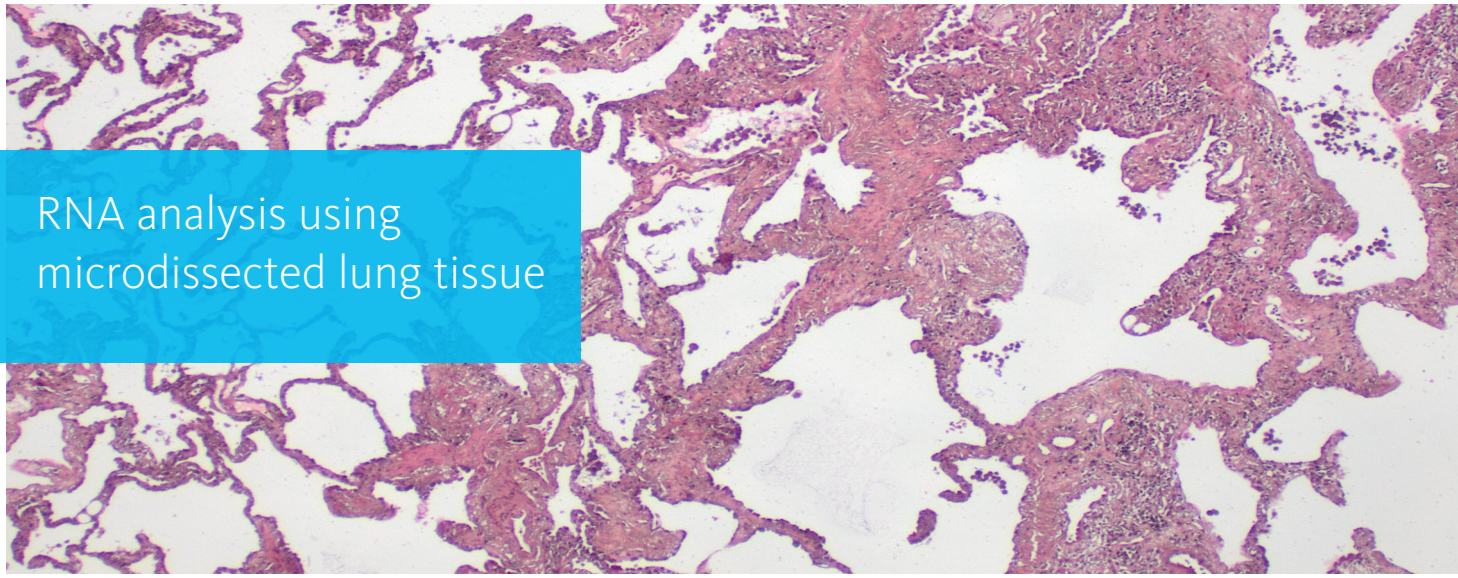
Application Note

# Comparative Analysis of Gene Expression in Fibroblastic Foci in Patients with Idiopathic Pulmonary Fibrosis and Pulmonary Sarcoidosis



RNA analysis using microdissected lung tissue

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## Abstract

Fibroblastic foci (FF) are characteristic features of usual interstitial pneumonia (UIP)/idiopathic pulmonary fibrosis (IPF) and one cardinal feature thought to represent a key mechanism of pathogenesis. Hence, FF have a high impact on UIP/IPF diagnosis in current guidelines. However, although less frequent, these histomorphological hallmarks also occur in other fibrotic pulmonary diseases. Currently, there is therefore a gap in knowledge regarding the underlying molecular similarities and differences of FF in different disease entities.

In this work, we used laser capture microdissection, mRNA and protein expression analysis to investigate the compartment-specific gene expression profiles of FF in IPF and sarcoidosis to elucidate similarities and differences as well as shared pathomechanisms.

