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# B-Scan Ultrasonographic Monitoring of Orthotopic Xenografted Plexiform Neurofibroma in Mice

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**Abstract.** *Background/Aim: Xenografted benign tumours in immunodeficient mice provide an in vivo model to study tumour biology and the effect of agents on tumour growth. Conventionally, these small grafts can only be monitored upon sacrificing the animals. We evaluated ultrasound biomicroscopy for monitoring such grafts in vivo. Materials and Methods: Small fragments (<10 mm$^3$) of a plexiform neurofibroma obtained from patients with established diagnosis of neurofibromatosis type-1 (NF1) were orthotopically-xenografted onto the sciatic nerve of immunodeficient mice and monitored using a high-resolution in vivo micro-imaging system. Results: Grafts were identified in most cases and were distinguished from the surrounding inflammatory host tissues by detailed ultrasonographic signals. Graft sizes could be calculated precisely from serial scan sections and monitored during the whole course of drug treatment. Conclusion: High frequency sonographic measurement is a superior non-invasive method for monitoring small grafts of slowly growing benign tumours in mice in vivo, e.g. plexiform neurofibroma, and is especially suitable for tracing the effects of drugs at multiple time-points, thus allowing a very cost-effective follow-up.*

Neurofibromatosis type-1 (NF1) is an autosomally-dominant inherited disease. Neurofibromas, the hallmark of NF1, are benign nerve sheath tumours of limited size, predominantly arising from the skin. A subtype of neurofibroma, termed plexiform neurofibroma (PNF), differs from cutaneous/dermal neurofibroma by its ability to invade and destroy surrounding tissues. PNF are tumours originating from major nerve fibres that can grow to extremely large sizes, causing various deficits (1). Treatment of PNF is still limited to surgical intervention. However, since tumours often infiltrate adjacent tissues, complete resection is usually not possible without damaging nerves and healthy tissues. Currently, there is no established medical therapy for PNF.

Recently, we successfully used an *in vivo* model to test for the efficacy of imatinib mesylate, a receptor tyrosine kinase inhibitor, for PNF treatment (2). Xenografting small pieces (<10 mm$^3$) of human benign PNF into immunodeficient mice provides an *in vivo* model for these tumours which is especially valuable for testing drugs. However, in our previous experimental study we had to sacrifice animals at various time-points in a preliminary experiment in order to determine the size changes of the grafts during a period of seven weeks of observation. For the treatment study, grafts could only be measured before the implantation and after sacrificing the animals at the very end of the experiment. Therefore, the effects of the drug could not be traced during the actual course of treatment. These limitations of our investigations are due to the benign nature of PNFs, being very slowly-growing tumours in humans. Indeed, the implants do not grow and remain very small or often even shrink in host mice (2). In addition, these orthotopic xenografts are implanted adjacent to the sciatic nerve, situated below the muscle layer, and therefore cannot be detected and measured from the outside of living animals as can subcutaneously-implanted xenografts. Consequently, the same implant cannot be traced entirely from the beginning to the end of an experiment and additional animals are needed for each additional measurement during the course of the treatment. Furthermore, host inflammatory tissue around the implant is recognized as a part of the latter when measured with callipers, leading to the misinterpretation of tumour growth (3). Finally, manual measurement with callipers itself is imprecise for small implants with volumes

