Advancing forensic RNA profiling

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Outline

of this thesis

In forensic criminal investigations, DNA profiling is an important and frequently used tool, as it has the ability to reveal the identity of a donor of a trace with high evidentiary value. In forensic settings, this technique was first applied in 1985 and has undergone major technological advancements since then, now allowing for the analysis of traces in which only minute amounts of often degraded cellular material are present. Additionally, the predictive value of DNA regarding donor ethnicity, visible features and age is increasingly explored for the forensic context.

Next to the question of *who* contributed to the trace (DNA analysis), knowledge regarding the cellular origin of the evidentiary trace, thus *what* cell type contributed to the trace, is often key to facilitate inference of activities. Previously, cell type inference of body fluid and organ tissue traces commonly relied on techniques such as microscopic, histological or immunological tests, which due their limited sensitivity and specificity are not ideal in forensic settings. Alternative methods for cell type inference include RNA-based approaches which have shown their up come in forensic investigations since 1999. RNA profiling techniques rely on the fact that different cell types, such as blood, express a characteristic pattern of genes, such as haemoglobin beta (HBB) expressed in the red blood cells. RNA profiling is generally performed simultaneously and alongside DNA profiling from the same trace of evidence.

The chapters of this thesis describe the work performed through the years aiming to expand and advance forensic RNA profiling. Details per chapter are outlined below. The ultimate goal of forensic research is application in casework. Regarding RNA typing, the Netherlands Forensic Institute (NFI) is one of few labs in the world actually applying RNA profiling in forensic casework. Since the first application in an NFI case in 2010 and to the time of writing (December 2017), RNA profiling has been considered in over 200 cases. In approximately 70% of the RNA cases, identification of body fluids is requested. This body fluid system, referred to as the "Cell-typer", allows for the inference of blood, saliva, vaginal mucosa, menstrual secretion, semen and skin. Three years later, an organ tissue profiling system, allowing for the inference of brain, lung, liver, skeletal muscle, heart, kidney and skin, was introduced to RNA casework at the NFI and is requested in 30% of the RNA cases. Body fluid inferring RNA cases mainly involve sexual assault cases in which the presence of vaginal mucosa cells is disputed. Inference of organ tissues is mainly requested on objects involved in violent crimes. Up to date, ten NFI RNA cases have been presented in national court, while presented twice internationally.

As RNA profiling is in many ways very distinct from DNA profiling, expertise in analysing RNA profiles, clear guidelines for data interpretation and awareness regarding potential interpretation pitfalls are essential when RNA profiling is applied in casework. When proceeding to application in casework, NFI developed such accompanying interpretation guidelines. **Chapter 1** describes an RNA profiling exercise conducted as a collaboration between nine partners of the European Forensic Genetics Network of

Excellence (EUROFORGEN-NoE) in order to assess both the value of RNA analysis for cell type inference and the accompanying interpretation guidelines in forensic context.

As bodily secretions may be encountered at a crime scene whilst not targeted by the body fluid multiplex, six additional secretion types were analysed to investigate the possibility of obtaining false positive signals in these non-target cell types. **Chapter 2** describes the results of this specificity study and additionally describes studies performed aiming to further forensic RNA profiling, such as the use of alternative markers to replace RNA markers for the identification of vaginal mucosa, or the search for a relation in sensitivity of DNA versus RNA profiling in single donor body fluids.

Alike for DNA profiling, low level noise signals and dye blobs may be detected in RNA profiles. Increased noise can difficult interpretation of amplified signals and lead to false positive scoring of absent markers. **Chapter 3** describes the various causes and solutions that were considered to reduce the level of noise signals in RNA profiles.

Especially when minute evidentiary traces are analysed, background cell material unrelated to the crime may contribute to detectable levels in the genetic analyses. **Chapter 4** aims to increase our understanding regarding the prevalence of human cell material in background and activity scenarios. This chapter describes the results of analysing 549 samples comprising various public objects, private samples, transfer-related samples and laundered samples, which were analysed using DNA and RNA profiling. Several research questions were proposed for this study, for example "Do increased DNA yields lead to increased numbers of contributors or to the detections of other cell types than skin?" and "Is the owner of a private item always the major contributor to the DNA profile?".

Unlike in the study described in Chapter 4, forensic casework scenarios do not allow for the analysis of pre-activity background samples from the exact same location as post-activity samples. **Chapter 5**, a sequel of Chapter 4, describes therefore the analysis of post-activity background samples taken from an untouched area. Chapter 5 presents how these control region specimens may be useful when investigating activity-related scenarios.

Chapters 1 to 5 mainly focus on the inference of body fluids, while in some forensic cases, such as the event of a violent crime, knowledge regarding the tissue type may be of aid in the reconstruction of the event surrounding the crime. In **Chapter 6** the development of an organ tissue inferring RNA-based multiplex is described. This multiplex, referred to as the "Organtyper", allows for the inference of seven previously mentioned organ tissue types. After considering multiple candidate RNA markers, a selection of 17 markers was combined in a multiplex comprising at least two distinct markers per target organ tissue.

Primate- and target-specificity of the organ tissue typing system described in Chapter 6 is essential when applied in forensic casework. Initially, primate-specificity

of the system was confirmed based on *in silico* analysis using sequence alignment software. In **Chapter 7** specificity of the assay to human tissues was physically assessed by subjecting the assay to a set of non-human organ tissues. Additionally, cross-reactivity of the Organtyper markers to body fluids and of body fluid markers to organ tissues was assessed.

When postmortem intervals increase such as with longer burial times, human remains suffer increasingly from the taphonomic effects of decomposition processes such as autolysis and putrefaction. It is often assumed that RNA is less stable than DNA and may therefore be unsuitable for analysis of degraded samples. In **Chapter 8** the stability of DNA and RNA in long-buried human remains was assessed to examine for trends in nucleic acid degradation and the postmortem interval. This study gives insight in the remarkable stability of nucleic acids in severely degraded tissues and may lead to a change in sampling policies in identification of degrading cadavers.

In the last experimental chapter, a novel application of RNA typing was explored, namely for the inference of sex. While the presence of male cell material can be readily inferred from Y-chromosome specific signals in DNA quantitation and DNA profiling results, the presence of female cell material is inferred only indirectly, *i.e.* from absence or unbalanced response of the Y-chromosomal marker. On DNA level, no forensic marker exists to positively identify female cell material. **Chapter 9** describes the search for male- and female-specific RNA markers. This is the first assay enabling positive identification of female cellular material, and the first overlapping information in DNA and RNA profiles.

Chapter 10 reflects on the outcomes described in the earlier chapters of this thesis and appoints aspects of future and alternative cell type inferring techniques used in forensic casework. These include the future developments that lie in the application of massively parallel sequencing (MPS) both to increase understanding and as an alternative cell typing method.