The Bruker In-Vivo Xtreme II System for Flexible and Sensitive Multimodal Preclinical Imaging

Todd A. Sasser[1], Joshua McHattan[2], Jens Waldeck[3].

Author Information: [1]Bruker Biospin Inc., 44 Manning Rd, Billerica, MA, 01821, USA; [2]Bruker Pty Ltd., 163 McEvoy St, Alexandria, NSW 2015, Australia ; [3]Bruker BioSpin MRI GmbH Rudolf-Plank-Str. 23 76275 Ettlingen, Germany.

Here we describe the Bruker In-Vivo Xtreme II system architecture, components and low noise charge-coupled device (CCD). The system provides for multimodal bioluminescent/Cherenkov luminescent imaging (BLI/CLI), direct radioisotopic imaging (DRI), multispectral fluorescent imaging (MS FLI), reflectance (REF), and X-ray imaging with 2D and 360 degree detection modes. The unique configuration combining a highly sensitive CCD detector, powerful xenon illuminator, patented wide-angle emission filters, patented phosphor screen technology, and microfocus X-ray source make the In-Vivo Xtreme II highly flexible and sensitive. Additionally, the system has advanced animal support options for enhanced analytical potential and cross-platform multimodal imaging.

System Architecture

The In-Vivo Xtreme II system operates on a novel inverted detection platform (Figure 1). This provides high uniformity imaging due to low cross-sample shadowing, a consistent flat focal plane, and reduction in the light path from sample to the CCD. The system utilizes a back-thinned/back-illuminated, 16-bit, -90 °C cooled, grade 1 CCD equipped with Hush® technology reducing read-noise even in high bin states. These characteristics culminate into a detector that provides leading minimal detectable radiance of < 60 photons/s/sr/cm^2. As a result, the Xtreme II achieves the highest standards of luminescent imaging for imaging applications employing luciferase (BLI), luminol, and PET radionuclides (CLI).

The In-Vivo Xtreme II system detector including CCD, lens, diopters, and emission filters are mounted on an elevator platform with 6 (7.2, 10, 12, 15, 18, 19 cm^2) preset FOV positions, allowing for imaging at high zoom for high resolution imaging and low zoom for multi-animal imaging. The system transitions sequentially between modalities without upsetting the sample animals' positions and with innate multimodal image registration between all FOVs for all modalities.

Figure 1

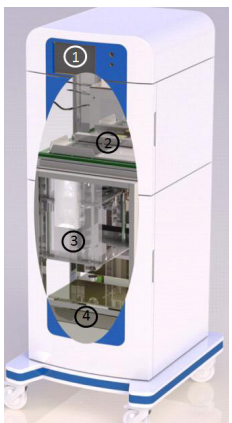

Figure 1: In-Vivo Xtreme II system architecture. (1) Microfocus X-ray source, including magnification stage, and 4-position aluminum filter wheel. (2) Imaging cabinet, including radiographic or radioisotopic phosphor screens. (3) CCD detector/lens, including diopters and 8-position emission filter wheel mounted on an elevator platform. (4) Xenon illumination lamp, including 28 position filter wheel.

The CCD Camera

Low-flux luminescent scientific applications, including areas of *in vivo* imaging utilising bioluminescence, DRI and fluorescence, may demand an exceptional level of sensor performance in order to achieve a useful signal-to-noise (SNR). There are a number of factors contributing to noise which can influence the performance and SNR of a CCD (Moomaw, 2013). These factors have a significant impact on the detection limit and therefore sensitivity of a detector. These variables are discussed below in the context of the Xtreme II detector.

- Dark Noise or Dark Current (DC) is due to thermal excitation of electrons and is generated even in the absence of any light, this is then collected within the wells of the CCD, and is read as signal independent of its source. Since thermally induced photons are the main contributor to dark current, cooling the camera reduces this unwanted electron source. In similarity to photon noise, dark noise follows a Poisson relationship to dark current, and is equivalent to the square-root of the number of thermal electrons generated within the image exposure time (Fellers & Davidson). The Xtreme II camera is deeply-cooled to -90 °C absolute which reduces the DC of 0.00018 e/p/s.

- Shot noise is incurred from the inherent statistical variation in the arrival rate of photons incident on the CCD. Photoelectrons generated within the semiconductor device constitute the signal, the magnitude of which fluctuates randomly with photon incidence at each measuring location (pixel) on the CCD. The interval between photon arrivals is governed by Poisson statistics, and therefore, the photon noise is equivalent to the square-root of the signal. Shot noise cannot be reduced via camera design (Fellers & Davidson).