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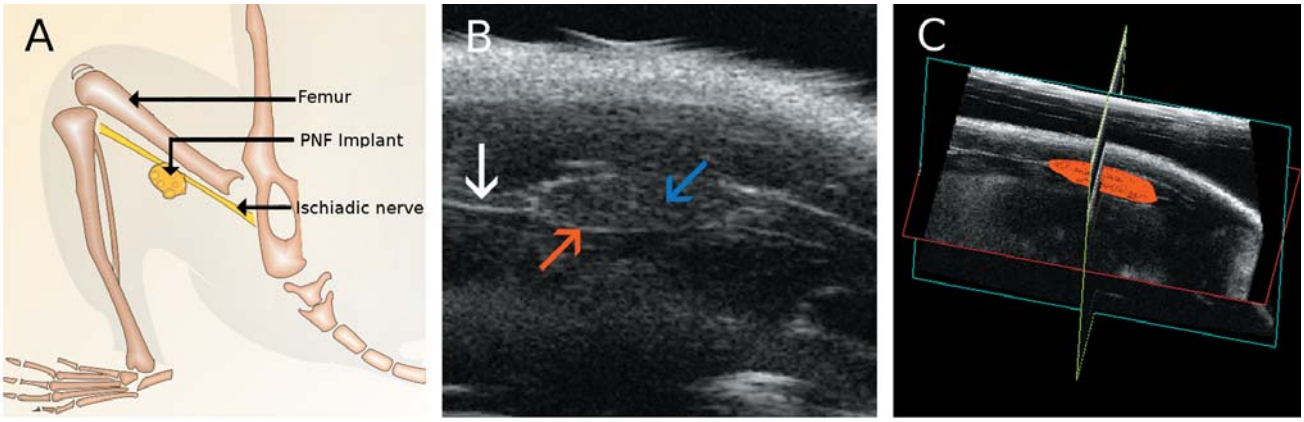


Figure 1. *A: Anatomical illustration of the implant on the sciatic nerve of a mouse. B: Ultrasound imaging of a plexiform neurofibroma graft (blue arrow) and surrounding echogenic inflammatory area (red arrow) on the sciatic nerve (white arrow). C: Three-dimensional reconstruction of the graft.*

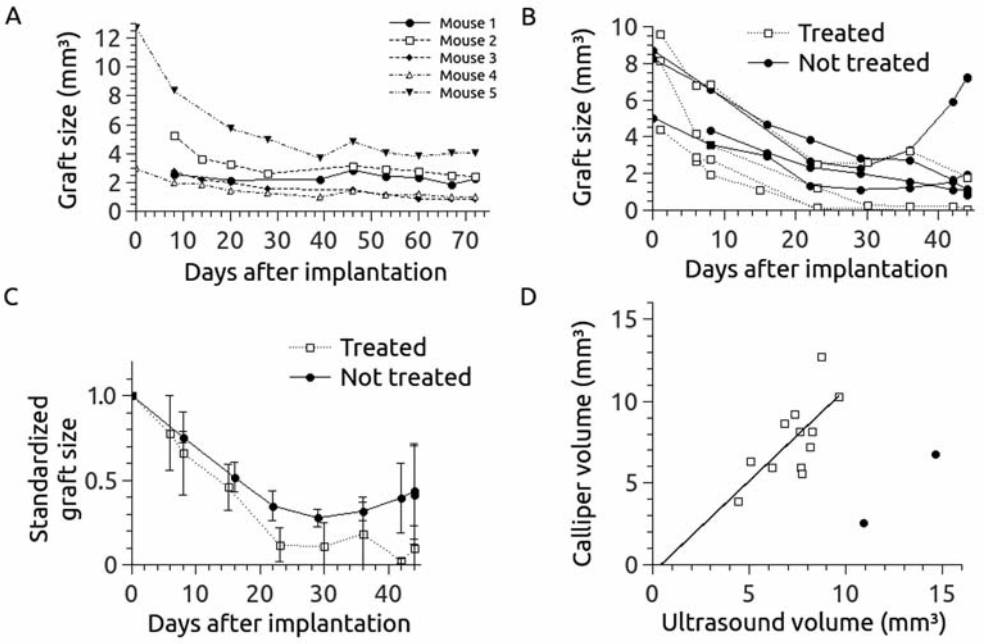


Figure 2. *Size changes of the grafts in nude mice (A) and in severe combined immunodeficiency (SCID) mice (B, C). Open circles are mice treated with imatinib (75 mg/kg daily) and solid circles are untreated mice. C: Means and standard deviation of individual mice shown in B. D: Correlation of sizes calculated from serial ultrasound sections with those from calliper measurements, the latter calculated using the two longest perpendicular axes with the assumption of an ellipsoidal body [V=(m1+m2)³π/48]. The two outliers (filled circles) were due to obvious non-ellipsoid forms of the grafts and therefore were not included in the calculation of linear regression.*

