Advancing forensic RNA profiling

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Chapter 10

General discussion

Challenges and opportunities in forensic RNA cell type inference

Introduction to cell type inference

In forensic criminal investigations, DNA profiling is an important and frequently used tool as it has the ability to reveal the identity of the donor of a trace, which is important for both investigative and evaluative purposes. In the hierarchical evaluation structure that goes from sub-source, source, activity to offence level, donor inference reflects sub-source level reporting [1-3]. In current days it is increasingly questioned how evidentiary traces got deposited (rather than by who) resulting in activity level reporting by forensic scientists. One of the aspects that can provide activity level information are the cell type(s) contributing to an evidentiary trace, although this in itself is source level information (a trace represents a certain cell type from a certain person). Conventional methods for cell type inference include chemical, enzymatic and histological assays that tend to be of limited specificity or sensitivity, which can be complex when analysing forensic samples that often comprise little cell material [4-9]. Additionally, these methods are often presumptive in nature and only suitable for identification of one cell type at a time [4][10]. More recently, genetic- and protein-based cell type inferring methods have been proposed that include the use of micro RNA (miRNA), DNA methylation, microbial species, proteins and messenger RNA (mRNA). Our choice to exploit mRNA markers for forensic cell type inference is mainly derived from the limitations regarding specific markers for the other methods. A summary of the advantages and limitations of the methods other than mRNA profiling can be found in **Box 1**.

mRNA plays a key role in cell function as it operates as a messenger between a DNA gene and the final protein product The amount and composition of mRNA molecules in a cell reflects the function of that cell. The human body is composed of trillions of cells, encompassing at least 400 different cell types [11-12], such as adipocytes, erythrocytes and monocytes, that have different morphologies and functions. Even though the genome information (DNA) is the same for practically all cells (except haploid gametes and anucleated cells such as erythrocytes or thrombocytes), different cells express only subsets of the ~21,000 coding genes that constitute the full human genome [10][13]. A gene is a region of the DNA that encodes function. It is transcribed from DNA into RNA, which can be mRNA (or other types of RNAs that have a role in protein synthesis, post-transcriptional modification, DNA replication or gene regulation). In a process called translation, mRNAs serve as a chemical blueprint for the synthesis of proteins. Cell types are defined during early embryonic development by their specific pattern of regulated gene expression [10][14]. Expression of genes may be restricted to a specific cell type, as for example genes that code for proteins involved in oxygen transport need to be transcribed in red blood cells and not in spermatozoa, while other genes may be expressed ubiquitously. While the DNA in one's blood is unique for that individual, the mRNA expressed in blood cells is similar between all individuals.

Box 1 – Alternative genetic- and protein-based methods for cell type inference

Both messenger RNA (mRNA) and **micro RNA** (miRNA) contribute to cell type differentiation [10] [70]. miRNAs are small non-coding regulatory RNA molecules that function in gene silencing and gene expression regulation [71]. Silencing is induced by base pairing with (partially) complementary sequences within the 3'UTR (untranslated region) of the targeted mRNA [72]. With a predominant length of 22 nucleotides, miRNAs are much shorter that mRNAs. Since miRNAs are *in vivo* embedded in protein complexes and protected against RNases (RNA-degrading enzyme) they have a higher resistance to degradation compared to mRNA, which could make miRNA markers advantageous in forensic analysis.

However, miRNAs may lack specificity as only partial base binding of miRNA-mRNA is required, which allows individual micro RNAs to have up to hundreds of targets. Additionally, individual mRNAs can contain binding sites for multiple and different miRNAs [73].

