL (20-60 µL for experiments evaluating the sample volume dependency) and the incubation time before starting photograph acquisition being 21 min to account for the volume reduction by blood cell separation and lower sample flow speeds due to increased viscosity, respectively.

Curve fitting procedure. In analogy to previously reported work,[1, 4] curve fitting of the experimentally obtained data (antibody concentration vs. hue values) was done using equation 1 shown below, where c_{50} indicates the antibody concentration resulting in 50% of the maximal hue change and Δhue_{max} and hue_0 represent the maximal change in the measured hue value and the hue value in the absence of target antibody, respectively.

$$\text{hue} = \frac{\Delta\text{hue}_{\max}[\text{antibody}]}{c_{50}+[\text{antibody}]} + \text{hue}_0 \qquad \text{(equation 1)}$$

Additional Results

Comparison of LUMABS response on paper substrates and in solution

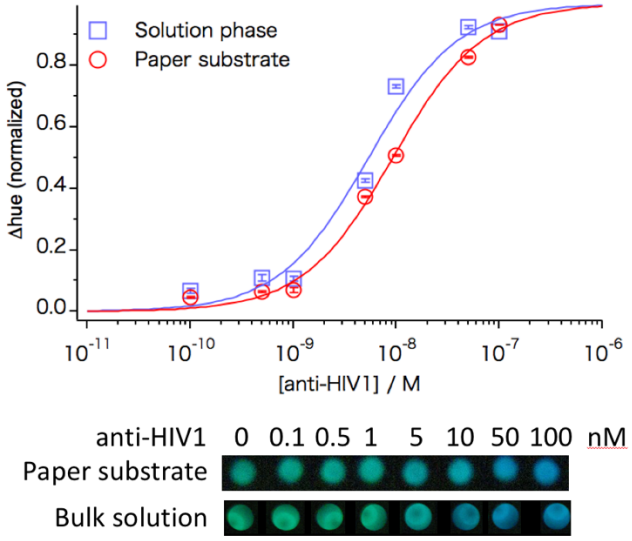

Figure S3. Hue-based response curves of anti-HIV-LUMABS towards the HIV1-p17 antibody on simple paper spots and in solution with equal concentrations of sensor protein. All experiments have been performed in triplicate. To adjust for differences in absolute hue values extracted from photographs of paper substrates and a 96-well microplate, the normalized Δhue values are plotted as a function of the target antibody concentration; top: antibody concentration-dependent response curves obtained with HIV1-p17 antibody targeting anti-HIV-LUMABS (25 nM) on wax-patterned paper or in bulk solution phase; error bars indicate the standard deviations for triplicate experiments; bottom: photographs of actually observed bioluminescence emission. All data recorded 15 min after sample application.

Working with 25 nM of sensor protein, the c_{50} values indicating the sample antibody concentration resulting in 50% of maximal hue change, were found to be 5.39 nM and 9.28 nM for the solution and the paper substrate, respectively. It should be noted, that for experiments on paper substrates, LUMABS concentrations are only approximative values, neglecting the effect of sample liquid evaporation.

Basic response behavior characterization of multi-layer 3D-µPADs towards antibodies in aqueous solution. A first series of experiments was performed with antibody samples applied in the form of pH-buffered (pH 7.4) aqueous solutions (PBS containing 1 mg/mL of BSA). Table S1 summarizes the basic data related to the response curves obtained with 3D-µPADs wherein all signal detection areas have been treated with identical amounts of one specific sensor protein (Figure 2a) and with 3D-µPADs with various amounts of a single sensor protein (Figure 2b).

Table S1. Relevant analytical parameters for response curves shown in Figures 2a and 2b of the main text.

LUMABS type (amount)[a]	c_{50} / nM[b]	hue_0 / degrees[b]	Mean relative st. dev. / % [c]
HIV (100 fmol) (Fig. 2a)	6.35	172.8±0.8	0.63
DEN (100 fmol) (Fig. 2a)	35.57	175.4±0.9	0.48
HA (250 fmol) (Fig. 2a)	8.48	171.5±0.6	0.47
HIV (100 fmol) (Fig. 2b)	5.65	172.1±2.1	0.86
HIV (500 fmol) (Fig. 2b)	55.66	171.1±0.3	0.58
HIV (1000 fmol) (Fig. 2b)	116.16	172.0±1.3	0.48

[a] Absolute amount of LUMABS deposited on a single signal detection area. [b] Standard deviations for measurements performed in triplicate. [c] Mean relative standard deviation of hue values obtained by triplicate measurements over the entire response curve.

Fabrication reproducibility of 3D-µPADs. Figure S4 shows the response curves recorded with 3 batches of 3D-µPADs targeting anti-HIV1, independently fabricated on different days.