of less than 10 $mm^3$ and the simplified geometric form derived from calliper measurements for volume calculation does not generally represent the real form of the grafts correctly, resulting in large data variability. Magnetic resonance tomography and positron-emission tomography can also be applied for detecting xenografts in small animals. However, these measurements require a large apparatus and special skills. Furthermore, the procedures are complex and the associated costs are high. High frequency B-scan ultrasound biomicroscopy visualizes tissues with high resolution and provides a fast, convenient, inexpensive and safe alternative imaging tool. On the other hand, high-frequency ultrasound lacks penetrating energy. For example, ultrasound at 40 MHz has a focus at 1 mm depth and reaches

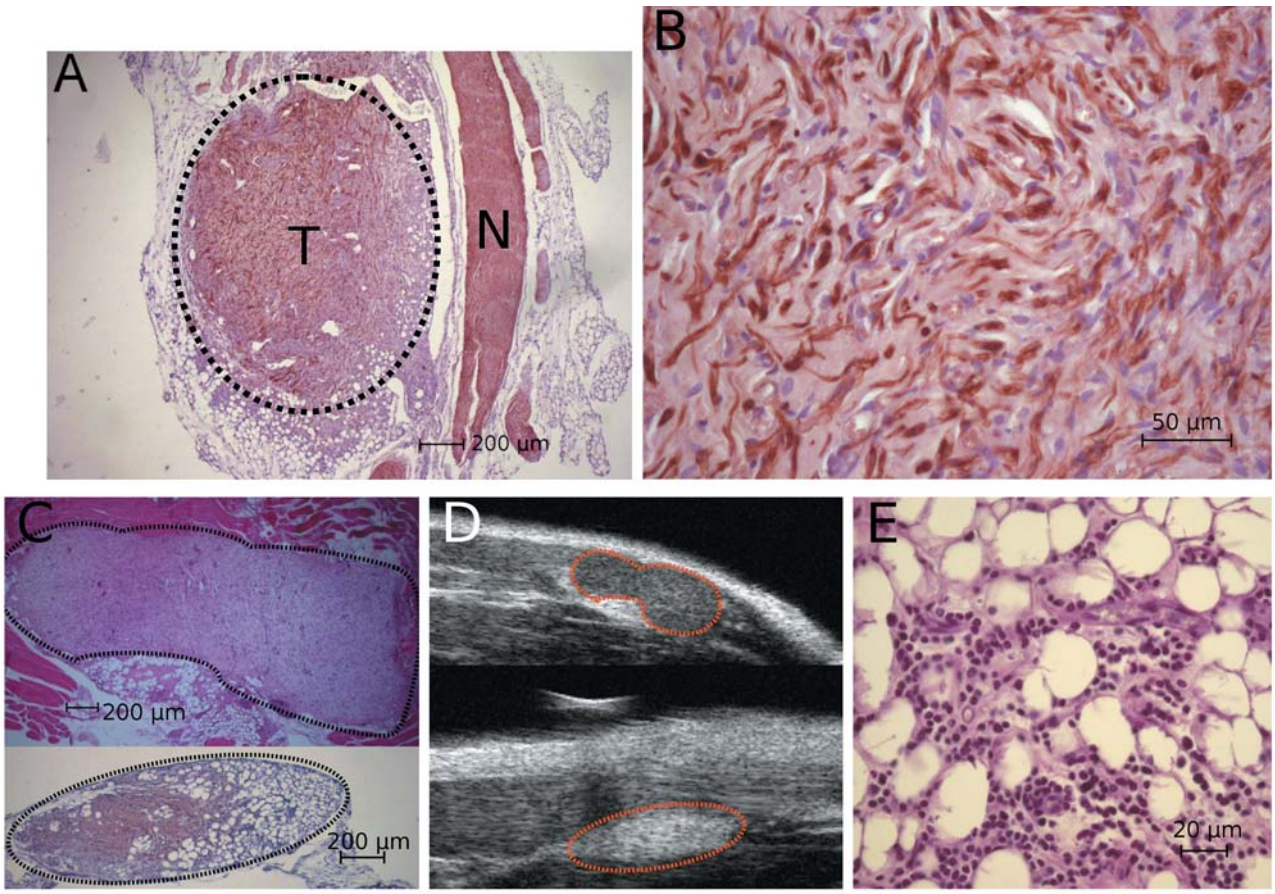


Figure 3. *Histology of the grafts. A: Tumour graft (T) on the sciatic nerve (N); B: S100 positively-stained Schwann cells in the tumour graft area. C, D: Good correspondence of echo image with the histological appearance of a graft. E: Periodic acid-Schiff reaction (PAS) staining revealed massive invasion of mast cells at the tumour boundaries.*

only to 1 cm. Therefore, clinical application of high-frequency ultrasound is limited to visualization of superficial structures, for example, in dermatology and ophthalmology. Since small animals such as mice are only 1/100 the size of human, high-frequency ultrasound reaches more organs in these organisms and thus provides a superior measuring technique for studies in cardiology, oncology and developmental biology (4-6). For tumour implants, serial vertical scanning enables three-dimensional reconstruction and precise volume calculation, an additional advantage over direct manual measurement (7). In the present study, we applied this imaging technology to monitor small implants of PNF on sciatic nerves of mice.

## Materials and Methods

PNF were obtained from two unrelated patients with NF1 who underwent surgery at the University Hospital Hamburg Eppendorf (REF). All patients gave informed written consent, and the local Ethics Committee approved the study protocol (No. 2863). After saving sufficient material for histological examination, parts of each specimen were used for xenografting into nude mice and severe combined immunodeficiency (SCID) mice. Insertion of the transplants and treatment with imatinib mesylate at daily dose of 75 mg/kg using osmotic minipumps defining a releasing rate of 0.2 µl/h was carried out as described previously (2). All animal experiments were approved by the local authority (No. 68/06).