Cell type information can also be obtained from DNA-based methods, although not through the actual genetic code but from changes occurring in the epigenome. The epigenome comprises the chemical changes to DNA and histones as a way to regulate gene expression [74]. One of these epigenetic modifications is called **DNA methylation**, which is the modification of DNA by the addition of a methyl group onto cytosine that can result in modification of gene function [75]. Various studies have revealed tissue-specific differentially methylated regions (tDMRs) that could provide an alternative to (m)RNA profiling [76-77]. DNA methylation-based assays employ either of two methods: the most applied method uses bisulphite conversion in which unmethylated cytosine nucleotides are converts into uracil, while the methylated cytosines remain unchanged. Data is analysed based on differential methylation levels, in which the ratio between methylated and unmethylated cytosines for each methylation site is determined (although SNaPshot CE-based visualisation results in "on-off" information rather than methylation rates, as the signal strength of different dyes differ [78]). A drawback of bisulphite conversion methods is that the conversion requires large quantities of DNA and goes hand-in-hand with DNA degradation [79]. The second method uses methylation-sensitive restriction enzymes (MSRE) [80-81] in a PCR targeting tDMRs. An advantage of this method is that it does not require bisulphite conversion, and therefore requires less DNA. This technique starts with digestion of genomic DNA using a methylation-sensitive restriction enzyme that digests the recognition site only if the cytosine is not methylated; when the cytosine is methylated the enzyme cannot cut. After digestion, either a PCR amplification is performed using primers that surround the digestion sites (only undigested *i.e.* methylated DNA fragments allow amplification) or a single base extension is performed (the extension primer will only be extended if the restriction site was not cut, *i.e.* the DNA was methylated). Suitable tDMRs with high methylation level differences have been discovered mainly for semen (most of which relate to hypermethylation). So far, DNA methylation is less successful for the inference of the remaining body fluids as markers for these body fluids show less distinct methylation levels. Additionally, methylation levels show inter-person variation and can be influenced by factors such as age, nutrition and environmental factors (*e.g.* stress, smoking, sun exposure) [82]. Furthermore, results can be affected by incomplete conversion or restriction, inhibition or degradation. One of the major advantages of DNA methylation markers compared to RNA markers is that it can be applied on DNA extracts. Therefore, it can readily be applied in virtually all forensic casework wherein DNA extracts are available, while RNA assays require additional procedures including the co-extraction of RNA. A recent study proposed the idea of implementing DNA methylation markers into currently existing STR kits [81]. This could be of aid for example for the semen fraction after differential lyses in which the presence of semen can be confirmed by detection of semen-specific methylation markers. Moreover, multiple independent studies have identified useful methylation markers for the purpose of age determination with improved predicting accuracy and practical applicability compared to alternative age predicting methods (such as traditional morphological-, chemical-, or genetic-based methods like mitochondrial DNA deletions and telomere shortening).

Another alternative marker for cell type inference lies in **microbial species**. Microorganisms can be found virtually everywhere on earth, including the trillions of microbial cells that can be found in the human body. They function both inside and outside the human body to secure human health and support food digestion. Different locations house distinct microbial communities and even though the makeup of these communities may vary depending on factors such as age or health issues, these communities show sufficient similarities between individuals to differentiate between certain body fluids or tissues [83-84]. For forensic cell type inference purposes especially the vaginal micro flora has received a lot of attention, mainly because of the difficulty in finding true vaginal mucosa specific (m)RNA markers [84-85]. The use

of bacterial markers has been proposed and especially the *Lactobacillus* species were found suitable for the identification of samples containing vaginal mucosa. There are however some drawbacks in the use of microbial markers, such as the variations that may be observed between persons, the fact that micro flora may change with disease (*e.g.* vaginosis), microbes may not be human-specific and the microbiome may transfer upon contact [86-87]. Additionally, multiple studies describe the detection of various microbial markers in samples that do not contain vaginal mucosa, such as other areas of the skin (including samples devoid of female DNA) [17][85][88]. Use of these markers for forensic body fluid identification settings may therefore not be desirable.

Rather than using mRNA markers that target uniquely expressed mRNAs, **proteomics** is based on the detection of specific protein biomarkers, such as Semenogelin for semen. Proteomes can be mapped using two-dimensional high-performance liquid chromatography (HPLC) [89-91]. After identifying potentially useful biomarkers, mass-spectrometry-based assays can be used to detect even low-abundance proteins in high resolution against a background of non-target molecules. This way, the proteomes of various body fluids such as menstrual blood, blood, semen, and saliva have readily been investigated and distinguishable proteomic profiles have been identified. The major challenge in proteomic analysis is, however, that it requires a separate extraction of proteins that has so far not been incorporable in DNA and/or DNA/RNA co-extraction protocols. Furthermore, the abundancy of certain proteins, such as keratins in skin, can impede the successful application of proteomic markers in forensic science.