Our results demonstrate that FF of end-stage IPF and sarcoidosis lungs, although different in initiation, are similar in gene and protein expression, encouraging further studies on the use of antifibrotic agents in sarcoidosis.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating chronic pulmonary disease, characterized by aberrant deposition of extracellular matrix and resulting in progressive and irreversible pulmonary remodelling [1]. The diagnosis of IPF is primarily based on a typical radiological and/or histological injury pattern, the usual interstitial pneumonia (UIP) [2]. In addition, one characteristic histological feature of UIP is the presence of fibroblastic foci (FF) which are found not solely, but also in early stages of disease development [3]. FF are composed of an accumulation of fibroblasts and/or myofibroblasts within a background of immature extracellular matrix and previous studies suggested an association of FF to disease genesis, progression and overall poor prognosis in UIP [4]. Although less frequent, FF can also manifest in a handful of other fibrosing pulmonary diseases like sarcoidosis [5], non-specific interstitial pneumonia (NSIP) [6], and chronic hypersensitivity pneumonitis [7]. In chronic pulmonary sarcoidosis, granuloma can undergo a fibrotic transition over time, resulting in an end-stage fibrotic lung disease, resembling a UIP pattern [8].

To date, data on the prevalence of FF in variant fibrosing pulmonary diseases is very limited. In addition, it is still unclear if the molecular signaling pathways and pathomechanisms contributing to their formation are similar across different entities. However, based on the comparable histomorphology, it can be hypothesized that there is a related molecular background of FF in variant fibrosing lung diseases. To approach this issue, we selected lungs from clinically well-characterized IPF and sarcoidosis patients as well as healthy controls and performed a molecular comparison of FF from both entities in a compartment-specific comprehensive analysis using the MMI CellCut laser microdissection system combined with molecular biology analyses.

The MMI CellCut laser microdissection system has been developed for the selective isolation of single cells or cell clusters from tissue sections for highly specific and meaningful molecular analyses such as RNA expression profiling.
though this method has been used in various research projects, detailed protocols are often not available. Thus, researchers are urged to spend their precious time and material to establish whole workflows and to optimize many parameters in order to obtain any results.

Fig. 1: MMI CellScan system on the Nikon Ts2R inverted microscope. The system is compatible with many microscope brands and models and can be combined with all MMI cell isolation systems for many research applications

This application note presents a step-by-step guideline as well as useful tips for laser microdissection work and subsequent RNA expression profiling using lung tissue from healthy people and diseased patients. In this example, FF are selectively cut to investigate their molecular footprint in different lung diseases.

Please note that this protocol has been established and optimized for this type of tissue and may not work the very same way for other tissue types. However, it provides a valid starting point for further optimization steps.

## Material and Methods

### Laser Microdissection

Standard formalin-fixed paraffin-embedded (FFPE) lung tissue blocks were prepared.

First, sections of approximately 10 µm thickness were cut and placed on RNAse free membrane slides (MMI MembraneSlides RNAse free, product number 50102; Molecular Machines and Industries GmbH, Eching, Germany). Following this, sections were uncoated from paraffin using Xylol (2 x 10 min) followed by a descending alcohol series (100%, 90%, 80%, 70%, 50% and water for 10 min each) and then stained for 3 sec by dipping the slide in filtered haemalum, (cave! not with eosin to prevent RNA damage. All steps were performed in compliance with the usual hygiene regulations in order to prevent contaminations. 30 slides in 2 sections were used for laser dissection from each case to yield about 30 x FF (~1 µm2). Laser microdissection was performed using the MMI CellCut system (Molecular Machines and Industries GmbH, Eching, Germany). Selection and encircling of FF was conducted using MMI CellTools V5.0 software (Molecular Machines and Industries GmbH, Eching, Germany). Finally, encircled regions were laser-cut and withdrawn in a sterile way using MMI IsolationCaps (0.5 ml, transparent, product number 50204; Molecular Machines and Industries GmbH, Eching, Germany). All samples from one patient were pooled in one tube. Samples were stored at -20°C until further use.