Ultrasonographic measurements were carried out weekly using a Vevo 770 high-resolution *in vivo* micro-imaging system (VisualSonics, Toronto, Canada). Mice were anaesthetized with isoflurane/medical oxygen at an initial dose of 4% and subsequently at 2% throughout the whole measuring period of approximately 10 minutes, and were fixed on a warming plate. B-Mode, a two-dimensional image composed of bright dots representing the ultrasound echoes, was chosen for our measurement. The single element scan-head RMV704 at 40 MHz has a focus at a depth of 1.5 mm, a focus range of 6 mm, and resolution of 40 and 30 µm in the two horizontal and 80 µm in the vertical directions. We started the scan with the applicator located closely to the longitudinal aspect of the femur. The surface of the bone can easily be detected by ultrasound due to its high echogenicity and well-defined structure. A small hypoechogenic structure with dense internal

echoes represented the PNF transplant appearing at the caudal side of the femur and on the sciatic nerve. The echogenic suture that was applied to fix the graft provided an additional landmark for identification of the transplant in the first two weeks post implantation. Once the implant was identified, vertical serial scanning was carried out automatically with a 30 μm step-wide movement of the scan-head, which lasted less than one minute. One complete measurement procedure took approximately 10 minutes, mainly spent on anaesthesia and identifying the transplant. The acquired data were saved and analysed later in the laboratory.

The volume of the transplant was calculated using the integrated software of the Vevo 770 imaging system (6, 7). To obtain an overview and impression of the transplant, the scanned area was first turned and inspected from various directions as well as at various sections. After becoming familiar with the size, form and localization of the implant, virtual sections with a thickness of 150 μm were defined. On each of the virtual sections, the area of the transplant was marked manually. This took approximately three to four minutes. The volume was calculated subsequently by adding transplant areas of all sections together and multiplying by the section thickness of 150 μm.