RNA profiling at the NFI

The forensic RNA-based cell type inferring assays at the NFI target genes that are predominantly expressed in forensically relevant body fluids (blood, saliva, vaginal mucosa, menstrual secretion, semen and nasal mucosa are targeted by the Cell-typer multiplex) and organ tissues (brain, lung, liver, skeletal muscle, heart, kidney and skin are targeted by the Organtyper multiplex). These assays allow for forensic assessments in for instance sexual assault cases where the presence of vaginal mucosa cells is questioned, or can be of aid in for example the reconstruction of a violent crime.

At the NFI, mRNA profiling for body fluid identification was first applied in casework in 2010. Three years later, mRNA-based organ typing was introduced in casework. In total, mRNA profiling has been considered in over 200 cases. Upon request of RNA profiling, the co-extracted DNA is first subjected to STR profiling to assess suitability and usefulness for RNA profiling. Depending on the case this may for example involve finding female DNA in a sampling from male skin areas or clothing or *vice versa*. In approximately 60% of the cases considered for RNA profiling, DNA profiling results led to the subsequent request of subjecting a sample to RNA profiling. About half of the DNA results of the samples that are sent through for Cell-typer requests are single-donor profiles, with the other half being mixed profiles. For the Organtyper requests, the majority of cases sent trough for RNA profiling are single-donor based on DNA profiling results (90%). For the Cell-typer multiplex the majority of samples selected for RNA profiling relate to sexual assault cases and include the analysis of samples obtained from *e.g.* the penis, underpants or fingers. For the Organtyper multiplex, mainly clothing or trauma-causing objects such as bullets or knives are examined. The Cell-typer multiplex is requested in approximately 70% of the RNA cases, compared to the Organtyper multiplex in approximately 30% of the cases. In two of the 200 cases, both the Cell-typer and the Organtyper multiplexes were requested for the same RNA extract. RNA profiling itself is generally performed unbiased – with no case context – after which results are handed to the reporting officers.

Over the years, the multiplexes have almost continuously been under optimisation to adapt to minor or major challenges that were encountered in casework or research [9][15-20]. For example, nasal mucosa was generally not considered a forensically relevant body fluid, and markers for the cell

type were therefore initially not included in the assay. The cross-reactivity of various Cell-typer markers in nasal mucosa and the observation that nasal samplings contain high amounts of human cell material, however, has led to the addition of a nasal mucosa marker in the Cell-typer multiplex [17]. This allows for alternative scenarios such as secondary transfer of nasal mucosa, blood from nose bleedings or expirated blood to be assessed. Overall, out of the 19 markers included in the Cell-typer multiplex that was first used in casework in 2010, only 11 remain in the currently used multiplex (although for all 11 markers the primers were redesigned to improve performance). Six markers were removed because they had limited informative value (*e.g.* general mucosa markers that are co-expressed in various cell types and skin markers that were found to provide limited added value in casework even when observed), two markers were replaced (a vaginal and a blood marker), and six markers were added. These new markers included three novel cell types: a female and male gender marker, and the previously-mentioned nasal mucosa marker that aids in distinguishing nasal mucosa from samples containing saliva and/or vaginal mucosa. The RNA-specific gender markers allow, for the first time, to establish a link between RNA and DNA profiles [19]. Also the Organtyper multiplex has had its improvements as it now targets a broader range of cell types that reside in the central nervous system (CNS), *i.e.* neuronal cells, astrocytes and oligodendrocytes and it carries the gender markers.

Challenges in forensic RNA profiling

There are various differences between DNA and RNA that challenge the workflow and data interpretation for RNA profiling. These differences can raise concerns or beliefs regarding the use of RNA in forensic settings that will be addressed below.

"RNA is less stable than DNA and therefore not suitable for use in forensic casework"

It is often disputed that RNA is less stable than DNA due to the hydroxyl group at the 2' position of the ribose sugar, which makes RNA more prone to hydrolysis than DNA. Some argue that, due to this lower stability, RNA is not suited for forensic casework. Yes, the hydroxyl group at the 2' position of the ribose sugar makes RNA more prone to hydrolysis than DNA [21-22]. This hydroxyl group, however, and the fact that G-U base pairing occurs within RNA molecules, allows RNA to form secondary and tertiary structures which probably adds to a remarkable RNA stability when fluids or tissues are in dried state [21-23]. *In vivo*, stability is known to be transcript-dependent based on the structure at the 3' region [24-25]. Also, RNA stability is provided from forming ribonucleoprotein complexes [26]. This stability has also been shown in Chapter 8 of this study, where RNA profiling was successfully applied on excavated samples that had been buried for over 40 years [18].