### Gene Expression Analysis

Compartment-specific RNA was isolated from the obtained tissue extracts using the RNeasy FFPE Kit (Qiagen, Venlo, The Netherlands). RNA content was measured using the Qubit RNA IQ Assay (Thermo Fisher Scientific, Waltham, MA, USA) guaranteeing a minimum of 100 ng in each sample.

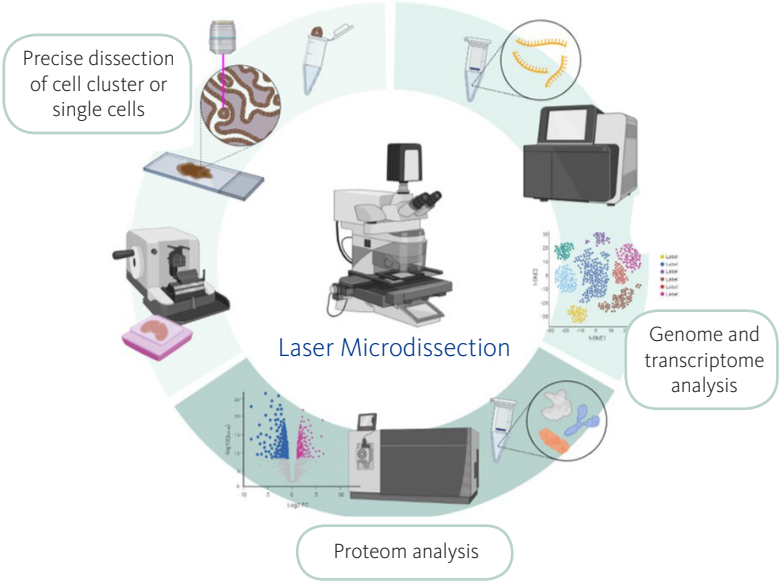


Fig. 2: Tissue processing for laser microdissection, generation of compartment-specific RNA isolates and further applications (Figure was designed using BioRender)

Samples were then analyzed using a commercial panel on 760 fibrosis-specific genes (nCounter Human Fibrosis V2 Panel) and the nCounter Analysis System (NanoString Technologies, Seattle, WA, USA, respectively). Afterwards, normalization of counts was performed using the nSolver analysis software version 3.0 (NanoString Technologies, Seattle, WA, USA). Therefore, 10 internal reference genes were used, as predefined by the manufacturer. Well established housekeeping genes (glucuronidase beta (GUSB) and phosphoglycerate kinase 1 (PGK1)) were designated as reference genes for standardization of measurements. Further analyses on the ascertained log2 mRNA counts were performed using R software version 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria) and the nCounter Advanced Analysis module version 1.1.5. The absolute gene expression results of both entities were analyzed and compared with each other. A Shapiro-Wilks test performed on all intragroup gene expressions showed a predominant normal distribution of data. Hence, t-tests were used for pairwise comparisons and ANOVA for

multi group comparisons. False discovery rates (fdr) were calculated and values < 0.05 were considered statistically significant. Statistical analysis was concluded by correction for multiple testing using the Holm Bonferroni method. Biological pathway analysis was performed using the GeneOntology database as well as gene-pathway associations supplied by the manufacturer.

General points to consider for your experiment:

- Use RNAse free water as well as sterile lab ware and equipment
- Always wear gloves (treated with RNAse inhibitor solution)
- Carefully open and close tubes or vessels to not contaminate any chemicals or solutions