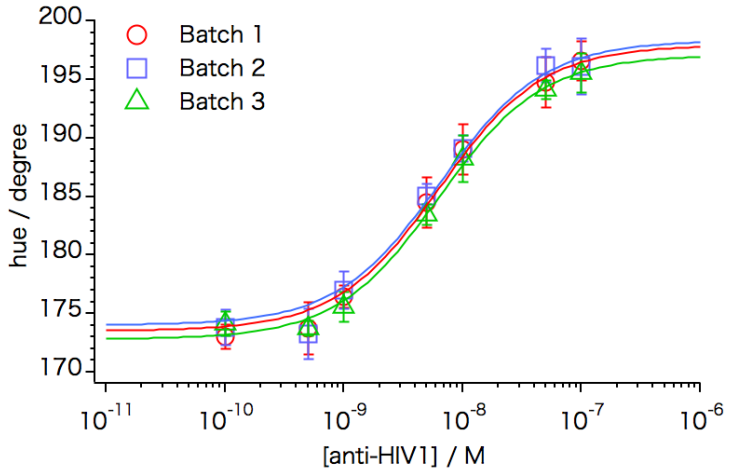

Figure S4. Response curves for 3 independently fabricated batches of anti-HIV1 detecting 3D-µPADs (signal detection areas modified with 100 fmol anti-HIV-LUMABS); error bars indicate the standard deviations for readouts from 3 different signal detection areas on one single device; data recorded 15 min after sample application.

The relative standard deviation of the mean of the c_{50} values obtained from the fitting curves shown in Figure S4 were found to be below 1% for the batches of devices fabricated on three different days (mean c_{50} = 6.40 ± 0.06 nM).

Time-dependency of bioluminescence hue values. The time-dependency of the colorimetric bioluminescence signal was evaluated by monitoring the hue values over 30 min immediately following aqueous antibody sample application to 3D-µPADs as the ones shown in Figure 2a. The resulting data for triplicate measurements is shown in Figure S5.

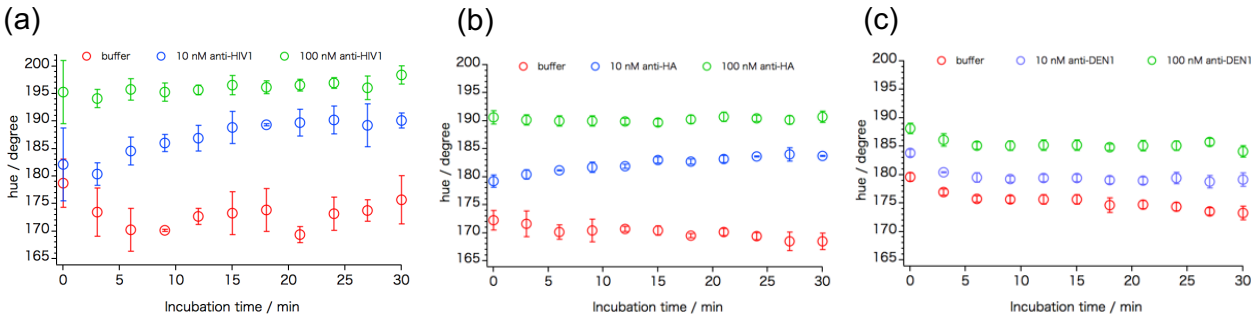

Figure S5. Time-dependent bioluminescence emission hue values recorded from 3D-µPADs after application of aqueous antibody sample solutions: (a) anti-HIV1 detected with anti-HIV-LUMABS, (b) anti-HA detected with anti-HA-LUMABS and (c) anti-DEN1 detected with anti-DEN-LUMABS; error bars indicate the standard deviations for readouts from 3 different signal detection areas on one single device. Please note that only data for selected antibody concentrations (0 nM, 10 nM and 100 nM) has been included in the graph for improved visibility.

The mean relative standard deviations in the hue signal averaged over the entire response curve (0, 0.1, 0.5, 1, 5, 10, 50 and 100 nM) for incubation times between 15 min and 30 min have been calculated as 0.79%, 0.32% and 0.37% for the anti-HIV1, anti-HA and anti-DEN1 detecting devices, respectively.

Storage stability evaluation. Quantitative evaluation of bioluminescence hue signals for 3D-μPADs stored over various periods of time (Figure S6). Antibody samples were applied in the form of pH-buffered (pH 7.4) aqueous solutions (PBS containing 1 mg/mL of BSA).

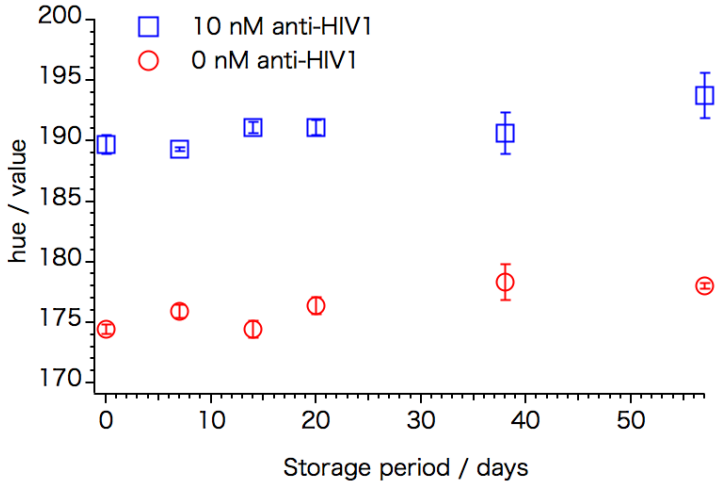

Figure S6. Storage period-dependent bioluminescence emission hue values recorded from 3D-μPADs after application of aqueous anti-HIV1 sample solutions; error bars indicate the standard deviations for readouts from 3 different signal detection areas on one single device.