To enable the analysis of degraded RNA, small amplicon sizes are used in our multiplexes. To prevent preferential amplification for a cell type, the targeted tissues have amplicons in the same size range. The main risk of low stability is that no RNA remains available, thus that no results are obtained and false negative interpretations may occur. Keeping in mind that absence of detection does not mean that a body fluid is absent(note that the stability of RNA may vary for body fluids, possibly related to the microbes present in a fluid [10][27]), RNA profiling is well suitable for use even in the challenging samples that can be encountered in forensic settings.

Other studies describe how the over-time stability changes of RNA can actually be used for other forensic purposes. For example, the degradation pattern of mRNA could be used to determine the time since deposition of a sample, or signals for circadian rhythm expressed RNA molecules could be used to determine at what time during the 24 hour day/night cycle a sample was deposited at a crime scene. More detailed information regarding alternative uses of mRNA is presented in **Box 2**.

Box 2 - Alternative applications using mRNA

There are various studies that use mRNA-based techniques for other purposes than cell type inference. Many of these studies involve the ex-vivo degradation of RNA. As the ex-vivo stability of RNA changes over time, the degradation pattern of mRNA can potentially be used to establish a system to determine the time a sample, such as a blood stain, was placed. Regarding **time of placement**, there are two distinct research focuses that are currently being explored. The first aims to determine the time since deposition (TSD), giving an indication of the time in hours/days/weeks since a trace was deposited. The ability to determine the TSD can provide information by establishing an approximation of the time a criminal offense took place, which could also be used to select/ensure crime-related stains or to assess a suspect's alibi [92-93]. More recently, a novel trace timing aspect was examined aiming to determine **at what time during the 24 hour day/night cycle** a sample was deposited at a crime scene [94-96]. This study builds on the intrinsic rhythms of core clock-controlled genes involved in the circadian rhythm, such as the hormones melatonin and cortisol. This method can be promising, however, more clock-controlled genes with different peak times in the 24-hour day/night cycle need to be identified to increase accuracy and reliability of this method. Furthermore, the robustness of the method in varying conditions (*e.g.* humidity, temperature, different substrates) and the effect of factors such as medication need to be assessed.

The determination of a **postmortem interval** (PMI) is an important aspect in forensic death investigations. Traditionally, the PMI is estimated based on various physical and biochemical changes occurring shortly after death, such as rigor, algor and livor mortis [97-98]. These factors can, however, only be used for estimating relatively short PMIs with a wide window of estimation [98-99]. More recently, studies have described the use of nucleic acid degradation patterns to estimate the PMI. The rationale behind this is that 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. Varying results are described in different studies [100-104] using either of two methods. An end-point PCR method can be used in which tissue specific mRNA markers (and STR markers) are used to examine for trends in degradation. Alternatively, a quantitative PCR approach is used in which RNA markers are selected based on their abundancy in various tissues that is either stable (as a reference) or becomes altered when the PMI elongates (as a target biomarker). The overall trend of these studies it that even though promising results can be obtained for mock-cases, PMI estimation may be much more complicated in real cases as the effects of many pre- and postmortem factors with a role in degradation are unknown. All of these studies do however show that RNA, which is often assumed to be much less stable than DNA, can be remarkably stable. While the previously described studies aim to investigate the postmortem decrease of mRNA expression levels, a more recent study examined the transcripts generated in a window up to 96 hours after death with varying peak abundances for different genes and the striking expression of genes that were not expressed before death. One of the explanations of the increase of these transcripts is that when the nucleosome structure unwinds, remnant transcription machinery can transcribe genes that were previously silenced by their chromatin structure. These so-called "zombie genes" appear to "wake up" after death and monitoring of their expression could potentially be of aid in determining the time of death more accurately [105-106].