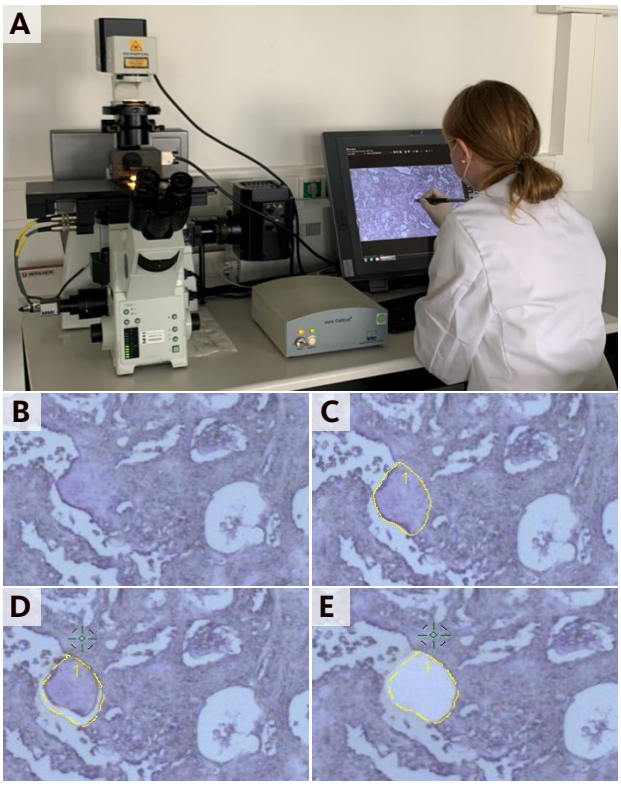


Fig. 3: Compartment-specific tissue extraction. (A) Laser-assisted microdissection using the IX71 microscope (Olympus Europa GmbH) with the CellCut system (MMI Molecular Machines & Industries GmbH, Eching, Germany). (B-E) Stages of the isolation of fibroblastic foci (FF) from lung tissue by laser-assisted microdissection (the laser cut is visible in D; E shows the dissected areal, isolated FF are collected in the cap [not shown]). Original magnification: ×100

## Results

The mRNA expression analysis using laser microdissected FF revealed a significantly altered expression signature for 375 out of 760 genes compared to controls. Of these, 264 showed similar regulation in FF from both disease entities. After Holm Bonferroni correction, 136 genes still showed a significantly altered expression compared to controls. Of these, 69 showed a similar expression in both groups, while 41 showed an altered expression solely in the sarcoidosis group and 26 solely in the IPF group (Figure 4). While directly comparing FF from sarcoidosis and IPF, there were no differentially regulated genes. The 12 genes which showed the strongest, although not significant difference between both entities (data not shown).

Based on the Holm Bonferroni corrected results, 67 of these 136 genes showed an altered expression compared to controls solely in sarcoidosis or in IPF. However, with regard to the fdr-values, only 2 of all these genes remained differentially expressed, i.e., decreased expression of calcium transport protein 1 (CAT1) and increased expression of SMAD specific E3 ubiquitin protein ligase 1 (SMURF1), both in the sarcoidosis group.

## Discussion

The results presented in this study indicate the molecular similarity of FF in sarcoidosis and IPF suggesting that the pathogenic mechanisms behind FF are independent from the underlying disease: a FF is a FF, regardless of the underlying disease. This in turn encourages the use of antifibrotic agents approved for IPF also in progressive fibrosing sarcoidosis. Moreover, in the light of the appearance of FF in other fibrotic lung disease entities, the impact of their presence on diagnosis should be re-evaluated in current guidelines. To draw a comparison, in a former work we could show that bronchiolitis obliterans (BO) and alveolar fibroelastosis (AFE) are based on the same molecular processes independent from the respective disease entities [9].