- Read Noise (RN) is a combination of system noise components inherent to the process of converting CCD charge carriers into a voltage signal for quantification, and the subsequent processing and analog-to-digital (A/D) conversion. The major contribution to read noise usually originates with the on-chip preamplifier, and this noise is added uniformly to every image pixel. Certain types of noise in the CCD's output amplifier are frequency dependent (and consequently the application for which the camera is intended) and the required read-out rate or frame rate partially determine the read noise specification and its practical effect on overall signal-to-noise level. Today, read noise is the main limiting factor in the CCD SNR since manufacturers are at the current limits of reducing DC (Fellers & Davidson). Therefore efforts to reduce read noise even at high bin states has been investigated, leading to the development of the Xtreme II camera with what has been termed HUSH technology, the result of which boasts the lowest read noise values to date, even at binning 32. Routinely these cameras are measuring well below the rating of 3 electrons (bin 1,2,4) and 5 electrons (bin 8,16,32).

Figure 2

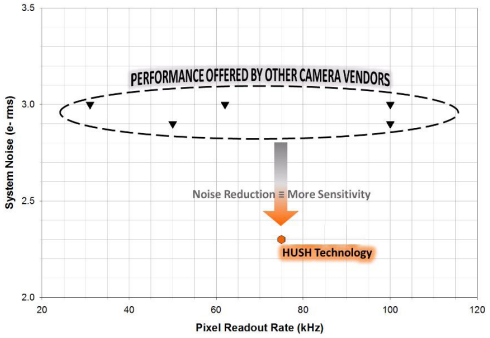

Figure 2: HUSH Technology provides for ultra-low CCD read noise.

- Binning is in itself is not a noise contributor, instead plays an important role in adjusting CCD sensitivity. This is achieved by a process of combining charge from adjacent pixels forming a "super-pixel" by a factor of the bin value selected, meaning there are less number of pixels (or steps) charge needs to travel to reach the readout amplifier, which would otherwise add the read noise value for each pixel movement shift. This process does reduce resolution, however since light scatter in tissues is the overriding factor in determining optical imaging resolution, it is often more useful to simply detect or not detect a signal of interest at the earliest possible time point. This makes binning a very useful tool to adjust for sensitivity for a particular specimen. The higher the binning available, whilst still maintaining a low RN such as found with the Xtreme II camera, the more SNR the user can obtain. Hence the Xtreme II offers a 32x32 binning option as standard, pushing the detection limits to as low as 41 p/s/cm^2/sr.

The Xtreme II camera delivers ultra-low noise, outperforming all cameras currently available that utilize either the same or similar detector arrays (Figure 2). This new camera development empowers researchers with the highest sensitivity available from a CCD sensor array in low-light imaging application.

Illuminator and Filters

The In-Vivo Xtreme II system is equipped with a high intensity broad spectrum (350 – 1000 nm) 400 Watt Xenon illuminator (Figure 3) with a fully populated 28 position (410 – 760 nm, 10 nm +/- bandpass filters with additional custom filters available) excitation filter wheel. The strong lumen flux provided by the Xenon illuminator supplies a constant illumination of fluorochromes over the whole spectrum. (This is not true of either Mercury/Halogen- or LED-based illuminators.) Peaks

are compensated by optical excitation based correction factors as well as calibration to enable comparisons and flatten illumination peaks. The bulb is equipped with a plug & play sled for ease of use upon bulb exchange.

Figure 3

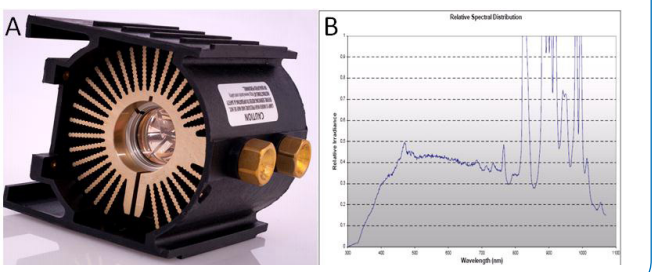

Figure 3: (A) 400 Watt Xenon Bulb with fast plug & play installation setting. (B) High lumen flux from visible to the near infrared wavelengths spectrum allows imaging of all common commercial and/or designed fluorochromes without limitation.