Alternatively, mRNA analysis could be of aid in determining the **cause of death** by examining alterations in mRNA expression patterns as a result of the traumatic effects surrounding death. For example, the amount of EPO (Erythropoietin) synthesized by the kidneys depends on the amount of oxygen present in blood. In case of for example drowning, strangulation or hanging, the expression level of EPO decreases. Similarly, various biomarkers are known to respond differently depending on the type of trauma that occurs. Although this field of study is relatively new in forensics, it may in the future be possible to distinguish traumas leading to death, such as hypoxia, injury (*e.g.* a different respond to brain, lung, or

cardiac damage), intoxication and drug adverse effects, or fire fatality based on changes in biomarker expression [107-108].

Wound age estimation is another issue in forensic science, that is routinely determined in forensic autopsy based macroscopic (*e.g.* skin colour changes) and immunohistochemical examinations [109-111]. Determination of the early stage (days) of wound age is especially important in case of suspected non-accidental injuries. Information regarding the age of the wound (dermal or intestinal) can give insight in the timing of the injury, the order of infliction, the survival time after injury and the relation between the time a trauma was inflicted and the wounds detected on a body. From a genomic point of view, the process of healing involves many biological changes that can be divided in three phases, namely inflammation, proliferation and maturation [110][112-113]. The timely changes in gene expression that occur during these three phases are caused by various biological mediators such as cytokines, growth factors and other bioactive molecules [110][114]. The key to mRNA-based wound age estimation is to identify a combination of genes that is indicative for specific time points or phases in the wound healing process. For this, mRNA expression of genes is monitored during the wound healing process to assess their suitability to be used to estimate wound age. Studies describe how especially mRNAs encoding cytokines and growth factors are useful markers for the determination of wound age [110][114]. Other studies have been performed aiming to determine the **age of bruises**, which is especially important in child abuse cases [115]. Alike for traditional wound age determination, morphological characteristics such as the transition of colour are important in determining the age of a bruise. There are however some factors that affect that affect the duration and appearance of a bruise, such as the location, size and skin colour [116]. Genes that play a role in bruise healing can aid in the determination of the age of a bruise. Various markers have been proposed that have the potential to be used to more accurately estimate the age of bruises and/or wounds [109-123]. However, aspects such as the effect of treatment like antiseptics, varying gene expression and the fact that most of the current bruise/wound age estimation methods are developed targeting animal models and are thus not fully representative for humans, difficult use of these assays in forensic casework [109][117][119][120].

"Lack of an RNA quantification system, determining input is not objective"

With DNA profiling, a quantitative real-time PCR approach targeting a human-specific sequence (nowadays a multicopy or even repetitive element to increase sensitivity [28]) is used. A similar method for RNA is complicated for two main reasons: 1) qPCR requires RNA to be copied into cDNA for which the input in the reverse transcription (RT) reaction needs to be inferred first (and one then assumes that this RT reaction is equally efficient for each sample) and 2) even when a ubiquitously expressed target sequence is used, this RNA is subject to expression differences for cell types, donors and in response to external factors [29-31]. At the NFI, we therefore utilize a serial input PCR approach which is a pragmatic solution to overcome these quantification difficulties [9]. For the RT reaction, we use a fixed amount of RNA following the rationale that the RT reaction is not readily overloaded with forensic samples (according to the manufacturer, up to 5 micrograms of total RNA can be reverse transcribed). Then, we use three cDNA inputs (*e.g.* 0.2, 1 and 4 µL) in the PCR and based on results of this serial input, an informative PCR input is selected which is subsequently used to generate replicate PCRs (generally four) [32]. Due to for example stochastic amplification effects (when template is limited), background expression (mRNA expression is enriched, not restricted in cell types) or spurious transcription (transcription is a relatively sloppy process with many cellular processes in place to destabilise aberrant transcripts) these PCR replicates may not be identical [10][32]. To overcome these issues, an approach alike the low-template DNA profile consensus approach is utilized [33], meaning the use of multiple PCR replicates per sample and scoring all markers for a cell type in all replicates jointly according to the "$x=n/2$" interpretation guideline. This method compares

the number of observed (*x*) to the number of theoretically possible peaks (*n*) in all replicates [32].