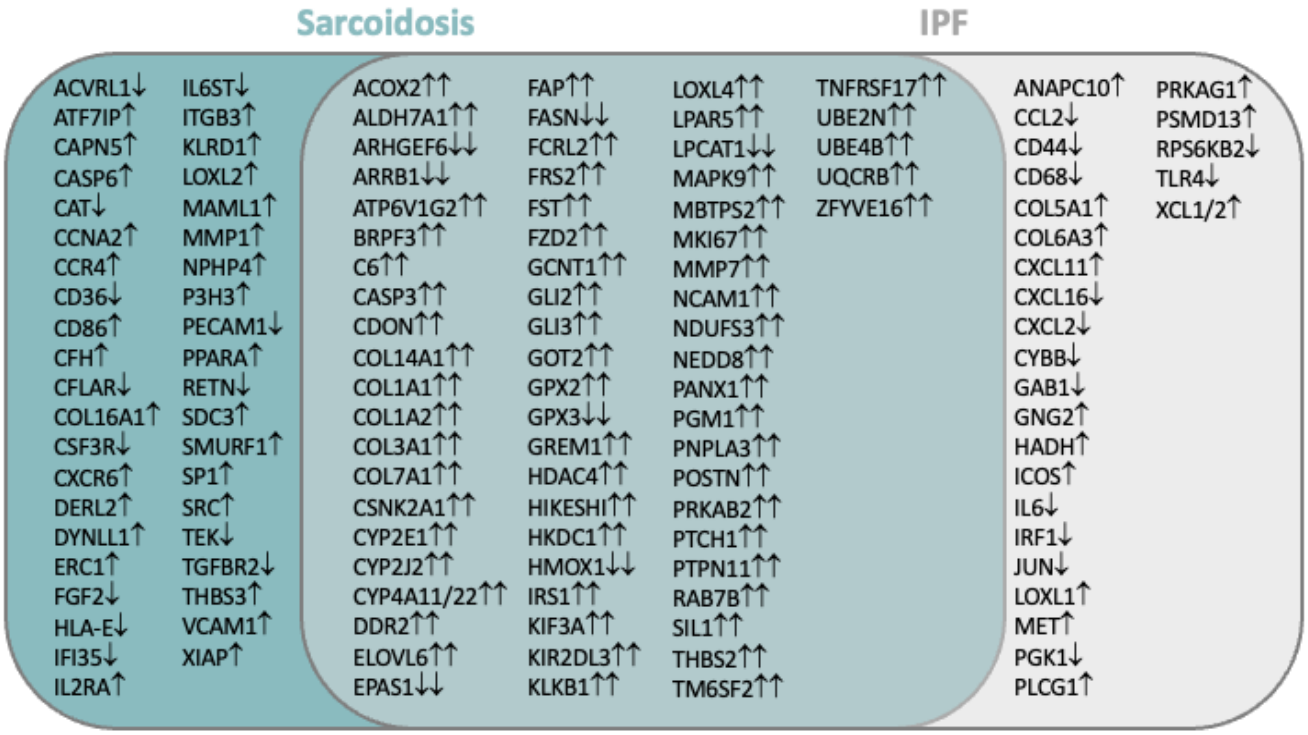


Fig. 4: Venn diagram showing 136 genes with significantly altered expression signature in Sarcoidosis and IPF compared to controls. 69 genes showed a similar expression in Sarcoidosis and IPF, while 41 showed an altered expression solely in the Sarcoidosis group and 26 solely in the IPF group

Interestingly, by analogy here we see again a divergence of histomorphological and clinical presentations emphasizing the importance of multidisciplinary boards for interstitial lung diseases. Limitations of this study are the small sample size and the monocentric design. Future studies are needed to explore the portability to other fibrotic lung disorders.

Using the MMI CellCut with its unique low-damage UV laser, we were able to specifically excise and analyse FF from IPF and sarcoidosis patients. Our protocol has been established to yield high quality RNA for gene expression analysis. This application will help other researchers providing a starting point for their own experiments with lung tissue or other tissue types.

## Reference

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## MMI CellCut - Your Benefits

- Ensure highest sample integrity for meaningful downstream applications
- Visual inspection of your cutting efficiency
- Cut almost any type of sample – even living cells
- Most flexible microdissection on the market: Compatible with many microscopes and almost all objective lenses
- Contamination-free cutting provides safe enviroment for operator and sample



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