## Results

Transplants of PNF on sciatic nerves of mice were found in most cases (Figure 1). However, at the beginning of our study, finding the implants and identifying them was not straightforward for an inexperienced operator.

In both nude and SCID mice, the sizes of grafts decreased in the first three weeks and became stable afterwards (Figure 2A-C). This initial size reduction was rapid for large grafts (Figure 2C). No growth of the grafts was observed throughout the whole experimental period of six weeks. At the end of the experiment, each graft was measured by both ultrasound, and after sacrificing the animals, with callipers. The sizes obtained by the two methods correlated well for ellipsoidal tumours (Figure 2D). Grafts seemed to shrink more in imatinib-treated mice (Figure 2C). However, the difference was not significant in this experimental setting where only four mice were included in each group.

Histological examinations at the end of the experiment revealed good integration of grafts into the host tissue on the sciatic nerve (Figure 3A). Tumour features, such as positive staining for S-100, were retained in mice (Figure 3B). Echogenic boundaries of the graft correlated well with fatty tissues in the histological section (Figure 3C and D). Massive invasion of mast cells at the boundaries suggested inflammation around the graft (Figure 3E). Grafts from nude or SCID mice did not differ in histological features.

## Discussion

We successfully applied high-frequency B-scan ultrasonography to detect orthotopic xenografts of PNF fragments on the sciatic nerve of mice, which enabled for monitoring of single grafts during drug treatment. By doing this, intra-graft variability can be excluded and the number of study animals required can be reduced. Furthermore, ultrasonographic measurement is simpler, safer, faster to perform and less expensive than other imaging techniques, such as magnetic resonance tomography and positron emission tomography, which also require bulky apparatuses.

Although not applied in the present study, perfusion within and surrounding the grafts can also be measured by Doppler ultrasonography with a special scanning head (8). Such measurement enables the assessment of angiogenesis inside the tumour grafts and consequently the demonstration of antiangiogenic effects of therapeutic agents.

Since mice need to be anaesthetized for measurements, the grafts should be found as quickly as possible. Knowledge about the murine anatomy and several training sessions with dead mice prior to undertaking *in vivo* studies are, therefore, advisable. Attention should also be paid to focussing of the serial scans on the region of interest, which is important for the subsequent volume calculation. Both growth behaviour and histological features of PNF grafts were similar in the more immunocompromised SCID mice, and in nude mice. Therefore, nude mice may be the animal model of choice because the lack of hair facilitates ultrasound sessions and these mice are less prone to infection.

In our previous study, a temporary enlargement of the grafts was observed in the first three weeks, which we assumed to correspond to inflammation of adjacent host tissue but not to growth of the grafts (2). However, due to the experimental design used there, this hypothesis could not be tested in the laboratory. In the present study, micro-ultrasound was easily able to distinguish surrounding inflammatory tissues from the grafts and therefore enabled size calculation of the latter exclusive of the former. No enlargement of the grafts was observed, confirming our previous assumption that grafts do not grow in mice in such a short period, of less than two months. Lack of size increase of the grafts is in concordance with our recent finding that PNFs do not enlarge in the majority of patients with NF1 and in patients where they did grow, they grew only very slowly (1). Therefore, our experimental setting appears to be of advantage compared to other study protocols not allowing for long-term measurement of the tumour volume in individual mice, both as far as the limiting impairment of the animals' well-being and the accuracy of measurements are concerned.

The shrinkage of the grafts can be explained by the presence of apoptotic cells in the original tumour fragment which are gradually absorbed by the host. The initial rapid reduction in size of larger grafts likely reflects massive cell death due to inflammation and insufficient nutritional supply. This observation and our previous experience suggest that grafts should be no larger than 4 μl in volume, although larger grafts are generally preferred since they are easier to detect and monitored.

In summary, high frequency ultrasound biomicroscopy provides a superior non-invasive method for monitoring and measuring xenografted benign tumour fragments, such as small PNF fragments, on the sciatic nerve of mice, and which can also be applied for *in vivo* models for other solid tumour types.

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