"Outcomes depend on interpretation approach"

The "*x*=*n*/2" guideline anticipates for the detection of background signals as cell types are only scored "observed" when ≥50% of the possible signals are detected [32]. In this approach, each marker has the same "weight" in data interpretation. This is theoretically not optimal as marker expression is known to vary between donors depending on various biological, physiological or environmental factors [32][34]. The exact composition of an evidentiary sample may vary as body fluids and organs are composed of various cell types. Thus, a specimen may contain fewer or more cells of the cell type in which a target marker is expressed. For example, at the NFI, brain is examined by a neuronal, astrocyte and oligodendrocyte mRNA marker. The use of multiple genes simultaneously in combination with the ≥50% value averages out these variations. This guideline has been shown to prevent false positive scorings [16]. This may however come at the cost of not inferring all cell types that are present, which is important to keep in mind during case interpretation (not seen ≠ absent). Results for cell types that are scored "observed" are reported as "fits with the presence of … cells". When no signals are seen the cell type is scored "not observed" which is reported as "no indication for the presence of … cells". When signals are seen for <50% of the possible signals, the scoring is "sporadically observed" which is reported as "no statement can be made regarding the presence of … cells" [32]. Thus, the ≥50% rule represents a fall of the cliff approach in which one observation more or less can make a categorical difference between "fits with the presence of" and "no statement can be made". Although challenging to achieve due to the many variables, reporting of RNA data could significantly benefit from probabilistic reporting, *i.e.* to assign weight of evidence to the results. An initial study shows both the outline and the challenges that come with this approach [35-36].

"Housekeeping markers are not used for normalization"

Clearly, the cell type specific markers are the most informative and important markers in RNA multiplexes, but there is debate regarding the importance of appropriate control/housekeeping markers. Housekeeping markers may be used to mark that RNA was present in the sample, but this requires that a housekeeping marker is always detected when RNA is present. This is not always the case with forensic samples (the expression of the housekeeping marker may vary or the housekeeping marker may not be detected due to a lower primer concentration that is selected to have a balanced end-point RT-PCR profile). Also, housekeeping markers can be used for normalisation when a quantitative RT-PCR approach is followed [29][36], but for the studies described in this thesis we apply end-point RT-PCR. The main purpose of the housekeeping markers in our analyses is to indicate absence of substances inhibiting cDNA synthesis and/or cDNA amplification for a sample [15][20]. This is especially informative when none of the other markers gives a signal. Thus, we use a housekeeping marker to confirm successful PCR amplification. Housekeeping markers in end-point PCRs should therefore be expressed ubiquitously, so that there is no issue on dependence for a cell type, which is the case in the assays described in this thesis. Various other controls are included throughout the RNA workflow, such as blank controls to confirm that no contamination has occurred, positive controls to confirm that all the used chemicals function, minus-RT controls (RT control lacking the reverse transcriptase enzyme) to assess for residuals of genomic DNA present in RNA extracts after DNase treatment.

"DNA and RNA results cannot be linked"

The use of multiple genes for different cell types in a multiplex allows for the analysis of cell type

mixtures. While DNA peaks are generally in line with the DNA contribution when contributions are not low template, RNA peaks may be unbalanced for a single contribution due to varying levels of expression per marker that can vary per individual depending on various biological, physiological or environmental factors [32][34]. Moreover, one contributor can donate more than one cell type and a single cell type can be given by multiple contributors. Up to now, profiling results are reported as DNA and RNA results, as currently DNA and RNA profiling outcomes cannot readily be linked. Only with certain body fluids, such as semen and vaginal mucosa, association of cell type and donor is possible based on gender (when the sample is inferred to comprise one female and one male donor) [19]. An alternative way of associating DNA and RNA based on peak heights has been assessed [37], but this was found to be impossible as the major in the DNA profile was found not to necessarily correspond to the highest RNA signals. The underlying reason may be that DNA and RNA profiling can have different sensitivities for cell types and donors.

Recently, the sequence of the RNA molecule has been proposed to provide information for association of DNA and RNA results [38]. Single nucleotide polymorphisms (SNPs) in transcribed regions are carried over to the RNA molecules. Clearly, within the open reading frame (ORF) there is selection against frameshifts, early stop codons and amino acid changes with deleterious effects on protein function and as a result SNPs may be less frequent in ORFs than in untranslated regions [39] (UTRs, note that 5'UTR changes may influence expression level and 3'UTR changes may affect stability [40-41]). Transcribed regions of marker genes can be examined for SNP locations in various databases such as the Ensembl database [42]. "Reference" SNP profiles can be obtained using DNA extracts; RNA extracts will confirm the cell type specific expression. To effectively associate donors (DNA) and cell types (RNA), multiple SNPs are required. This can be a collection of SNPs with varying minor allele frequencies (MAFs), in which the SNPs with a higher MAF are more common and thus more likely to be observed, while SNPs with a lower MAF are less common and thus more discriminative. Initially, RNA SNP profiles could be used to determine whether a suspect can have contributed to a trace. In the future, it may be possible to assign a weight of evidence to the RNA SNP profile, which enables moving towards distinguishing between individuals. Previous studies have reported that SNP mixtures can be statistically evaluated in a similar way as STR mixtures [43-44]. In contrary to STRs, SNPs do not stutter, which simplifies statistical analysis. For performing such statistical analyses, frequency data of the individual SNPs are required.