The large excitation filter selection allows for flexible filter selection for visible (VIS) to near infrared (NIR) imaging as well as excitation based deconvolution/spectral unmixing (MS FLI) (Alexander et al., 2008). The system is supplied with a fully populated 8 positon emission filter wheel (535 – 830 nm, 20 nm +/- bandpass filters with additional custom filters available e.g. for upconversion nanoparticles. The system employs patented wide-angle emission filters that show consistent blocking efficiency even outside the center of the filter, improving the SNR ratio and overall image uniformity (Figure 4).

Figure 4

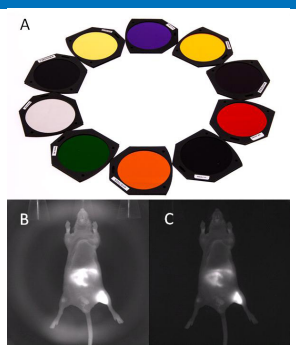

Figure 4: (A) Selection of patented wide-angle emission filters. (B) Example of *in vivo* FLI with a standard emission filter. The ring artifact observed is due to poor blocking at the peripheral of the filter. (C) Example of *in vivo* FLI with a patented wide-angle emission filter.

Radiographic Screen and X-ray Source

For DRI and X-ray imaging, a radioisotopic or radiographic phosphor scintillation screen (respectively) slide between the sample and the detector, converting gamma energy to photons for detection by the CCD detector (Figure 5). Enabling the scintillation screens to position underneath samples during image acquisition is advantageous, since there is no limitation to either animal height (imaging of larger research animals is possible) or the selected FOV, unlike an overhead configuration that requires a reduced specific distance between the samples whilst in an active mode, often leading to poor resolution, shadowing and mismatches in overlays.

Figure 5

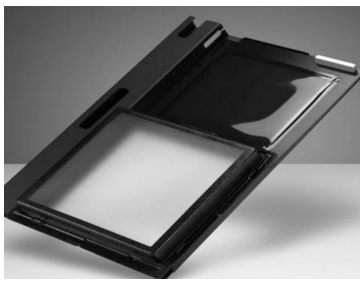

Figure 5: Radiographic phosphor screen installed in the In-Vivo Xtreme II Animal Management Center (AMC) frame. (The AMC is shown outside the In-Vivo Xtreme II cabinet). The open imaging window is shown to the left. The radiographic screen is shown to the right in the inactive position.

The 500 µA, adjustable 20-45 kVp, <60 µm spot size micro focus X-ray head provides leading digital X-ray resolution, low dose (~0.3 mGy) and high temporal (0.2 s) image acquisition that also supports dynamic imaging utilizing X-ray contrast agents, and a range of specimens with varied degrees of densities. Coupled with a geometric magnification stage for 3x magnification for reaching ≥ 21 lp/mm resolution (Figure 6), a 5-position aluminum filter wheel for variable beam hardening, and automated X-ray energy calibrations that along with the optional plug-in Bone Density Software allow for absolute long bone $CaPO^4$ (g/cm^3) measurements (Vizard et al., 2010). X-ray not only supplies an anatomical map for molecular imaging, but is also a useful research tool in its own right for absolute quantitative studies where a change in density is observed.

Figure 6

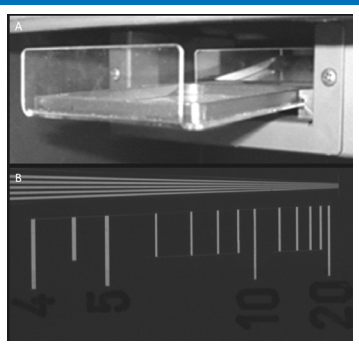

Figure 6: (A) Geometric magnification stage providing X-ray magnification for specimen imaging. (B) Line-pair gauge imaged using X-ray with the In-Vivo Xtreme II.