Response in antibody-spiked porcine serum. Figure S7 shows the response of various 3D-µPADs towards antibodies in spiked porcine serum.

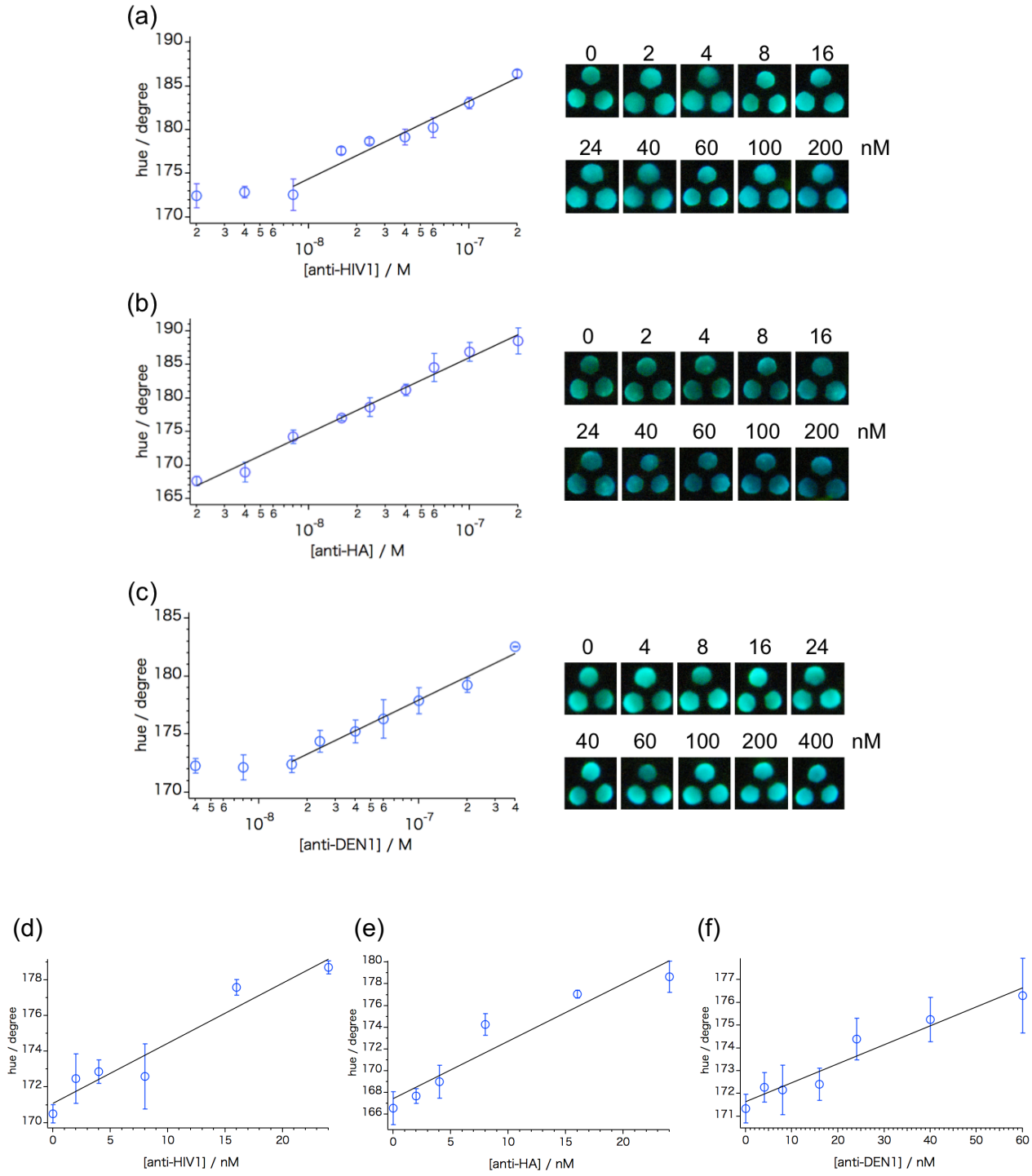

Figure S7. 3D-µPADS applied to antibody assays in spiked porcine serum. Typical response curves and photos showing the actual colorimetric response as recorded by the camera for 3D-µPADs after application of 20 µL of porcine serum samples spiked with various antibodies: (a) anti-HIV1 detected with anti-HIV-LUMABS, (b) anti-HA detected with anti-HA-LUMABS and (c) anti-DEN1 detected with anti-DEN-LUMABS; low concentration-range response curves: (d) anti-HIV1 detected with anti-HIV-LUMABS, (e) anti-HA detected with anti-HA-LUMABS and (f) anti-DEN1 detected with anti-DEN-LUMABS; error bars indicate the standard deviations for triplicate readouts from 3 signal detection areas on one single device; data recorded 15 min after sample application.

References

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