"RNA profiling is time consuming and labour intensive with value in limited numbers of cases"

Yes, RNA profiling described in this thesis represents a labour intensive approach and should be applied in cases only where the labour is likely to merit the information for the case. Notwithstanding, there may be strategies to use a simplified form of RNA typing in many more forensic cases when the method is adapted to befit pre-screening of crime stains (as also outlined in the ParaDNA Body Fluid ID Test [45-46]). Then, the method could replace and extend conventional presumptive and/or confirmatory tests (the first giving an indication regarding the identity of a sample, the latter being more specific). In forensic settings these tests, such as the RSID (Rapid Stain Identification) or prostate specific antigen (PSA) test, are applied mainly for the indicative identification of seminal fluid, blood and saliva [4]. The major drawback of these tests is that they can only be used to identify a limited number of cell types and false positive and negative scorings can occur [4][10]. With RNA profiling, the number of cell types available for pre-screening would significantly increase and encompass for example vaginal mucosa and gender (note that the XIST RNA marker is the only true female marker described in forensic literature [19]). A direct RT-qPCR approach is proposed to this aim. The approach is highly similar to that used in the DNA6hours procedure, which is currently used for rapid STR profiling [47-48]. A lot of time is gained by omitting steps such as cell lysis, co-extraction and DNase treatment. From an evidentiary sample a small amount

is collected by tape-lifting using a mini-stub [47-48]; the remainder of the sample stays dry and intact. The cell material on the mini-tape is submitted to a short lysis, and the lysate is used as input for a one-step RT-qPCR which, in one reaction, combines two enzymes for reverse transcription of RNA to cDNA and the cDNA amplification of genes of interest. These genes can be the same as the ones used in currently-used mRNA-based assays, thus using the knowledge obtained in all earlier RNA work. Alike the commonly used RNA assays, interpretation of the direct RT-qPCR assay will provide information on the presence of a body fluid. Furthermore, the obtained lysates could additionally be used for direct STR-profiling assays. This rapid assay, that is more sensitive, human-specific and additionally allows to simultaneously target more body fluids, could potentially replace currently-used presumptive and confirmatory tests. In forensic casework, this rapid tool can serve as a selection tool, in which knowledge regarding the cell type including the gender of the donor contributing to a trace can aid in selecting the traces for which to proceed with regular DNA and/or RNA profiling. This tool can for example be of aid in identifying a male blood stain in a collection of mainly female blood spatters. This rapid RNA assay is more investigative in nature while current RNA profiling is foremost evaluative. Thus, the assays will not replace each other.

"MPS is the future of forensics, including RNA profiling"

In the past few years, massively parallel sequencing (MPS) has made its rise in forensic science. MPS is a high-throughput sequencing technique that allows for the simultaneous determination of individual sequences of large numbers of molecules [49]. Conventional methods for DNA and/or RNA analysis mostly focus on profiling via PCR amplification and capillary electrophoresis (CE). Although the CE-approach is highly useful for analysis of samples that vary in lengths, MPS additionally provides information regarding the actual sequence of the amplified fragment, which greatly increases discriminating power [49].