Radioisotopic Screen

For DRI, a radioisotopic phosphor screen (thicker compared to the radiographic phosphor screen to provide higher sensitivity) converts both PET and SPECT radionuclide gamma energy to photons. The In-Vivo Xtreme II Is the only preclinical multimodal system equipped for both CLI and DRI. DRI allows researchers to image some radionuclides commonly used in nuclear medicine (e.g. ^{99m}Tc) that are not detectable using CLI and allows for deep tissue penetration imaging. The CLI/DRI combination provides for highly flexible radionuclide tracer imaging with excellent temporal resolution. This imaging compliments dedicated PET/SPECT platforms, due to its multi-animal throughput, relative short exposure times (milliseconds to seconds) and high sensitivity, e.g. down to 0.05 µCi of ^{99m}Tc (Doney et al., 2014). Large animal cohorts with therapeutic combinations can be screened for research importance using DRI, reducing cost and demand for more complex PET/SPECT analysis. Additionally, DRI allows preclinical researchers to use small radionuclide reporters that do not typically upset the normal biodistribution of the tagged compound. This is particularly useful for researchers working with small compounds or developing/validating PET and SPECT tracers (Kularatne et al., 2009 a, b).

Animal Chambers and Beds

The In-Vivo Xtreme II system is offered with a range of animal chambers/beds. These solutions provide for optimum animal care, end-user safety, and analytical and multimodal potential.

The In-Vivo Xtreme II system is available with an advanced anesthesia system. This is a combined anesthesia and evacuation system that includes several unique features:

- Provides exact anesthesia delivery and collection.
- Includes an animal chamber that isolates gas when closed and is configured with HEPA filters to support SPF facilities (Figure 7A).
- Includes an EquaFlow 5-animal manifold that provides even distribution of anesthesia gas to all animals (Figure 7B & C). (Necessary for stable/safe maintenance of anesthesia for all animals and consistent luminescence enzyme kinetics for quantitative analysis).
- Includes an active gas collection system at each nose cone to isolate gas near the source outlet.
- Optional 3-animal and rat manifolds are available.
- Includes a series of scavenging vacuum lines in the animal chamber to isolate gas that escapes the nose cone collection system.
- Includes a series of vacuum lines at the system cabinet door, scavenging isoflurane that might escape the nose cone and chamber collection systems, thus providing an up to 99.9 % security for the operator to be exposed to toxic isoflurane or other gas components.
- Includes a scavenger line at the lip of the induction box opening to secure effective and efficient isoflurane clearance after anesthetic induction.

The animal chamber is temperature-controlled by regulated warm air flow. In contrast to temperature control designs using heated platforms, the In-Vivo Xtreme II system design provides even temperature across the anatomy of the animal. Additionally, the optional Multimodal Animal Transport

Figure 7

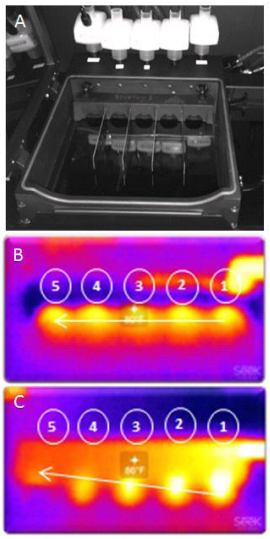

Figure 7: (A) Animal chamber installed with the 5-nose cone EquaFlow manifold. (B) Warm airflow/thermal imaging test results showing even airflow in the 5-nose cone EquaFlow manifold, and (C) graded airflow a 5-nose cone manifold supplied with an alternative optical imaging instrument. (In each thermal image heat was inserted in the manufacturer's anesthesia inlet line at the right. Heat signature is shown in fire.)

System (MATS) provides for 4-animal transport between the In-Vivo Xtreme II and the Albira PET/SPECT/CT system, facilitating throughput and cross-platform imaging (Wathen et al., 2014). Alternatively, the optional Multimodal Animal Bed (MMAB) allows for animal transport between the In-Vivo Xtreme and any of the MMAB compatible Bruker Preclinical BioSpec and Icon MRI, SkyScan microCT, and Albira PET/SPECT/CT systems (Figure 8A-E) (Sasser et al., 2015). This facilitates imaging using platforms offering the highest possible performance. For example, cross-platform imaging using a whole body high resolution SkyScan microCT scanner and In-Vivo Xtreme II provides for optical imaging with microCT resolution/features not available with any commercial integrated microCT system (Figure 8F).