MPS for forensic RNA applications is relatively new and so far, only few approaches have been used. Of course, whole transcriptome sequencing (RNA-Seq) can provide massive amounts of information regarding the full transcriptome of organisms and tissue types [50]. RNA-Seq data can be used in many ways, for example to identify expressed transcripts, examine the transcriptional structure of genes (such as start sites, 5' and 3' ends, (alternative) splicing patterns and RNA editing) and to examine changes in expression levels [51]. For forensic cell type inferring purposes, whole transcriptome sequencing is mainly used to identify novel markers. This can be complicated when a forensically relevant sample contains a low percentages of human material (for example saliva and vaginal samples comprise mainly microbes; only 5-10% of sequences are reported to map the human genome [27]). Previously, microarray studies were performed to examine gene expression by using a selection of sequences with known identities. MPS allows analysis of the complete transcriptome including both known and unknown genes. Once genes of interest are identified, targeted sequencing can be applied. In the future, this could be used to solve the earlier described drawback regarding the deconvolution of RNA profiles to associate cell types with individual donors [52]. Although MPS may indeed be the future of forensics on DNA level, its added value on RNA level will depend on the feasibility to associate DNA and RNA results based on RNA SNPs. Should RNA SNPs be of insufficient or limited added value, RNA profiling may well be performed using capillary electrophoresis (CE), as then MPS provides similar information compared to CE-based methods. If RNA marker analysis is to be incorporated in MPS multiplexes targeting DNA (which can be various types of markers such as autosomal-, Y- and X-STRs, or SNPs for identification, ancestry or externally visible characteristics), the issue of reverse transcription needs to be accommodated in the protocol.

Alternatively, MPS could be considered to establish a more quantitative analysis to provide insight in the gene expression variations that can be observed for different markers. This could additionally provide insight when analysing mixtures of multiple cell types. This could be achieved by using a two-step PCR, targeted sequencing approach [53]. In the first PCR, random tag primers are incorporated to the target

that consist of 1) a gene-specific primer (thus targeted); 2) a unique tag of *e.g.* 10 random nucleotides in length (in this example yielding 410 or 1,048,576 unique tags); 3) a common barcode, that is the target of the second PCR. During the first PCR, these random tag primers are merely annealed and extended (no exponential amplification). A second enrichment PCR is performed that uses primers targeting the common barcodes (3). This way, each original target of interest is tagged by a unique tag (2). After sequencing, the number of unique tags per target represents the number of original molecules, providing quantitative information for each target. Up to now, this approach has mainly been tested and promoted by commercial companies. One of the major drawbacks of this method up to date is the low sensitivity of the assay due to which it is not yet applicable in forensic settings.

Concluding remarks

Since the first publication in 1999 [54], RNA profiling for the purpose of body fluid identification has been investigated almost continuously [55- 69]. While RNA profiling can be applied also for alternative aims (such as determining the time since deposition or estimating the postmortem interval), in forensic casework, its main application at the moment is inference of cell types. Up to date, there are at least two forensic laboratories that routinely apply RNA profiling for the purpose of cell type inference in casework. These are ESR (the institute of Environmental Science and Research) in New Zealand, that applies RNA profiling for the inference of body fluids since 2010, and the NFI in The Netherlands, that uses both body fluid and organ tissue inferring assays since 2010 and 2012, respectively. Organ typing has not been implemented at ESR because of a low rate of shootings (SallyAnn Harbison, personal communication). Besides, some laboratories use RNA profiling occasionally, often as a follow-up of collaborative exercises in which NFI primer mixes were shared. Other laboratories seem reluctant in implementing RNA profiling in forensic casework, which may be due to the labour intensity of both the implementation procedure and the RNA profiling itself and the low frequency of cases in which RNA profiling is requested in smaller laboratories. Although RNA profiling is not subject to DNA law, absence of acceptance in court in a country may withhold laboratories to proceed to RNA profiling especially in countries that run by the adversarial system. The fact that RNA profiling is routinely used for forensic casework in both a country in which the adversarial is used and a country run by the inquisitorial legal system may be helpful. In our experience, a steady inflow of cases is required to retain the essential awareness and expertise in analysing RNA data. The use of a uniform set of markers and interpretation guidelines may be necessary in making RNA profiling more approachable. Also, when RNA markers would be incorporated into commercial MPS kits with clear protocols and interpretation software, the implementation hurdle might be lessened for many laboratories. Major future advances could lie with rapid RNA-based screening, which could serve not only as a replacement or extension for currently-used presumptive tests, but also as a selection tool for identifying traces of interest. Furthermore, when incorporated in MPS platforms and thereby extending the analysis from amplicon length-based to sequence-based, RNA may in the future provide information regarding the cell type as well as information to associate cell type to a donor. This could provide the missing link between RNA and DNA profiling.

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