Figure 8

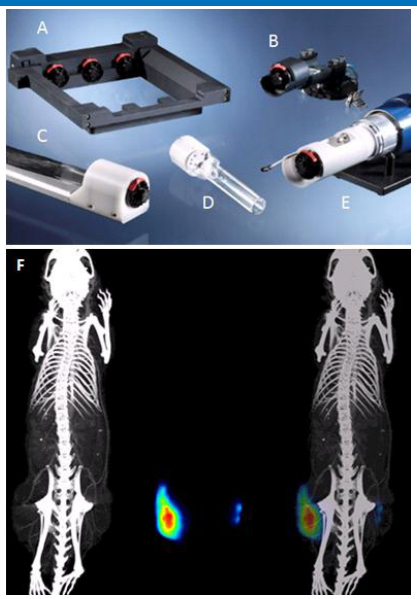

Figure 8: MMAB and adapters. (A) In-Vivo Xtreme II adapter. (B) SkyScan microCT adapter. (C) Albira PET/SPECT/CT adapter. (D) MMAB chamber. (E) ICON MRI adapter. (F) Cross-platform BLI (rainbow) and µCT *in vivo* imaging of tumor xenograft model using In-Vivo Xtreme II and SkyScan 1176.)

Rotation Imaging

The optional Multimodal Animal Rotation System (MARS) facilitates automatic incremental animal rotation for multi-projection/segmented imaging. MARS imaging provides for maximum sensitivity by optimizing detections for the shortest tissue light path (Figure 9).

Figure 9

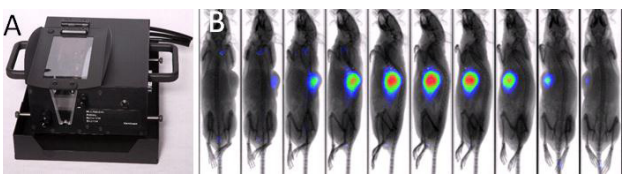

Figure 9: (A) The MARS module facilitates automated multimodal rotation acquisitions. The MARS employs a rotation bed that allows for full 360° axial rotations. (B) Example multimodal FLI (rainbow) and X-ray (gray) *in vivo* rotation acquisitions of a mouse with a fluorescent xenograft tumor. Lateral projections show maximum sensitivity due to the optimized tissue light path for detections at these angles.

Software

The In-Vivo Xtreme II is provided with a comprehensive software package. The Bruker Molecular Imaging (BMI) Software controls acquisitions and provides for a range of image preparation and data analysis features (https://youtu.be/5lVRiNKCmb4). The Capture menu includes an intuitive "foreground/background" interface with several presets for all modalities. More complex longitudinal and multiplex acquisition can be easily programmed using the software Protocol tool. Post-acquisition image overlays and contrast tools produce publication quality displays. The BMI Software data analysis features include highly flexible unit conversion, ROI analysis, and data reporting of single or multiple images. The images can also be analyzed in "batch mode". The BMI Software can be installed on multiple remote computers to liberate the control computer for use making system acquisitions.

The In-Vivo Xtreme II system is the only multimodal imaging system able to provide absolute $CaPO^4$ (g/cm^3) long bone density measurements. The system employs a unique X-ray energy calibration and bone cylinder model for these measurements. The measurements are made using the patented, optional Bruker Bone Density Software (https://youtu.be/FPfd6_5rung).

All In-Vivo Xtreme II acquisitions are driven via the BMI Software, including multispectral (MS) and rotation (MARS) acquisitions. Post-acquisition processing for MS and MARS acquisitions are performed using the Bruker Multispectral Software (https://youtu.be/iEyLl3qAzpA) and Bruker Rotation Software modules (https://youtu.be/V49qHYAqWsY). These software modules allow for customized MS modeling and display for multispectral and rotation data. The Bruker MS software employs an excitation based modeling approach that, relative to emission based approaches, is more suitable for distinguishing fluorescent spectrums.

Optional/Custom Items

Sometimes special research needs require special solutions. Bruker works to enable researchers with adapted solutions upon request, e.g. by offering non-standard emission filters to researchers focusing on upconversion nanomaterials, DRI collimators for improved radioisotopic imaging resolution, NIST traceable calibration phantoms, or non-standard HEPA filters.

Conclusion

The In-Vivo Xtreme II system offers the leading detector technology providing for unparalleled imaging. The unique inverted imaging geometry allows for excellent image quality across the full FOV, imaging and registration of all modalities at all FOVs, and X-ray imaging of large samples. Patented wide-angle emission filters ensure that florescent imaging across the FOV is artifact free, and the large selection of excitation filters and multispectral software enable advanced fluorescent deconvolutions. The phosphor screen technology provides radiographic and flexible radioisotopic imaging. The In-Vivo Xtreme II is the new gold standard in CCD-based non-invasive, small animal imaging technology.

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Bruker BioSpin

info@bruker.com
www.bruker.com